A Murine Model of HUS: Shiga Toxin with Lipopolysaccharide Mimics the Renal Damage and Physiologic Response of Human Disease

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Hemolytic uremic syndrome (HUS), which is caused by Shiga toxin–producing Escherichia coli infection, is the leading cause of acute renal failure in children. At present, there is no complete small animal model of this disease. This study investigated a mouse model using intraperitoneal co-injection of purified Shiga toxin 2 (Stx2) plus LPS. Through microarray, biochemical, and histologic analysis, it was found to be a valid model of the human disease. Biochemical and microarray analysis of mouse kidneys revealed the Stx2 plus LPS challenge to be distinct from the effects of either agent alone. Microarrays identified differentially expressed genes that were demonstrated previously to play a role in this disease. Blood and serum analysis of these mice showed neutrophilia, thrombocytopenia, red cell hemolysis, and increased serum creatinine and blood urea nitrogen. In addition, histologic analysis and electron microscopy of mouse kidneys demonstrated glomerular fibrin deposition, red cell congestion, microthrombi formation, and glomerular ultrastructural changes. It was established that this C57BL/6 mouse is a complete model of HUS that includes the thrombocytopenia, hemolytic anemia, and renal failure that define the human disease. In addition, a time course of HUS disease progression that will be useful for identification of therapeutic targets and development of new treatments for HUS is described.


Materials and Methods
Shiga Toxin Purification

Stx2 was purified by immunoaffinity chromatography from cell lysates (provided by Alison O’Brien) of E. coli DH5α that contained the Stx2-producing pES120 plasmid (7). Briefly, Stx2 was purified using 11E10 antibody (8) that was immobilized using an AminoLink Plus Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Endotoxin was removed using De-toxi-Gel (Pierce Biotechnology) as per the manufacturer’s instructions. Stx2 purity was assessed by SDS-PAGE and determined to be endotoxin-free, and activity was measured in a Vero cell cytotoxicity assay. Stx2 was chosen because it is far more frequently associated with HUS clinical isolates than Stx1 (9,10).

Animal Studies

C57BL/6 male mice that weighed 22 to 24 g were purchased from Charles River Laboratories (Wilmington, MA). Mice received an intraperitoneal injection of a low sublethal dose of LPS at 300 µg/kg (O55:B5; Sigma-Aldrich, St. Louis, MO), 225 ng/kg Stx2 (two times the LD50), or both. Saline injection was used for control mice. At 0, 2, 4, 6, 8, 12, 24, 48, and 72 h after injection, two mice per time point were killed and kidneys were processed as described next. These experiments were repeated three times. In separate experiments, blood samples were obtained from mice at 0, 4, 8, 12, 24, 36, 48, 60, and 72 h after injection. Mice were weighed every 12 h for 3 d to determine the percentage of weight loss. All animal procedures were done in accordance with University of Virginia Animal Care and Use Committee policies.

Blood Analysis

Blood was collected from mice with EDTA-treated or with non–EDTA-treated capillary tubes via retroorbital bleed. Blood that was collected without anticoagulant was either smeared on microscope
treated mice was compared with GeneChip Expression Analysis using the manufacturer’s instructions. Total kidney RNA from control and experimental mice was extracted using the RNeasy Midi Kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s instructions. Blood urea nitrogen (BUN) was determined spectrophotometrically with VetScan (Idexx Corp., Westbrook, ME). For reticulocyte counts, three drops of EDTA-treated blood was mixed with two drops of Reticulocyte Stain (Sigma-Aldrich) for 10 min at room temperature. Mixtures then were smeared on a microscope slide, dried, and coverslipped. Percentage of reticulocytes was determined by counting the number of reticulocytes per 1000 red blood cells (RBC). A complete blood count (CBC) was performed on 20 μl of the EDTA-treated blood using MASCOT HEMAVET 850 (CDC Technologies, Oxford, CT) according to the manufacturer’s instructions.

Immunohistochemistry

One half of a mouse kidney was fixed in 4% paraformaldehyde for 24 h, processed, and embedded in paraffin. Three-micron-thick sections were cut and placed onto charged slides. Martius Yellow-Brilliant Crystal Scarlet-Aniline Blue (MSB) differential staining procedure was performed as described previously (11). Martius yellow and phosphotungstic acid in alcoholic solution stain red cells, brilliant crystal scarlet stains muscle and mature fibrin, and aniline blue stains collagen. Glomeruli that were positive for fibrin staining were quantified by counting three sets of 20 glomeruli per slide and averaging the percentage positive for fibrin at each time point. Immunohistochemistry for platelets was performed using polyclonal goat anti-human integrin-β3 antibody that cross-reacts with the mouse protein (Santa Cruz Biotechnology, Santa Cruz, CA) (12). Primary antibody was detected using an avidin/biotin horseradish peroxidase system (Vector Laboratories, Burlingame, CA) and diaminobenzidine. Sections then were counterstained in hematoxylin, dehydrated, and mounted.

Electron Microscopy

Kidneys were harvested from killed mice 0, 24, 48, and 72 h after Stx2 plus LPS challenge, cut into blocks approximately 1 to 2 mm³, and fixed overnight at 4°C in 4% paraformaldehyde and 2.5% glutaraldehyde in 1× PBS. The fixed tissue subsequently was processed by the University of Virginia Advanced Microscopy Facility. The tissue blocks were washed with PBS at 24°C and postfixed for 1 h in 1.0% osmium tetroxide. The blocks then were washed in distilled water, dehydrated using a graded acetone series, embedded in epoxy resin (EMBED 812; Electron Microscopy Sciences, Hatfield, PA), and polymerized for 2 days at 60°C. Ultrathin sections approximately 70 nm in thickness, obtained with a diamond knife (Diatome, Hatfield, PA) on a Leica Ultracut UCT ultramicrotome (Leica, Bannockburn, IL), were collected on 200-mesh copper grids, contrast-stained with uranyl acetate and lead citrate according to routine procedures, and examined in a JEOL 1230 transmission electron microscope (JEOL, Peabody, MA). Digital images were acquired using an SIA L3-C digital camera (Scientific Instruments and Applications, Duluth, GA). At least four glomeruli from each of three mice were examined per time point.

cRNA Synthesis and Microarray Hybridization

One half of a mouse kidney was stored in 2 ml of RNALater (Ambion, Austin, TX) at 4°C until RNA extraction. Total RNA was isolated using the RNeasy Midi Kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s instructions. Total kidney RNA from control and treated mice was compared with GeneChip Expression Analysis using Mouse Genome 430A 2.0 Arrays (Affymetrix, Santa Clara, CA) by the Biomolecular Research Core Facility (University of Virginia, http://www.healthsystem.virginia.edu/internet/biomolec/).

Microarray Analysis and Quantitative Reverse Transciptase–PCR

Microarray probe-level determinations were made by the Probe Logarithmic Intensity Error estimation method, part of the ArrayAssist Lite software package (Stratagene, La Jolla, CA). DNA-Chip Analyzer (dChip) (13) model-based estimation was used to normalize the signal levels to the median arrays for each series of experiments. Multiclass significance analysis across the time course was performed with the Significance Analysis of Microarrays software (14). Genes with q values (false discovery rate) of <5% were determined to be altered signifi-

Figure 1. Survival and weight loss of mice that were challenged with Shiga toxin 2 (Stx2), LPS, and Stx2 plus LPS. Mice received an injection of 225 ng/kg Stx2, 300 μg/kg LPS, or both. (A) Survival curve is representative of three experiments. Data contain six mice per group. (B) Mice were weighed every 12 h after injection for 72 h. Data contain 10 to 12 mice per group. *P < 0.002 significantly decreased from weight at time 0 h; #P < 0.002 significantly increased from weight at time 0 h evaluated by ANOVA and t test.

cantly by challenge. From this set, only genes that were altered 2.0-fold or greater at any time point compared with controls were used for further analysis. Cluster analysis was performed on these data set with GeneCluster 2.0 software (15), and dChip was used for gene ontology analysis of the clusters (13). Expression patterns of select genes from each cluster were verified by quantitative real-time PCR using the iScript cDNA Synthesis Kit, iQ SYBR Green Supermix and iCycler Thermal Cycler (BioRad, Hercules, CA).

Statistical Analyses
All statistics (excluding those that were used for microarray analysis) were performed using single-factor ANOVA followed by two-sample t test, and P < 0.05 was considered significant.

Results
Stx2 Plus LPS Causes Diminished Renal Function
Mice an intraperitoneal injection of a low sublethal dosage of LPS (300 μg/kg), 225 ng/kg Stx2, or both agents. The dosage of Stx2 chosen is the minimum 100% lethal dosage (two times the LD50), resulting in lethality within 4.5 d (Figure 1A). When this dosage of Stx2 was combined with the sublethal dosage of LPS, the time to death was decreased by 1 d. Mice were weighed every 12 h to determine percentage of weight loss caused by the toxins (Figure 1B). LPS induced weight loss early in the time course, whereas Stx2 induced weight loss late in the time course. Stx2 plus LPS caused weight loss both early and late in the time course. Mice were evaluated for kidney function, peripheral cell count abnormalities, and structural kidney changes. These results are summarized in Table 1. We determined that only mice that received Stx2 plus LPS exhibited all of the signs of clinical HUS. Therefore, we chose to present the data that are relevant to the complete Stx2 plus LPS mouse model of HUS. Stx2 plus LPS co-administration intraperitoneally at these dosages was used for all subsequent experiments except where noted.

Increases in serum levels of creatinine and BUN suggest decreased glomerular filtration and were used as indicators of abnormal renal function (Figure 2). After administration of Stx2 plus LPS, creatinine levels were increased significantly compared with saline at 12 h and continued to rise with a maximal average concentration of 0.92 mg/dl at 72 h. Similarly, BUN levels were increased in mice that received Stx2 plus LPS in a time-dependent manner. BUN was elevated significantly at 8 h after injection and continued to increase until death, with a maximal average BUN concentration of 114.33 mg/dl by 72 h.

Stx2 Plus LPS Alters White Blood Cells
A CBC was performed on mice that received Stx2 plus LPS throughout the 72-h time course (Figure 3, A through D). Mice exhibited neutropenia, lymphocytopenia, and mild monocytosis. Rise in peripheral neutrophil levels was significant at 4 h and maximal at 8 h after Stx2 plus LPS injection, after which levels gradually returned to normal at 72 h (Figure 3A). Neutrophils expanded from 0.67 ± 0.17 to 2.09 ± 0.48 K/μl and increased from 12.4 ± 2.1% of the total white blood cell (WBC) population to 56.8 ± 8.9%. Consequently, the lymphocyte population decreased from 82.9 ± 4.7% of total WBC with saline injection to 37.7 ± 5.8% at 8 h after Stx2 plus LPS injection (Figure 3B). Similarly, total lymphocyte levels were minimal at 4 to 8 h after injection with a significant drop from 4.69 ± 0.92 to 1.30 ± 0.28 K/μl. Lymphocyte levels then gradually increased throughout the time course with a recovery at 72 h to approximately half the normal level. The monocye cell population expanded with respect to the other WBC populations as reflected by the increase in the percentage of monocytes of total WBC throughout the time course. Whereas the total number of monocytes was not altered significantly after Stx2 plus LPS injection, the percentage of monocytes increased from 3.17 ± 1.0% with saline injection to 5.92 ± 2.8% 8 h after Stx2 plus LPS injection (Figure 3C).

Blood smears from mice at various time points after Stx2 plus LPS administration demonstrated changes in WBC morphology. Figure 3F is a blood smear that was taken 24 h after Stx2 plus LPS injection and depicts a segmented neutrophil as well

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<tr>
<td></td>
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<td>Increased serum creatinine</td>
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<td>Weight loss</td>
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*Mice received an injection of 225 ng/kg Shiga toxin 2 (Stx2), 300 μg/kg LPS, or both and were evaluated for the conditions listed throughout the 72-h time course as described in Materials and Methods. A ‘+’ indicates relative severity of the condition, with each additional ‘+’ indicating greater change from normal. Temporally, changes that occurred from 0 to 12 h were designated ‘early,’ 12 to 36 h as ‘mid,’ and 36 to 72 h ‘late.’
as an activated monocyte. Monocytes and neutrophils increased in smears beginning at 12 h after injection and thereafter were present throughout the time course. Monocytes displayed evidence of activation, including increased cell size, linearized chromatin, granules, and pseudopod formation. Neutrophil activation was indicated by increased cell size, granules, and segmentation as well as hypersegmentation. Plasmacytoid lymphocytes also were present in blood smears.

**Stx2 Plus LPS Causes Signs of Hemolytic Anemia**

Although a CBC of Stx2 plus LPS mice during the 72-h time course showed moderately increased hematocrit and hemoglobin levels (data not shown), blood smears demonstrated signs of anemia and hemolysis. Hematocrit reached a plateau at 36 h after injection of 52.0 ± 2.6% compared with the control of 44.3 ± 4.3%. However, manual peripheral reticulocyte counts were increased at 12 h after Stx2 plus LPS injection and remained elevated throughout the time course (Figure 3, D and G). Reticulocytes are newly developed immature RBC that appear blue/purple and are a sign of anemia. It also was noted that serum that was collected from mice that received Stx2 plus LPS had a red discoloration, presumably from hemoglobin as a result of RBC hemolysis.

In addition, blood smears revealed several RBC morphologic abnormalities (Figure 3, G and H) compared with normal (Figure 3E). Howell-Jolly bodies are nuclear fragmentations of DNA that appear as small, round, blue structures in erythrocytes in anemic states. These were found in smears beginning at 8 h (Figure 3G, arrowhead), were more numerous at 12 h (Figure 3H, arrows), and continued to increase through 72 h after Stx2 plus LPS injection. Echinocytes are a morphologic change in which the RBC has uniform spikes or burrs on its surface, indicating uremia. At 24 h after Stx2 plus LPS injection, echinocytes were evident in blood smears.

**Stx2 Plus LPS Causes Thrombocytopenia in Mice**

Platelet levels significantly decreased in mice that were administered Stx2 plus LPS beginning at 4 h after injection and continued to decline through the time course (Figure 4A). The minimal platelet level was 392.7 ± 104.9 K/μl at 36 h after injection compared with 820.8 ± 134.6 K/μl with saline injection. In addition, platelet clumping in blood smears was most apparent at 72 h after Stx2 plus LPS injection. In the kidney, glomerular platelet aggregation was increased at 2 h after Stx2 plus LPS injection (Figure 4B). After a slight decline at 12 h, platelet clumping began to increase again until the end of the time course (Figure 4, B through D).

**Stx2 Plus LPS Causes Changes in the Mouse Kidney**

The MSB differential stain for RBC, fibrin, and collagen was performed on fixed mouse kidney tissue during the time course (Figure 5). In normal kidney tissue (Figure 5A), collagen was distributed throughout the inner tubules of the cortex, medulla, and papilla as indicated by light blue staining. There was no significant change in collagen levels after Stx2 plus LPS injection. RBC, stained yellow, were widely dispersed throughout the normal kidney in small numbers. There was a general increase in RBC in the entire kidney and RBC clustering in the cortex beginning at 4 h after Stx2 plus LPS injection (Figure 5B). RBC congestion in glomeruli, capillaries, and intertubular spaces continued to increase during the time course. Specifically, RBC began to accumulate in the medulla at 6 h after injection, glomerular RBC congestion was evident at 8 h after injection, and large areas of RBC congestion and clumping were evident throughout the cortex at 24, 48, and 72 h after injection.

Significant changes in fibrin staining were evident at 8 h after Stx2 plus LPS injection. Fibrin was increased in the cortex of the kidney in the intertubular spaces, glomeruli, and capillaries. It was dispersed throughout the papilla and medulla. Glomeruli that were positive for fibrin also increased at this time point from 23.3% positive at 0 h to 75.0% positive for fibrin at 8 h after injection. Fibrin continued to accumulate with maximal staining at 48 h after Stx2 plus LPS challenge (Figure 5C). At this time, the medulla and cortex showed a rise in the number and in

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**Figure 2.** Serum creatinine and blood urea nitrogen (BUN) analysis. Serum from mice that received Stx2 plus LPS was analyzed for creatinine (A) and BUN (B) concentration. Each circle represents one mouse, and data represent three separate experiments. Black bars are the average of all mouse samples. *P < 0.05 significantly increased over saline by ANOVA and t test.
the size of concentrated areas of fibrin deposition, and 93.3% of glomeruli were positive for fibrin. In addition, thrombi of RBC and fibrin were seen in the glomerular arterioles (Figure 5D).

Transmission electron microscopy of glomeruli from mice that received Stx2 plus LPS demonstrated significant ultrastructural change to the capillary loops, endothelial cells, and podocytes compared with controls (Figure 6). RBC congestion and electron-dense flocculent material, which likely is a combination of collected serum proteins and fibrin, was present from 24 to 72 h (Figure 6, B and C). Increasingly large extranuclear endothelial inclusions were observed beginning at 24 h and extending through 72 h after challenge (Figure 6B). These inclusions are bounded by two membranes and are anuclear, which suggests that they are remnants of endocytosed RBC, platelets, or other cellular debris. Furthermore, endothelial cell fenestrations were irregular and occasionally partially detached from the glomerular basement membrane (Figure 6C). Podocytes appeared swollen, diminishing the urinary space, and some contained extensive vacuoles of unknown significance (Figure 6D). None of the pathologic findings was observed in control mouse kidneys (Figure 6A).

**Stx2, LPS, and Stx2 Plus LPS Challenges Alter Renal Gene Expression**

Microarrays were used to analyze gene expression in the whole mouse kidney in response to Stx2, LPS, or Stx2 plus LPS.
challenges. Analysis of the data revealed 136, 737, and 722 significant differentially expressed genes for the Stx2, LPS, and Stx2 plus LPS challenges, respectively (Figure 7). These genes represented approximately 1% (Stx2) and 5% (LPS and Stx2 plus LPS) of the 14,000 genes that were included on the array. The Venn diagram in Figure 7 demonstrates that, although many of the genes whose expression was altered by each challenge were affected by the other challenges, there also was a distinct set of genes that were uniquely significant for each challenge. For example, of the 136 genes that were differentially expressed in response to Stx2 alone, 74 also were differentially expressed in response to LPS alone, 75 also in response to Stx2 plus LPS, and 57 also were differentially regulated by both LPS and Stx2 plus LPS whereas 44 were unique to Stx2 alone.

Stx2, LPS, and Combination Challenges Provoke Distinct Renal Expression Patterns

The Stx2, LPS, and Stx2 plus LPS challenges exhibited distinct temporal alterations in gene expression over the time course. Figure 8A is a plot of the number of genes whose transcription was altered, either increased or decreased, at each time point by each challenge. LPS affected a rapid, early change in gene expression, whereas Stx2 caused a later, gradual change. The differentially expressed genes from Stx2 plus LPS challenge created a pattern that shared the respective temporally early and late changes in gene expression from the individual agents. To discover classes of genes with similar expression patterns, we performed self-organizing map clustering, an unsupervised learning method, with the GeneCluster program. Within each challenge, genes were grouped together on the basis of the similarity of their expression patterns within the 72-h time course (Figure 8B). These groups were labeled on the basis of the time point of maximum expression change over the course of the pattern. Six major gene expression patterns were observed for LPS challenge. Genes whose maximum differential expression occurred at 2 to 4 h after injection were termed “immediate,” those at 6 to 8 h were termed “early,” and those at 12 to 24 h were termed “late.” In contrast, Stx2 challenge created only an upregulated and downregulated pattern, each reaching maximum differential expression at 48 to 72 h. These clusters therefore were termed “very late” to contextualize...
them in light of the LPS expression patterns. The Stx2 plus LPS challenge creates an arrangement of gene expression patterns that combines each of the Stx2 plus LPS patterns together (data not shown). Supplemental Table 1 contains a list of the most differentially expressed genes in each cluster from Figure 8B. Many of these gene products are upregulated in patients with HUS, most notably IL-6 and monocyte chemoattractant protein-1, thereby adding validity to the HUS mouse model (16,17).

Stx2 and LPS Alter Overlapping and Unique Groups of Genes

To discern the functions of the genes that were differentially expressed in response to challenge, we subjected each cluster that was described in Figure 8B to gene ontology analysis (Table 2). dChip categorizes genes on the basis of molecular function, biologic process, and cellular component using GeneOntology (http://www.geneontology.org/) terms and information from the National Center for Biotechnology Information LocusLink database and calculates a $P$ value for overrepresentation of that cluster in the gene set. The LPS-upregulated gene clusters contained significantly large alterations in genes that are involved in the following biologic processes: Inflammatory response, immune response, and regulation of transcription, with more minor changes of genes involved in apoptosis, cell differentiation, proliferation, and regulation of cell cycle. Molecular function analysis of the LPS-upregulated genes identified numerous cytokines, transcription factors, cell surface ligands, and complement components. In contrast, the gene biologic processes that are upregulated by Stx2 did not include the inflammatory and immune responses, although Stx2 did cause an upregulation of genes that are involved in cell proliferation, differentiation, and regulation of cell cycle and transcription. Molecular function analysis of the Stx2-upregulated genes included some cytokines and transcription factors but not any complement components. Downregulated by both challenges were solute and macromolecule transporters that are

Figure 5. Fibrin deposition and RBC congestion accumulates over time in mice that were administered Stx2 plus LPS. Martius Yellow-Brilliant Crystal Scarlet-Aniline Blue staining of mouse kidneys at 0 h (A), 4 h (B), 48 h (C), and 72 h (D) after injection (arrow indicates thrombi in arteriole). Bright red staining is fibrin deposition, RBC are stained yellow, nuclei are stained dark blue/purple, and collagen is stained blue. Magnifications: ×200 in A through C; ×400 in D.

Figure 6. Stx2 plus LPS administration causes glomerular ultrastructural changes in mice. Representative transmission electron micrographs of mouse glomeruli from control (A), and 72 h after challenge (B through D). (B) Endothelial cell with arrows indicating extranuclear inclusions. (C) Glomerular capillary loop with endothelial detachment (arrow). (D) Abnormal podocyte with cytoplasmic vacuolation (arrows). Bars = 2 μm. E, endothelial cell; P, podocyte; R, RBC. Magnifications: ×5000 in A; ×10,000 in B through D.

Figure 7. Venn diagram of genes that were altered 2.0-fold or greater by each challenge with $q < 5\%$ in multiclass ANOVA analysis. The total genes used from each challenge are outside the circles. Each challenge alters both an overlapping set of genes and a distinct gene set, compared with the other challenges.
necessary for normal renal function. The Stx2 plus LPS challenge altered the combined functional groups from each of the individual challenges (data not shown).

Discussion

This Stx2 plus LPS mouse model of HUS recapitulates the human disease in both its signs and symptoms, including the clinical diagnostic triad of renal failure, thrombocytopenia, and hemolytic anemia (1–3). Increased serum creatinine and BUN in these mice demonstrate kidney dysfunction. Low platelet counts that were found in our model are the definition of thrombocytopenia. Reticulocytes and Howell-Jolly bodies that were found in the blood smears, an increase in the percentage of reticulocytes, and red discoloration of the serum that was caused by free hemoglobin are evidence of hemolytic anemia. Although no schistocytes were seen in the blood smears, we found that, for unknown reasons, in published mouse models of hemolytic anemia, schistocytes are an uncommon finding (18–20). The mice also exhibited the neutrophilia and monocytosis that is found clinically (21), and fibrin and red cell staining of kidneys show thrombus formation in the microvasculature, as seen in patients with HUS (22). Furthermore, electron microscopy showed ultrastructural changes in the glomeruli that are consistent with HUS. As indicated in Table 1, neither Stx2 nor LPS alone was able to mimic HUS in the mouse; however, injection of both agents together elicited the diagnostic triad as well as the other associated clinical signs. This reinforces evidence from human patients that both Stx and LPS are involved in the development of HUS (6,23).

Microarray expression analysis reinforces the mouse as a model for human disease and provides new insight into the details of HUS pathogenesis. Specific gene products that are known to be increased in patients with HUS are upregulated in these mice, such as IL-6 and monocyte chemoattractant protein-1, and these were shown to be part of a general inflammatory response by evaluation of gene expression clusters. Furthermore, global expression analysis demonstrates that there are temporal waves of transcriptional response for distinct types of genes. A closer look at the specific differentially expressed genes allows formation of multiple testable hypotheses that should be useful to the scientific community, such as that the formation of the fibrin-rich clots that are typical of HUS may be heavily affected by the upregulation of all three types of fibrinogen in the kidney (Supplemental Table 1). Although these gene clusters were formed in an unbiased manner by grouping similar expression patterns, it is noteworthy that the quoted classifications of these clusters do not necessarily describe the complete functional potential of these genes. Nevertheless, the classifications cited in Table 2 do provide insight into what a transcript or group of transcripts does. It also is important to note that the numbers of genes that are altered by each challenge does not reflect the importance of those genes or of the challenge. For instance, even though Stx2 alters fewer transcripts than LPS in this model (Figure 7), much of the HUS pathology in Table 1 can be attributed to Stx2. In addition, this leads to the hypothesis that pharmacologic or other alteration of relatively few gene products may be able to alter significantly the course of this disease. Overall, the gene expression analysis should serve as a starting point for numerous avenues of future research.

This model may be complicated by dehydration of the mice, a consequence that does not occur in the human disease (2). Although patients with HUS normally have decreased hematocrit, mice that receive Stx2 plus LPS display moderately increased hematocrit and hemoglobin. In the mouse, this likely is hemoconcentration as a result of dehydration, as suggested by the weight loss that is sustained after injection (Figure 1). We contend that all of our significant findings are evidence of HUS as caused by Stx2 plus LPS and are distinct from the vascular volume depletion. Specifically, even though the mice that are challenged with LPS alone lose as much initial weight as the
Stx2 plus LPS mice, they exhibit no increased serum creatinine and no signs of hemolytic anemia (Table 1). Furthermore, the mice that are given Stx2 alone develop increased serum creatinine and reticulocytosis at 4 to 12 h after injection but do not lose weight until 48 h after injection. Because LPS induces early weight loss without a rise in creatinine and Stx2 causes a rise in creatinine 36 h before weight loss, we conclude that the dehydration that results from combinatorial challenge with Stx2 and LPS is distinct from the signs of HUS.

On the basis of our model, we propose a time course of disease progression in the mouse. Two hours after Stx2 plus LPS injection, platelet levels rise in the kidney followed by peripheral thrombocytopenia that persists throughout the time course. After the rise in renal platelets, RBC infiltrate the kidney, form clumps, and congest the glomeruli and vasculature of the kidney. Fibrin deposition follows RBC infiltration and leads to thrombus formation in the renal microvasculature. This thrombosis likely causes glomerular filtration failure, uremia, and eventual death of the mouse. During this time course, we also ascertained the progression of gene expression in the affected kidney tissue. Inflammatory signaling that is induced primarily by LPS and cellular damage and repair pathways that are induced by both Stx2 and LPS are activated in these model HUS kidneys. Furthermore, the downregulation of genes that are necessary for normal renal function is evidence for activation of a de-differentiation and repair pathway that has been described in response to other renal insults (24). Modulation of upregulated genes that are involved in cell proliferation, apoptosis, the cell cycle, and repair could be able to alter this disease course. This precise establishment of the physiologic and molecular progression of HUS in the mouse model will allow identification of novel therapeutic strategies to treat this disease.

Although other animal models have been reported, this mouse model of HUS is the most economical, practical, and complete model of HUS so far described. Although the baboon (25) and canine (26) models of HUS mimic the human disease, these large animal models are expensive and impractical for the common researcher. Other rodent models of HUS have been described (27–32), but none has completely investigated the full pathophysiology of the disease. Mouse models using oral bacterial inoculation require antibiotic pretreatment and do not

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Table 2. Significant gene ontology clusters as identified by dChip for each gene expression cluster described in Figure 8

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<td>chemokine activity</td>
<td>9(^b)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>cytokine activity</td>
<td>13(^b)</td>
<td>9(^b)</td>
<td>1</td>
</tr>
<tr>
<td>DNA binding activity</td>
<td>29(^b)</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>receptor binding activity</td>
<td>15(^b)</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>transcription regulator activity</td>
<td>12</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>transporter activity</td>
<td>2</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^{a}\)LPS and Stx induce both distinct and overlapping gene expression responses in the mouse kidney. LPS induces a large inflammatory and defense response that is not affected by Stx2, whereas they share many other aspects, including increased apoptosis and proliferation, cell-cycle regulation, and transcription factor changes.

\(^{b}\)P < 0.05 as described in Materials and Methods.
result in a 100% rate of infection (33). Hence, these models can be inefficient and impractical. In addition, HUS is a toxemia and not a bacteremia; therefore, a model does not require live bacteria (2). In summary, we have shown that concurrent intraperitoneal injection of both Stx2 and LPS is sufficient to reproduce HUS in the C57BL/6 mouse. The data indicate this offer a reproducible model with which to study HUS and identify potential therapeutic targets and for testing of new therapies.

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