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Shiga toxins and damage-associated molecular patterns leading to endothelial dysfunction

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SHIGA TOXINS AND DAMAGE-ASSOCIATED MOLECULAR PATTERNS
LEADING TO ENDOTHELIAL DYSFUNCTION

by

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DEDICATION

I would like to dedicate this work to my patient spouse Whitney, to my father Richard, my mother Judith, my sister Soliz, and to the rest of my family and friends for all the support they have given me throughout my life, and for enabling me to take advantage of this opportunity.
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SHIGA TOXINS AND DAMAGE-ASSOCIATED MOLECULAR PATTERNS
LEADING TO ENDOTHELIAL DYSFUNCTION

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ABSTRACT

Enterohemorrhagic *E. coli* (EHEC) infection is a leading cause of acute kidney failure in otherwise healthy children, and a leading cause of foodborne illness with an outsized economic impact from outbreaks. EHEC secrete two Shiga-like toxins (Stx1 and Stx2) which are AB₅ holotoxins that inhibit protein synthesis in cells expressing the toxin receptor Gb₃. Infection with EHEC typically begins with a diarrheal prodrome that can progress in 5-15% of cases to hemolytic uremic syndrome (HUS), a clinical diagnosis characterized by thrombocytopenia, hemolytic anemia, and thrombotic microangiopathy. Historically, strains of EHEC expressing Stx2 have been associated with more severe disease. We hypothesized that tissue injury due to the toxins leads to the release of damage-associated molecular patterns (DAMPs), which act through inflammatory receptors to promote the endothelial dysfunction that drives this disease alongside the inciting Shiga toxins. Here we demonstrate that two well-characterized DAMPs,
extracellular histones and HMGB1, are produced in two different mouse models when Stx2 is present; one model represents challenge with the toxin alone, and the second model introduces toxin through secretion with a lysogenized bacterium, \textit{C. rodentium}, mimicking EHEC colonization. We investigate whether Stx1, Stx2, or histones affect the endothelial expression of well-characterized members of the protein C pathway, namely the endothelial protein C receptor (ECPR), protease-activated receptor 1 (PAR1), and thrombomodulin (TM), on human aortic (HAEC) and human renal glomerular endothelial cells (HRGEC). We show that Stx and/or histones reduce endothelial expression of these anti-coagulant molecules and histones dramatically increase expression of pro-thrombotic PAR-1. These changes lead to physiologically important decreases in activated protein C (APC), a critical anti-coagulant and cytoprotective molecule. Finally, we demonstrate that histones exacerbate thrombin’s barrier-disruptive effects on the endothelium, and prevent APC’s protective effects. These data provide novel mechanistic insight into the endothelial dysfunction that characterizes HUS and also provide a new perspective on systemic consequences of the bacterial Shiga toxins that might drive organ injury in susceptible patients.
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LIST OF ABBREVIATIONS

Ab ........................................................................................................................... Antibody
A/E .......................................................................................................... Attaching/Effacing
APC ........................................................................................................ Activated Protein C
DAMPs ................................................................. Damage Associated Molecular Patterns
D+HUS ....................................................... Diarrhea positive Hemolytic Uremic Syndrom
EDTA ................................................................................ Ethylenediaminetetraacetic Acid
ELISA ................................................................. Enzyme-Linked Immunosorbent Assay
EHEC ................................................................. Enterohemorrhagic Escherichia coli
EPCR .................................................................................. Endothelial Protein C Receptor
G ........................................................................................................................ gram
Gb3 ..................................................................................................... globotriaosylceramide
HAEC ................................................................. Human Aortic Endothelial Cells
HBSS ........................................................................................ Hanks Buffered Salt Solution
H/His/Hist ........................................................................................ Histones
HRGEC ................................................................. Human Renal Glomerular Endothelial Cells
HUS ........................................................................................ Hemolytic Uremic Syndrome
LAL ..................................................................................................... Limulus Amebocyte Lysate
Med ................................................................................................................ Media
mL ................................................................................................................ milliliter
ng ................................................................................................................ nanogram
nm ................................................................................................................ nanometer
PAMPs ............................................................... Pathogen Associated Molecular Patterns
PAR1 ............................................................ Protease-Activated Receptor 1
PBS ............................................................... Phosphate Buffered Saline
RNA ............................................................. Ribonucleic Acid
Stx1 .............................................................. Shiga Toxin 1
Stx2 .............................................................. Shiga Toxin 2
TM ............................................................... Thrombomodulin
CHAPTER ONE: BACKGROUND AND INTRODUCTION

**Enterohemorrhagic E. coli and HUS**

Enterohemorrhagic *Escherichia coli* are enteropathogens that cause foodborne illness and hemorrhagic colitis. In 5-15% of patients disease can progress to a potentially life-threatening syndrome known as diarrhea-associated hemolytic uremic syndrome (D+HUS), characterized by thrombotic microangiopathy, thrombocytopenia, and hemolytic anemia, all of which contribute to acute kidney injury (1). In the United States, D+HUS is a leading cause of acute kidney failure in otherwise healthy children (2). EHEC produce and secrete Shiga toxin 1 (Stx1), Shiga toxin 2 (Stx2), or both, with serotypes that secrete Stx2 historically being associated with more clinically severe disease (3). These toxins are primary virulence factors of EHEC, and much of the pathogenesis observed during EHEC infection is attributed to them. The toxins are secreted using a type III secretion system (4, 5) after the bacteria have formed attaching/effacing (A/E) lesions in the gut. After the toxins reach cells that express their receptor (globotriaosylceramide [Gb3]) such as renal tubular epithelial cells and endothelial cells, the toxins bind and are internalized. Inside the cell, the toxin molecule is transported to the endoplasmic reticulum, where the A subunit is cleaved by furin (6) to generate catalytic activity. The active A1 subunit, which has RNA N-glycosidase activity, then is transported to the cytosol, where it cleaves an adenine
residue from 28S ribosomal RNA, preventing protein synthesis (7-9) and initiating the ribotoxic (10, 11) and endoplasmic reticular-stress responses (12) that lead to apoptosis and inflammation (13). How these toxins cause D+HUS, a syndrome driven by thrombotic microangiopathy, is a process that remains to be fully explained. A full understanding of the mechanism is crucial for development of novel therapies.

The Endothelial Protein C Pathway

Shiga toxin receptors are located on the endothelium, and endothelial dysfunction characterizes D+HUS. Endothelial cells are crucial to maintaining blood in a fluid state and preventing leakage through the vessel walls, and they do this through a variety of pathways. The protein C pathway is a critical one involved in endothelial cell anticoagulant function, and is composed of receptors and membrane proteins located on the apical surface of endothelial cells. The pathway accomplishes its role in anticoagulation by the generation of activated protein C (APC) by the thrombin/thrombomodulin complex. This activation is further facilitated by the endothelial protein C receptor (EPCR) (14, 15).

In addition to anticoagulant properties, the endothelium is critical as a barrier lining the entire vasculature, and the protein C pathway plays a role here as well. The barrier-protective effects of APC are mediated by the occupation of EPCR and by cleavage of the thrombin receptor, protease-activated receptor 1 (PAR1),
through which APC elicits an opposite and barrier-protective effect to that of thrombin (16). PAR1 is a receptor intimately involved in endothelial barrier function, coagulation, and platelet activation, and mechanisms behind this receptor's differential signaling depend on whether it undergoes activation by APC, thrombin, or other proteases (16, 17), and whether EPCR is occupied during this cleavage. Dysfunction in the endothelial protein C pathway could lead to the thrombotic microangiopathy and tissue damage that characterizes HUS.

**Damage-Associated Molecular Patterns**

A relatively new paradigm in inflammation and disease is the role of damage-associated molecular patterns (DAMPs) (18). The release of DAMPs from damaged tissue has been well documented in many models of sepsis, injury, and trauma (19-23). DAMPs are normal endogenous molecules that, when extruded from the cell into the blood or tissue either as a defense mechanism (histones in NETs (24), HMGB1 from monocytes (25)) or as a result of apoptosis or necrosis, can activate the same receptors as pathogen-associated molecular patterns (PAMPs) (21, 26), propagating inflammation and tissue damage. DAMPs have already been shown to cause endothelial dysfunction manifested by increased permeability (27) or increased platelet adhesion (24). If DAMPs are produced in D+HUS it is possible that they contribute to the microangiopathy in the small vessels that drives disease. D+HUS patients and non-human primate models of Stx-induced HUS show extensive
tissue damage after toxin challenge (4, 28, 29) and due to this observed tissue
damage, we hypothesized that Shiga toxins produce DAMPs that together with the
toxins compromise the antithrombotic and barrier protective properties of
endothelial cells to contribute to the pathogenesis in EHEC infection.
CHAPTER TWO: MATERIALS AND METHODS

Reagents

Plasma levels of HMGB1 and extracellular histones were measured using ELISAs for HMGB1 (IBL-international, Toronto, ON, and Chondrex Inc., Redmond, WA) and cell-death detection (Roche, Indianapolis, IN). Antibodies were purchased against EPCR (JRK 1494, both unconjugated and conjugated to Alexa 488), PAR1 (ATAP2, Santa Cruz Biotechnology, Dallas, TX, un/conjugated to Alexa 488), and thrombomodulin (1029, conjugated to Oregon green, for flow cytometry, and clone 1A4 for on-cell western; Becton Dickinson, Franklin Lakes, NJ). Goat anti-mouse IRDye 800CW for use in on-cell westerns was purchased from Li-COR (Lincoln, NE). Stx1 and Stx2 were purchased from Tufts University and endotoxin was removed by incubation with polymyxin B-agarose (Sigma Aldrich, St. Louis, MO). Residual endotoxin levels were < 0.015ng/mL (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, Rockford, IL). Anti-histone IgG clone BWA3 (30) was kindly provided by Dr. Ann Rothstein (Department of Immunology and Virology, University of Massachusetts Medical School, Worcester, MA). Calf thymus histones, TNFα, and polymyxin B-agarose were purchased from Sigma Aldrich. The QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) was used to determine mouse BUN. Mouse Cystatin-C duoset (R&D Systems, Minneapolis, MN) was used to determine plasma levels of Cystatin-C according to the manufacturer’s instructions.
Mice

Mice for all experiments were purchased from the Jackson Laboratories (Bar Harbor, ME). 6-week old male C57BL6/J mice were used for the Stx2 alone model (n=70) (see Chapter 3), while 6-week old C57BL6/J females were used for the *Citrobacter rodentium* bacterial model (n=30) (Chapter 4). Mice were acclimated to living conditions prior to the start of each experiment and given normal chow and water *ad libitum*. Criteria for euthanasia included huddled posture, shaking, ruffled fur, audible distress, and weight below 85% of starting weight. Euthanasia was performed by isoflurane anesthesia followed by cervical dislocation and pneumothorax.

Mouse model of Stx2 toxemia

All animal studies were approved by the Boston University Institutional Animal Care and Use Committee. Mice were injected intraperitoneally with 1~1.2ng Stx2 per 20g bodyweight on day 0 and 3. Control mice received equally proportional volumes of normal saline by intraperitoneal injection. Blood was collected prior to challenge and periodically until euthanasia for downstream analyses. Animals were monitored and weighed daily and blood urea nitrogen (BUN) was calculated using a urea assay kit (BioAssay Systems) and used as a marker of acute kidney injury.
**Mouse model of EHEC infection**

Mice were inoculated with *Citrobacter rodentium*, a Gram-negative mouse pathogen lysogenized with an Stx2 phage (Strain DBS770) for toxin expression, or wild-type does not express the toxin (strain DBS771). *C.rodentium* strains were kindly provided by Dr. John M. Leong (Department of Molecular Biology and Microbiology, Tufts University Medical Center, Boston, MA) (31). Briefly, DBS770 bacteria were grown in LB broth containing chloramphenicol (10mg/mL), and DBS771 bacteria were grown in LB broth containing both chloramphenicol and kanamycin (25ug/mL) to an OD$_{600}$ of 0.75-0.90, corresponding to ~1x10$^{10}$ CFU/mL (by standard curve). Bacteria were washed, re-suspended in sterile saline and mice were challenged with 1x10$^9$ CFU by oral gavage on Day 0. Dosing concentration was confirmed by standard plating methods. Weight was monitored daily and plasma collected by facial vein bleed periodically. CFU/g feces was determined by mixing feces with 10 volumes PBS, homogenizing with a sterile toothpick, centrifuging 30 seconds at 1500 x g, and plating serially diluted supernatants on LB agar with appropriate restrictive antibiotics. Plates were grown overnight at 32$^\circ$C and colonies were counted the next day.
**TLR2-Knockout Mice**

TLR2-knockout (TLR2-KO) mice (n=4) were a gift from the laboratory of Dr. Lee Wetzler (Department of Microbiology, Boston University School of Medicine) and were generated by targeting insertion of a neo-cassette into the exon of TLR2 using mouse ES cells from the C57BL6 background. Cells transfected with the targeting vector were grown in the presence of neomycin and ganciclovir. Cells that grew under restrictive conditions were microinjected into C57BL6 blastocysts and heterozygous offspring were crossed to produce homozygous TLR2-deficient mice (32).

**Cell culture**

Human aortic endothelial cells (Cascade Biologics, Grand Island, NY) or human renal glomerular endothelial cells (Sciencell, Carlsbad, CA) were purchased and grown in endothelial cell medium (Sciencell) supplemented with 5% fetal bovine serum, 100U/mL penicillin, 100µg/mL streptomycin, with endothelial cell growth supplements according to the manufacturer’s instructions. Culture and experiments were performed on plasticware coated with 0.1% gelatin (Sigma). All experiments were performed between passage 2 and 6.
**BWA3 Production**

Hybridomas producing anti-histone antibodies (clone BWA3) were kindly provided by the Rothstein Lab. Hybridomas were grown in RPMI, 10% FBS, 10mM HEPES, 0.05mM $\beta$-mercaptoethanol, 1% penicillin/streptomycin, 2mM L-glutamine, 0.22$\mu$m-filtered, until viability was >90%, and then gradually transitioned to SFM4MAb (HyClone) serum-free production medium over 5 days, increasing the percentage of serum-free medium by 20% each day. Once cells were in 100% serum-free medium, they were allowed to culture without media changes until viability had dropped to <10% by trypan-blue staining. At this point cells were pelleted by centrifugation at 1000 rpm for 20 minutes and clarified serum-free media was buffered with 10mM HEPES, pH 7.4 before being filtered through a 0.45 micron filter and frozen for later purification.

**BWA3 Purification**

To purify the antibody, a 1mL HiTrap Protein G column (GE Healthcare) was washed with 7 column volumes of PBS before serum-free media containing BWA3 was loaded onto the column at a flow rate of 0.5mL/min. After sample had been loaded, the column was again washed with 7-10 column volumes of PBS, and sample was eluted using 30mM glycine, pH 3.0, collecting 1mL fractions into tubes containing 100uL Tris-HCl, pH 9.0. A Nanodrop spectrophotometer (ThermoScientific) was used to check the absorbance of each sample at 280nm to
ensure that no fraction exceeded 10mg/mL of protein, and then high-yield fractions were pooled and dialyzed in 3500 MWCO dialysis tubing (Pierce) against 100mM NaCl and 10mM HEPES. 3 changes of dialysis buffer >6 hours were performed, and the final samples were filtered through a 0.22 micron syringe filter into sterile 1.5mL microcentrifuge tubes before being stored at 4°C.

Flow cytometry

Human aortic endothelial cells between passage 2 and 6 were seeded on 0.1% gelatin-coated plates at 1.5x10^4 cells/cm^2. The next day media was replaced with fresh media containing Stx1 or Stx2 (100ng/mL), extracellular histones (50ug/mL), or combinations. Cells were incubated for 24 hours, washed with PBS (ThermoScientific), then detached using Versene (Invitrogen) and passed through a 40µm nylon mesh to ensure a single-cell suspension. All subsequent steps were done on ice. Cells were washed in PBS with 25mm HEPES, 0.5% BSA, and 5mM EDTA, pH 7.4 (flow buffer) before staining with primary conjugated antibodies (2ug/mL) for 30 minutes. Cells were washed again and re-suspended in flow buffer prior to acquisition. Dead cells were excluded during acquisition by propidium iodide staining and gating. Cell associated fluorescence was determined using a FACSCalibur flow cytometer (Becton Dickinson) and data analyzed using FlowJo X (Treestar Inc., Ashland, OR) software. One-way ANOVA with a Dunnett posttest was used to determine significance with significance for α set at p<0.05.
On-cell Western

Black clear-bottomed 96-well tissue culture plates (Costar) were coated with 0.1% gelatin and then seeded with human renal glomerular endothelial cells (Sciencell) grown in endothelial cell media. When cells were near confluent, media was replaced and challenges were added. After 24 hours, cells were washed once with PBS then fixed for 20 minutes at room temperature in PBS with 3.7% formaldehyde. After fixation, cells were washed twice with TBST (50mM Tris, pH 7.4, 150mM NaCl, 0.05% Tween-20) and blocked with Odyssey blocking buffer (Li-Cor) at room temperature with gentle shaking for 1.5 hours. Following blocking, cells were washed twice with TBST and incubated with primary unconjugated antibodies for EPCR (JRK 1494), PAR1 (ATAP2), or thrombomodulin (1A4) at 2µg/mL in Odyssey blocking buffer (50uL for 2.5 hours). Cells were washed twice with TBST and incubated with goat anti-mouse IRDye 800CW (Li-Cor) diluted 1:800 in Odyssey blocking buffer and Draq5 (5mM stock, Thermo Scientific) diluted 1:10,000 for 1 hour at room temperature. Plates were washed with TBST, and all liquid aspirated before reading fluorescent values on a Li-Cor Odyssey dual-channel infrared imager at 700 and 800nm. Images were analyzed with Odyssey 2.1 software (LiCor), and fluorescent intensities at 800nm were normalized to cell number based on Draq5 staining at 700nm. Data was analyzed using GraphPad Prism 5.01 and one-way ANOVA with a Dunnett posttest was used to determine significance.
Protein C activation assay

Confluent human aortic endothelial cells (HAEC) were incubated with Stx1 (100ng/mL), Stx2 (100ng/mL), calf thymus histones (50µg/mL), or combinations. Confluent human renal glomerular endothelial cells (HRGEC) were challenged with Stx1 (50ng/mL), Stx2 (100ng/mL), or histones (50µg/mL). For experiments with BWA3, endothelial cells were challenged with 30µg/mL histones with or without BWA3 at 20µg/mL. All challenges were done for 24 hours at 37ºC in 5% CO2. Cells were washed with Hank’s balanced salt solution (HBSS; Thermo) and protein C (a kind gift of Kaketsuken, Tokyo, Japan) was added at a final concentration of 0.4µM in HBSS, 0.1 mM HEPES, pH 7.4, 0.6mM MgCl₂, and 1mM CaCl₂. Human α-thrombin (10nM final, Haematologic Technologies, Inc.) was added and the reaction was stopped after 30 minutes at 37ºC with HBSS, 0.1mM HEPES, 0.5mg/mL human ATIII, and 1.4U/mL heparin. Spectrozyme PCa (American Diagnostica, Lexington, MA) was added (0.2µM final) and the change in absorbance 405nm with time was performed on a VersaMax ELISA Microplate Reader and analyzed with SoftMax Pro 5.4 (Molecular Devices, Sunnyvale, CA). Activated protein C levels were determined by reference to a standard curve prepared with purified activated protein C generated from plasma-derived protein C (Kaketsuen, Tokyo, Japan). Cell numbers were determined using Draq5 staining of a parallel plate. One-way ANOVA with a Dunnett posttest was used to determine significance comparing values to those
generated by cells incubated with media alone.

**Trans-Epithelial Electrical Resistance Assay**

Changes in endothelial cell monolayer permeability was done by quantifying changes in electrical resistance across a human aortic endothelial monolayer were monitored by electric cell-substrate impedance sensing using an ECIS® ZΘ array station (Applied Biophysics, Troy, NY). For each experiment, two 40 electrode/well 8W10E+ electrode chamber slides (Applied Biophysics) were coated with 10mM L-cysteine per well for 10 min, washed 2 times with HBSS, coated with 5μM fibronectin in HBSS for 10min, then washed 2 times more with HBSS and allowed to dry. HAEC between passages 2 and 6 were seeded at a density of 2.5x10^5 cells per chamber and allowed to adhere and form a monolayer (30-45 minutes) as determined by the stabilizing capacitance at 64kHz. For experiments with pre-treatments, histones (25μg/mL) were added to the appropriate wells 12 hours before thrombin addition. Thrombin was added to 1U/mL and the experiment was followed for at least 12 hours after thrombin addition. For experiments using activated protein C, concentrations of thrombin were lowered to 2nM in order for the protective effect of APC on PAR1 to be observed (16). Pre-incubation with activated protein C (10nM) was done for 3 hours prior to thrombin challenge.
CHAPTER THREE: STX2-TOXEMIA MOUSE MODEL

To begin answering the question of whether Shiga toxin challenge leads to DAMP production *in vivo*, we developed a mouse model of Stx2 toxemia. Briefly, 6-week old male C57BL6/J mice were purchased from Jackson Labs and challenged with Stx2 by peritoneal injection. Endotoxin contamination of Stx2 was determined to be <0.015ng/mL by LAL assay. Mice were monitored and weighed daily, with blood being collected by periodic facial vein bleed on day 0 prior to Stx2 injection, on day 2, on day 3 before performing Stx2 injection, and at day 5 or when mice had reached euthanasia criteria. We tested for the presence of DAMPs by using ELISA kits designed to detect extracellular histones or HMGB1 in the plasma of mice. The mice challenged with Stx2 exhibited decreased survival (Fig 1) when compared to saline controls. Mice challenged with Stx2 also demonstrated increased weight loss (Fig 2), with a significant drop in weight by day 2 when compared to saline controls. Finally, Stx2-challenged mice exhibited kidney injury as demonstrated by increasing BUN compared to saline controls, and this was significant on day 4 of the experiment (Fig 3A). Cystatin-C levels were also determined (Fig 3B), but as these appeared to follow the same general trend as BUN, the assay was discontinued and BUN used alone as a plasma marker of kidney injury. These changes in survival, weight, and BUN were consistent in direction with those expected based on prior models using Stx2 alone (33).
We next wanted to ascertain whether Stx2 challenge had led to the generation of DAMPs in the mice, and we quantified two well known DAMPs, extracellular histones and HMGB1, in the plasma of the mice using ELISA. We observed that in the plasma of mice challenged with Stx2, extracellular histones rose significantly on day 3 when compared to day 0 values (Fig 4A), and that plasma HGMB1 rose significantly as early as day 2 when compared to day 0 values (Fig 4B).

Having demonstrated that Stx2 challenge did lead to elevated plasma histones in our Stx2-toxemia model, we decided to test whether an anti-DAMP therapeutic might alter outcomes and plasma DAMP values in the model. We decided to target extracellular histones, and in order to target histones we treated the mice with a single bolus of 400 ug of an anti-histone mouse IgG (clone BWA3) on day 0 prior to injection of Stx2. Mice given BWA3 prior to Stx2 injection demonstrated no change in survival (Fig 5) or weight loss (Fig 6) but demonstrated a rise in BUN that appeared to exceed that of animals given Stx2 alone, although any differences were not statistically significant (Fig 7). Using ELISA to quantify levels of extracellular histones and HMGB1 in the plasma of mice given BWA3 prior to Stx2 challenge demonstrated that the antibody treatment succeeded in preventing significant rises in histones (Fig 8A) or HMGB1 (Fig 8B) compared to day 0.

There are many DAMPs, many with overlapping receptors on the endothelium. In order to begin testing whether targeting the DAMP receptors might be a better approach than single-DAMP therapeutics, we acquired 4 mice with targeted mutations of TLR2 that deleted this DAMP receptor, and challenged them
with Stx2 as before. TLR2-KO mice demonstrated no significant difference in survival (Fig 9), but they were significantly different in weight from wild-type C57BL6/J mice challenged with Stx2 on both day 3 and 4 (Fig 10). Finally, plasma BUN of TLR2-KO mice challenged with Stx2 was significantly higher than wild-type C57BL6/J mice on day 5 (Fig 11). Levels of plasma DAMPs were not determined in these animals.

In conclusion, in a mouse model of Stx2-toxemia essentially free of LPS contamination, we have demonstrated that challenge with Stx2 is sufficient to cause decreased survival, weight loss, kidney injury, and increased plasma DAMPs, and we have shown that an anti-DAMP (anti-histone) IgG therapeutic antibody can lower plasma DAMP levels but does not change outcomes. In addition, a mouse that does not express TLR2 also shows no difference in outcome measures, except for slightly delayed weight loss. Thus in this mouse model of Stx2-toxemia, DAMPs are elicited by toxin challenge but do not appear to play a significant role in pathogenesis.
Figure 1: Stx2 Mouse Model Survival
**Figure 1:** Mice (6 week old C57BL6/J, n=45) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g body weight on day 0 and 3 by intraperitoneal injection (dashed line). Control mice received vehicle alone (solid line, n=10). Injection with Stx2 caused significantly decreased survival when compared to saline control. Statistical significance was determined by Log-rank test of Kaplan-Meier curves. ***p<0.001
Figure 2: Stx2 Model Weight Loss
**Figure 2:** Mice (6 week old C57BL6/J, n=48) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (■). Control mice (n=12) received vehicle alone (●). Injection with Stx2 caused significantly decreased weight as early as day 2 when compared to saline control. Significance was determined by 2-way ANOVA with a Bonferroni post-test. **p<0.01, ***p<0.001**
Figure 3: Stx2 Model Rise in BUN and Cystatin-C
Figure 3: Mice (6 week old C57BL6/J, n=18) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (■). Control mice (n=11) received vehicle alone (●). Injection with Stx2 caused significantly increased plasma BUN by day 4 when compared to saline control. Cystatin C levels were also determined for Stx2 challenged mice (n=10 on days 0 and 3) and mice given vehicle control (n=5). Significance for panel (A) was determined by 2-way ANOVA with a Bonferroni post-test, and significance was determined for day 0 and 3 in panel (B) by 2-way ANOVA with a Bonferroni post-test. ***p<0.001
Figure 4: Stx2 Mouse Model DAMPs
**Figure 4:** DAMP levels in plasma collected from mice challenged with Stx2 were quantified using ELISAs. Extracellular histones rose significantly in mice (n=24) challenged with Stx2 by day 3 (A), while plasma HMGB1 rose significantly (n=13) on day 2 (B). Statistical significance was determined by 1-way ANOVA with a Dunnett post-test. *p<0.05, **p<0.01
Figure 5: Stx2 Model Survival with BWA3
**Figure 5:** Mice (6 week old C57BL6/J, n=45) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (long dashed line). Control mice (n=10) received vehicle alone (solid line). A third group of mice received a single bolus injection of 400ug anti-histone IgG (clone BWA3, short dashes) (n=5) suspended in normal saline on day 0 prior to injection with Stx2. Injection with BWA3 caused no significant change in survival when compared to mice challenged with Stx2 alone, and mice given BWA3 still exhibited survival significantly different from saline controls. Statistical significance was determined by Log-rank test on Kaplan-Meier curves. ***p<0.001
Figure 6: Stx2 Model Weight Loss with BWA3
Figure 6: Mice (6 week old C57BL6/J, n=48) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (●). Control mice (n=12) received vehicle alone (■). A third group of mice received a single bolus injection of 400ug anti-histone IgG (clone BWA3, ▲) (n=5) suspended in normal saline on day 0 prior to injection with Stx2. Injection with BWA3 did not prevent a significant loss in weight when compared to mice challenged with Stx2 alone, and mice given BWA3 still exhibited weight loss that was significantly different from saline controls but not from mice given Stx2 alone. Statistical significance was determined by 2-way ANOVA with a Bonferroni post-test. **p<0.01, ***p<0.001
Figure 7: Stx2 Model Rise in BUN with BWA3
Figure 7: Mice (6 week old C57BL6/J, n=18) were challenged with 1-1.2ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (●). Control mice (n=11) received vehicle alone (■). A third group of mice received a single bolus injection of 400ug anti-histone IgG (clone BWA3, ▲) (n=5) suspended in normal saline on day 0 prior to injection with Stx2. Injection with BWA3 did not prevent a significant rise in BUN when compared to mice challenged with Stx2 alone, and mice given BWA3 appeared to show higher levels of BUN compared to Stx2 and saline challenged mice, although they were not statistically significant. Statistical significance was determined by 2-way ANOVA with a Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
Figure 8: Stx2 Model DAMP Levels with BWA3
Figure 8: DAMP levels in plasma collected from mice challenged with Stx2 after being injected with a bolus of anti-histone IgG (clone BWA3) were quantified using ELISAs. Extracellular histones (n=5) showed no significant rise in mouse plasma compared to day 0 values (A). Similarly, plasma values of HMGB1 (n=4) showed no significant difference when compared to day 0 values (B). Statistical significance was determined by 1-way ANOVA with a Dunnett post-test.
Figure 9: Survival of TLR2 KO Mice Given Stx2
Figure 9: 6-week old female mice with a knockout mutation in TLR2 (n=4) were challenged with 1ng Stx2 per 20g bodyweight on day 0 and 3. Compared to saline challenged wild-type mice (n=10; solid line), both TLR2-knockout mice (dashed line) and wild-type mice given Stx2 (dotted line, n=45) exhibited significantly decreased survival; however, TLR2-knockout mice showed no significant difference in survival compared with wild-type mice given Stx2 alone. Statistical significance was determined by Log-rank test of Kaplan-Meier curves. ***p<0.001
Figure 10: Weight Loss in TLR2 Knockout Mice Given Stx2
**Figure 10:** 6-week old female mice with a knockout mutation of TLR2 (n=4) were challenged with 1ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (▼). Wild-type 6-week old C57BL6/J mice received the same challenge (●) (n=48) and demonstrated significant weight loss (***). TLR2-knockout mice challenged with Stx2 exhibited weight loss but it was significantly less when compared to wild-type mice given Stx2 on day 3 and 4 (### and #). By day 5 there was no difference between TLR2-knockout mice and wild-type C57BL6/J mice with respect to weight loss post-Stx2 challenge. Significance was determined by 2-way ANOVA with a Bonferroni post-test. #p<0.05, ###p<0.001, ***p<0.001
Figure 11: TLR2-knockouts Exhibit Rising BUN When Given Stx2
**Figure 11:** 6-week old female mice with a knockout mutation of TLR2 (n=4) were challenged with 1 ng Stx2 diluted in normal saline per 20g bodyweight on day 0 and 3 by intraperitoneal injection (▼). Wild-type C57BL6/J mice were given the same challenge (●) (n=18) and showed significantly elevated BUN on day 4 and 6 (***). When compared to wild-type control mice (n=11) receiving vehicle alone (■), TLR2-knockout mice also exhibited rising BUN, and it was significantly different compared to wild-type mice given Stx2 alone on day 5 (#). Statistical significance was determined by 2-way ANOVA with a Bonferroni post-test. #p<0.05, ***p<0.001
CHAPTER FOUR: CITROBACTER RODENTIUM MODEL OF EHEC INFECTION

In order to more closely mimic human disease, we utilized a model in which female C57BL6/J mice are inoculated with strains of *Citrobacter rodentium* that have been engineered to either express (DBS770) or not express (DBS771) Stx2 (31). The advantage of this model lies in the fact that it uses a mouse pathogen, *Citrobacter rodentium*, to achieve colonization with whole bacteria that produce the characteristic attaching & effacing lesions with the intestinal epithelium, and there is no pre-treatment of the mice with antibiotics or manipulation through starvation to achieve stable colonization(34). Inoculating mice (6-week old females, strain C57BL6/J) by oral gavage with $1 \times 10^9$ CFU DBS770 resulted in decreased survival (Fig 12), decreased weight (Fig 13), and kidney injury evidenced by rising BUN (Fig 14) when compared to mice given the non-Stx2 producing strain (DBS771). In order to determine whether colonization differed between the two strains, we plated feces collected from the mice on LB agar plates with appropriate selective antibiotics (chloramphenicol for DBS770, and chloramphenicol with kanamycin for DBS771). We observed that colonization was similar between the two groups (Fig 15).

We collected plasma from the mice by facial vein bleed and measured DAMPs in the plasma at different time points. We found that the mice colonized with non-toxin expressing DBS771 showed no significant rise in plasma histones when compared to day 0 (Fig 16A), while mice given Stx2+DBS770 had significantly elevated histones by day 3 when compared to day 0 (Fig 16B). In mice challenged
with DBS771, we also observed no significant rise in plasma levels of HMGB1 (Fig 16C) when compared to day 0, but we did observe elevated levels of HMGB1 by day 5 when compared to day 0 (Fig 16D).

In order to determine whether targeting DAMPs in this model would have any effect on outcomes, we injected a group of mice with a single bolus of 400ug anti-histone antibody (clone BWA3) prior to gavage with Stx2+DBS770 on day 0. Mice given this antibody prior to challenge showed no significant difference in survival compared to mice given Stx2+DBS770 alone (Fig 17). Mice pre-treated with BWA3 also showed significantly decreased weight, reaching significance on day 7 of challenge, and showing no difference when compared to mice given Stx2+DBS770 without antibody pre-treatment (Fig 18). Mice given BWA3 also showed kidney injury as represented by a rise in plasma BUN, with BUN values significantly higher than those of mice given Stx2+DBS770 alone on day 8 (Fig 19). There was, however, no significant difference in colonization with Stx2+DBS770 in mice pre-treated with BWA3 when compared to mice given either strain of C. rodentium alone (Fig 20).

By using ELISA to quantify levels of histones or HMGB1 in the plasma of mice pre-treated with BWA3, we observed that unlike mice given Stx2+DBS770 alone, mice pre-treated with anti-histone antibody exhibited no significant rise in histones (Fig 21A) or HMGB1 (Fig 21B) when compared to day 0 values.

In conclusion, we have demonstrated in a mouse model of Stx2-expressing enteric infection that Stx2 is necessary and sufficient to induce decreases in survival
and weight, and increase in BUN, and elevations in plasma histones and HMGB1. We have also demonstrated that pretreatment with an anti-DAMP anti-histone IgG does not change outcome measures, but does prevent the rises in plasma DAMPs induced by Stx2. Thus we have again demonstrated that Stx2 induced tissue injury causes production of DAMPs in a mouse model, but that the DAMPs produced are more likely markers of injury than drivers of pathogenesis in these models.
Figure 12: *C. rodentium* Mouse Model Survival
Figure 12: Mice (6 week old female C57BL6/J) were challenged with $1 \times 10^9$ CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. Mice challenged with strain DBS770 (dashed line) showed decreased survival (A) when compared to mice colonized with DBS771 strain (solid line). Statistical significance was determined by Log-rank test of Kaplan-Meier curves. ***p<0.001
Figure 13: *C. rodentium* Mouse Model Weight Loss
Figure 13: Mice (6 week old female C57BL6/J) were challenged with 1x10⁹ CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. Mice challenged with strain DBS770 (●) showed decreased weight by day 7 when compared to mice colonized with DBS771 strain (■). Statistical significance was determined by 2-way ANOVA with a Bonferroni post-test. ***p<0.001
Figure 14: *C. rodentium* Mouse Model Rise in BUN
Figure 14: Mice (6 week old female C57BL6/J) were challenged with 1x10^9 CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. Mice challenged with strain DBS770 (●) showed increasing BUN by day 7 of challenge when compared to mice colonized with DBS771 strain (■). Statistical significance was determined by a 2-way ANOVA with a Bonferroni post-test. ***p<0.001
Figure 15: *C. rodentium* Mouse Model Colonization
Figure 15: Mice (6 week old female C57BL6/J) were challenged with 1x10^9 CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. Mice challenged with strain DBS770 (●) showed increased colonization with DBS770 by day 3 of challenge when compared to mice colonized with DBS771 strain (■). Statistical significance was determined by 2-way ANOVA with a Bonferroni post-test. *p<0.05*
Figure 16: *C. rodentium* Mouse Model DAMPs
Figure 16: Mice (6 week C57BL6/J) were challenged with 1x10⁹ CFU C. rodentium on day 0 that either did (DBS770) or did not (DBS771) express Stx2 by oral gavage. Mice challenged with DBS771 strain (■) showed no significant rise in plasma levels of extracellular histones (A) (n=10), while mice challenged with DBS770 strain (●) showed a significant rise in plasma levels of extracellular histones on day 3 (B) (n=15). Mice challenged with DBS771 strain showed no significant increase in plasma levels of HMGB1 (C) (n=9), while mice challenged with DBS770 strain showed increased HMGB1 on day 5 (D) (n=14). Statistical significance was determined by 1-way ANOVA with a Dunnett post-test. *p<0.05, **p<0.01
Figure 17: *C. rodentium* Mouse Model Survival with BWA3
Figure 17: Mice (6 week old female C57BL6/J) were challenged with 1x10⁹ CFU C. rodentium on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. A third group was given a single bolus of 400ug anti-histone IgG (clone BWA3) prior to receiving strain DBS770 on day 0 (n=4). Mice challenged with strain DBS770 (dashed line) showed decreased survival when compared to mice colonized with DBS771 strain (solid line), while mice given BWA3 prior to DBS700 gavage (short dashes) also showed significantly decreased survival compared to mice given non-Stx2 expression DSB771. Additionally, mice given BWA3 showed no significant difference in survival compared to mice given DBS770 alone. Significance was determined by Log-rank test on Kaplan-Meier curves.

***p<0.001
Figure 18: *C. rodentium* Mouse Model Weight Loss with BWA3
**Figure 18:** Mice (6 week old female C57BL6/J) were challenged with 1x10⁹ CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. A third group was given a single bolus of 400ug anti-histone IgG (clone BWA3) prior to receiving strain DBS770 on day 0 (n=4). Mice challenged with strain DBS770 (●) showed decreased weight by day 7 when compared to mice colonized with DBS771 strain (■). Mice given BWA3 (▲) showed no significant difference in weight loss when compared to mice given DBS770 alone, and also demonstrated significant weight loss by day 7 when compared to mice given DBS771 alone. Significance was determined by 2-way ANOVA with a Bonferroni post-test. ***p<0.001
Figure 19: *C. rodentium* Mouse Model Rising BUN with BWA3
**Figure 19:** Mice (6 week old female C57BL6/J) were challenged with $1 \times 10^9$ CFU *C. rodentium* on day 0 that either did (DBS770, ●) (n=14) or did not express (DBS771, ■) (n=10) Stx2 by oral gavage. A third group was given a single bolus of 400ug anti-histone IgG (clone BWA3, ▲) prior to receiving strain DBS770 on day 0 (n=4). Mice challenged with strain DBS770 showed increasing BUN by day 7 of challenge when compared to mice colonized with DBS771 strain (**). Mice given BWA3 prior to DBS770 showed increasing BUN that was significantly higher than mice given DBS770 alone on day 8 (##). Significance was determined by 2-way ANOVA with a Bonferroni post-test. #p<0.05, ***p<0.001.
Figure 20: *C. rodentium* Mouse Model Colonization with BWA3
Figure 20: Mice (6 week old female C57BL6/J) were challenged with 1x10⁹ CFU *C. rodentium* on day 0 that either did (DBS770) (n=14) or did not express (DBS771) (n=10) Stx2 by oral gavage. A third group was given a single bolus of 400ug anti-histone IgG (clone BWA3) prior to receiving strain DBS770 on day 0 (n=4). Mice challenged with strain DBS770 (●) showed increased colonization with DBS770 by day 3 of challenge when compared to mice colonized with DBS771 strain (■), while mice pre-treated with BWA3 prior to DBS770 challenge (▲) exhibited no significant difference in CFU counts when compared either group. Significance was determined by 2-way ANOVA with a Bonferroni post-test. *p<0.05
Figure 21: *C. rodentium* Mouse Model DAMPs with BWA3

A

![Graph A](image)

B

![Graph B](image)
**Figure 21:** Mice (6-week C57BL6/J females) given a single dose of 400ug anti-histone IgG (clone BWA3) were gavaged with $1 \times 10^9$ CFU of DBS770, a strain of *Citrobacter rodentium* that has been engineered to express Stx2 (●). Blood collected by facial vein bleed was centrifuged and the plasma levels of extracellular histones and HMGB1 were quantified by ELISA. Mice exhibited no significant rise in plasma levels of extracellular histones (A) (n=5) or HMGB1 (B) (n=3) when compared to day 0. Statistical significance was determined by 1-way ANOVA with a Dunnett post-test.
CHAPTER FIVE: DYSFUNCTION IN THE ENDOTHELIAL PROTEIN C PATHWAY IN HAEC

Human aortic endothelial cells (HAEC) were cultured in 6-well format and challenged with Shiga toxins (100ng/mL) and/or histones (50ug/mL) at 37°C. After 24 hours, challenged cells were gently detached with Versene, washed, and stained with antibodies directed against human EPCR, PAR1, or TM. After a final wash, cell-bound fluorescence was analyzed with a BD FACS Calibur (Becton Dickinson), using proidium iodide as an exclusion dye for dead cells (Fig 22). The geometric mean of the peaks was compared to that observed with unchallenged cells to determine whether the surface receptors involved in the protein C pathway had changed their expression after challenge. It was observed that challenge with histones increased PAR1 (Fig 23A), while challenge with Shiga toxins led to the opposite result and lowered PAR1 in a subset (M1) of cells (Fig 23B). Challenging HAEC with histones or Shiga toxins led to a decrease in surface EPCR (Fig 23C and D). Challenge with histones lead to a decrease in surface TM (Fig 23E), while Shiga toxins showed no evident effect on surface TM expression (Fig 23F).

To confirm the results we observed by flow cytometry, on-cell western technique was used with HAEC looking at the same antigens of interest: EPCR, PAR1, and TM. Briefly after on-cell western had been performed, the fluorescence in the 800nm channel (antigen expression; Fig 24A) and the fluorescence in the 700nm channel (nuclear staining; Fig 24B) were acquired and the 800nm values normalized to the 700nm values to account for cell number (Fig 24C). Data were
analyzed using GraphPad Prism 5.0 and means were compared using ANOVA to
determine significance. Although the magnitude of changes differed, the trends
were similar, with Shiga toxins and histones lowering EPCR, Shiga toxins lowering
PAR1 but histones increasing PAR1, and histones but not Shiga toxins lowering TM
expression (Fig 25). When Stx1 or Stx2 were combined with histones during a
challenge, EPCR and TM were lowered in both cases, but only Stx1 with histones
lowered PAR1, while Stx2 with histones showed no significant change in PAR1.

In conclusion, we have demonstrated that Shiga toxins and extracellular
histones (a DAMP) can have significant effects on the human aortic endothelial
surface expression of receptors involved in the protein C pathway: namely EPCR,
TM, and PAR1. Shiga toxins and/or histones cause significant decreases in all three
(except that histones increase PAR1) and it is possible that there are functional
consequences in the amount of activated protein C produced by endothelium after
Shiga toxin and/or histone challenge.
Figure 22: Example Gating of HAEC and PI Exclusion
Figure 22: HAEC were run on a BD FACS Calibur and gated on forward and side-scatter (A). PI exclusion was done in the FL3 channel (B) to gate for live cells, and the fluorescence in the FL1 channel for these live cells (C) was used to generate histograms (D) for overlays.
Figure 23: Changes in Surface Antigens on HAEC
**Figure 23:** Primary cultured HAEC were challenged with Shiga toxins or histones before being detached, washed, and stained for EPCR, PAR1, and TM. Flow cytometry was performed on a BD FACS Calibur using PI exclusion of dead cells, and the geometric mean of FL1 channel was used to determine differences in surface antigen expression when comparing to unchallenged cells. Antigens of interest were PAR1 (A and B), EPCR (C and D), and TM (E and F). Cells challenged with histones evinced a significant rise in surface PAR1 (A) while challenging with Shiga toxins causes dramatic decrease in PAR1 in an M1 subpopulation (B). Challenging with histones lowered EPCR (C) and Shiga toxin challenged demonstrated a similar effect (D). Finally, histones lowered TM (E) but Shiga toxins had no significant effect on surface TM expression (F). Statistical significance was determined by 1-way ANOVA with a Dunnett post-test. ***p<0.001
Figure 24: On-Cell Western Technique

A

B

C
Figure 24: HAEC were seeded at 10,000 cells per well on a black-walled clear-bottomed 96-well 0.1% gelatin coated tissue culture plate and allowed to grow overnight. The next day cells were challenged for 24 hours in 100uL fresh endothelial cell media per well. After 24 hours, cells were washed with PBS, fixed, and blocked with Odyssey Blocking Buffer before being probed with 2ug/mL of primary antibody: JRK 1494 for EPCR, ATAP2 for PAR1, and αCD141 for thrombomodulin. Afterwards cells were washed with TBST and goat anti-mouse secondary conjugated to an infrared dye diluted 1:800 in Odyssey Blocking Buffer was added in conjunction with Draq5 (5mM) diluted 1:10,000 in the same buffer. After secondary staining, cells were washed again with TBST before all liquid was aspirated and the plate read dry on an Odyssey imager. The fluorescence in the 800nm channel (A), which represents the probes of interest, was corrected by dividing by the fluorescence in the 700nm channel (B) which represents staining of nucleated cells by Draq5, with (C) representing the overlay of both channels. Finally, values were compared to unchallenged cells and statistical significance determined using GraphPad Prism 5.0 software.
Figure 25: Confirmation of Surface Antigen Changes on HAEC
**Figure 25:** HAEC grown in 96-well format were challenged with Shiga toxins and/or histones for 24 hours. After challenge, cells were fixed and stained for EPCR, PAR1, and TM and detected using goat anti-mouse secondary conjugated to an 800nm emitter. Nucleated cells were stained with Draq5, a 700nm emitter, and surface antigen signal was corrected to total cell number in each well, as well as normalized to values for unchallenged cells. Similar changes in surface expression of EPCR, PAR1, and TM were observed as those seen by flow cytometry. Significance was determined by 2-way ANOVA with a Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
CHAPTER SIX: STX AND HISTONES CAUSE A DECLINE IN THE ACTIVATION OF PROTEIN C BY HAEC

In order to ascertain whether the decreases in protein C pathway surface proteins had functional consequences, thrombin-dependent activation of protein C zymogen on an endothelial monolayer was measured by quantifying the resultant activated protein C activity with a kinetic-based chromogenic assay after the activation reaction has been stopped. We observed a significant decrease in the amount of activated protein C generated after cells were challenged with Stx1 or histones when compared to unchallenged cells (Fig 26). This is consistent with the prior observation that Stx and histones decreased surface expression of EPCR and TM antigen.

To see if the histone-induced decrease in protein C activation was preventable, we added anti-histone antibody BWA3 concomitantly with histone challenge and alone for 24 hours and observed that while BWA3 alone did not change the amount of protein C activated when compared to unchallenged cells, BWA3 prevented histones from causing a significant decrease in the amount of activated protein C generated by HAEC (Fig 27).

In conclusion, using human aortic endothelial cells and an in vitro model of Shiga toxin and DAMP (histones) challenge, we have demonstrated that both Stx1 and histones can cause significant decreases in the amount of protein C converted to activated protein C by endothelial thrombin/TM and EPCR. Stx2 tended to decrease protein C activation, but this did not reach significance. As we have previously
demonstrated that both of these receptors are decreased by DAMPs and that EPCR is also decreased by Shiga toxins, these experiments demonstrate that the significant differences observed in levels of surface expression translate into significant functional differences in the amount of protein C activated by thrombin.
Figure 26: Activation of Protein C by HAEC
Figure 26: HAEC challenged with Stx1, Stx2, or histones for 24 hours were used to perform a protein C activation assay. Cells were washed and a buffered solution containing protein C zymogen was added to the cells, and the cleavage of protein C was accomplished by adding human α-thrombin and incubating the reaction at 37°C for 30min. Afterwards, the reaction was stopped by adding portion to a solution anti-thrombin III and heparin, and the amount of activated protein C was determined by adding Spectrozyme PCa substrate and measuring the change in mOD/sec at 405nm. Challenge with Stx1 or with histones caused a significant decrease in the amount of activated protein C generated. Challenge with Stx2 lowered the amount of protein C activated but the difference was not significant. Statistical significance was determined by 1-way ANOVA with a Dunnett post-test. *p<0.05, **p<0.01
Figure 27: Histone Inhibition of Activated Protein C Formation is Rescued by BWA3
Figure 27: HAEC were challenged with histones or histones in combination with BWA3 for 24 hours before a protein C activation assay was performed. While challenge with histones lowered the activation of protein C, co-incubation with anti-histones IgG restored activation of protein C to approximately that of unchallenged cells. Significance was determined by 1-way ANOVA with a Dunnett post-test.

*p<0.05
CHAPTER SEVEN: DYSFUNCTION IN THE ENDOTHELIAL PROTEIN C PATHWAY IN HRGEC

As much of the pathology in human D+HUS occurs in the microvasculature of the kidney in children and the elderly, we repeated our previous in vitro experiments in human renal glomerular endothelial cells (HRGEC). HRGEC were seeded directly from frozen aliquot stocks into 96-well format and on-cell western was used to quantify the amount of EPCR, PAR1, and TM expressed on the surface of the cells. Cells were challenged with Stx1 (50ng/mL), Stx2 (100ng/mL), or histones (50ug/mL) for 24 hours before the changes in surface receptors were quantified. Similar to what was observed in HAEC, both toxins and histones lowered EPCR (Fig 28A), while toxins lowered PAR1 and histones had no significant effect on surface PAR1 (Fig 28B). Both Stx1 and Stx2 significantly lowered HRGEC TM, while histones had no significant effect on TM expression (Fig 28C).

In order to determine whether there was functional significance in the changes of surface antigens on HRGEC, we again performed a protein C activation assay on challenged HRGECs. We observed that challenge with toxins had no significant effect on the activation of protein C, while challenge with histones caused a significant decrease in protein C activation (Fig 29). Co-incubation with anti-histone IgG was again able to partially restore the activation of protein C (Fig 30).

Although we observed similar changes in endothelial protein C pathway components, HUS is known for causing thrombotic microangiopathy in the kidney capillaries, not in major vessels like the aorta, and so we compared the amounts of
total EPCR, PAR1, and TM expressed on unchallenged HAEC or HRGEC. Significantly more EPCR (7.4 fold), PAR1 (1.8 fold), and TM (3.2 fold) were found on HAEC when compared to HRGEC, with HAEC expressing eight-fold the amount of EPCR, twice the amount of PAR1, and three-fold the amount of TM that HRGEC do (Fig 31). In addition, at baseline, HAEC activate significantly more APC than HRGEC (2.83 fold).

In conclusion, we have now demonstrated that on HRGEC microvascular cells the components of the protein C pathway are again downregulated by challenge with Stx or histones, and that this downregulation results in functional differences in the amount of protein C activation accomplished by the endothelium. Furthermore, we have shown that compared to large vessel endothelium such as HAEC, the microvasculature in the kidneys as represented by HRGEC expresses significantly lower levels of protein C pathway components at baseline. Conceivably, an insult such as Stx and/or histones which further lowers expression of these molecules and reduces protein C activation could have a proportionally larger effect on thrombus production, which provides some insight into why the kidney is a particularly susceptible organ for injury in D+HUS.
Figure 28: Changes in Protein C Pathway Receptors in HRGEC
**Figure 28:** HRGEC seeded into 96-well format were challenged with Stx1, Stx2, or histones for 24 hours prior to fixation and the performance of an on-cell western looking at surface expression of EPCR (A), PAR1 (B), and TM (C). Challenge with either Stx1, Stx2, or histones elicited a decrease in EPCR expression, but only challenge with Shiga toxins lowered PAR1 expression. Histones had no effect on surface PAR1 expression and slightly raised surface TM, while challenge with Shiga toxins elicited no observable difference in the surface expression of TM on HRGECs. Significance was determined by 1-way ANOVA with a Dunnett post-test.

***p<0.001
Figure 29: Protein C Activation in HRGEC
**Figure 29:** HRGEC were seeded in 96 well format and challenged with Stx1 (50ng/mL), Stx2 (50ng/mL), or histones (50ug/mL) for 24 hours prior to performance of a protein C activation assay. Challenge with either toxin alone did not alter the activation of protein C when compared to unchallenged cells, while challenge with histones caused a significant decline in the amount of protein C activated when compared to unchallenged cells. Significance was determined by 1-way ANOVA with a Dunnett post-test. ***p<0.001
Figure 30: BWA3 Reverses Histone-Induced Drop in HRGEC Protein C Activation
Figure 30: HRGEC seeded in 96-well format were challenged with histones (30μg/mL), BWA3 (20μg/mL), or a combination of both for 24 hours prior to the performance of a protein C activation assay. BWA3 alone had no effect on the activation of protein C when compared to unchallenged control (media), while histones significantly inhibited the activation of protein C. Co-incubation with histones and anti-histone IgG (BWA3) partially reversed the inhibition of protein C activation seen with histones alone. Significance was determined by 1-way ANOVA with a Dunnett post-test. **p<0.01
Figure 31: Differences Between Large- and Small-vessel Endothelial Cells

A

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Normalized Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPCR</td>
<td>1.0</td>
</tr>
<tr>
<td>PAR1</td>
<td>0.2</td>
</tr>
<tr>
<td>TM</td>
<td>0.3</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Endothelial Cell Source</th>
<th>nM APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>3.0</td>
</tr>
<tr>
<td>Glomerulus</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*** indicates statistical significance at p < 0.001
Figure 31: HAEC or HRGEC were seeded in 96-well plates and on-cell western was performed to determine the relative amounts of ECPR, PAR1, and TM by correcting the fluorescence generated by detecting antibodies in the 800nm channel to cell number as quantified by fluorescence in the 700nm channel. We observed that HAEC (solid bars) have eight times the amount of EPCR, twice the amount of PAR1, and five times the amount of TM as do HRGECs (checkered bars) (A). In addition, comparing the amount of protein C activated by either cell type demonstrated that human aortic endothelial cells activation a significantly higher amount of protein C than human renal glomerular endothelial cells (B). Significance for panel (A) was determined by 2-way ANOVA and for panel (B) by a one-tailed t-test. **p<0.01, ***p<0.001
CHAPTER EIGHT: DAMP-INDUCED BARRIER DYSFUNCTION IN HAEC

In order to determine whether Shiga toxins and DAMPs caused barrier dysfunction in endothelial cells, we utilized trans-epithelial electrical resistance assays to measure the response to thrombin challenge in endothelial cells. By plating cells on cysteine and fibronectin coated electrodes, very sensitive continuous measurements in electrical impedance can be made across a cellular monolayer, with 40 samples taken from each well in an 8-well chamber slide. Using this method, we found that thrombin (1U/mL) produces a rapid and transient decrease in resistance, corresponding to an increase in permeability of the monolayer (Fig 32A). Pretreating a monolayer with histones (25ug/mL) slowed the recovery from thrombin challenge, prolonging the decreased resistance (Fig 32B).

APC is known to switch thrombin from a permeability-inducing to a barrier-protective effect and so we pretreated monolayers with 10nM APC prior to challenge with thrombin (2nM) and observed that when compared to the normal decrease in resistance induced by thrombin, APC led to a smaller decrease and a faster recovery to baseline resistance for the endothelial barrier (Fig 32C). However, pretreatment of the cells with histones 9 hours prior to APC pretreatment abrogated the protective effect of APC on thrombin challenge, and restored the degree of change in resistance seen with thrombin alone.

As both Shiga toxins and histones decrease EPCR but only histones raise PAR1 on HAEC, we replaced histone pretreatment with Stx1 (100ng/mL) pretreatment to observe whether the action of APC on thrombin’s effects was
prevented. We observed that even in the presence of Stx1, APC was able to prevent thrombin-induced barrier dysfunction to the same degree as a 3 hour APC pretreatment alone (Fig 32D). Comparing the areas under the curves generated by trans-epithelial electrical resistance assay software demonstrated that thrombin causes a significant decrease in the resistance of an endothelial monolayer, and that this decrease in resistance is augmented by pretreatment with histones, but decreased by APC. Finally, pretreatment with histones followed by APC negates APC’s effect, restoring thrombin’s ability to decrease resistance to a significant level (Fig 33).

In conclusion, the increase in surface PAR1 we have previously demonstrated by challenging HAEC with histones translates functionally into increased permeability and delayed recovery of endothelium. Normally, occupied EPCR switches the signaling of PAR1 from permeability-enhancing to barrier-protective, and we observed that pre-treatment of an endothelial monolayer with APC indeed attenuated the barrier disruption that challenge with thrombin caused. However, by lowering the amount of available EPCR and increasing the amount of available thrombin receptor (PAR1) through histone challenge, we were able to prevent the protective effects of APC pre-treatment and restore thrombin’s barrier-disruptive effects. Additionally, we demonstrate that Stx1 cannot attenuate APC’s protective effects, indicating that DAMPs produced by Stx-mediated tissue injury could have a more important effect on the action of generated thrombin in the vasculature.
Figure 32: Changes in Trans-Epithelial Electrical Resistance of a HAEC Monolayer
**Figure 32:** Treatment of a HAEC monolayer at 12 hours post-seeding with media (○) produced a steady state of electrical resistance, while thrombin (●) challenge (1U/mL) produced a decrease in the resistance across the monolayer (A). 12 hour pretreatment with histones (x) (B) prolonged thrombin-induced reduction in resistance of the monolayer. 3 hour pretreatment with APC (★) (C and D) attenuated thrombin's barrier disruptive effects; however, 12 hour pre-incubation with histones followed by subsequent addition of APC (▲) resulted in APC's protective effects being inhibited (C). This result was not seen when Stx1 was substituted for histones in combination with APC (▼) (D).
Figure 33: Areas Under the Trans-Epithelial Electrical Resistance Curves

![Graph showing areas under curves for different challenges: Media, Thr, H/Thr, APC/Thr, H/APC/Thr, Stx/APC/Thr. The graph indicates statistical significance with * and *** symbols.](image)
Figure 33: Calculating the area under the curves for trans-epithelial electrical resistance measurements of a HAEC monolayer showed a significant decrease in barrier integrity when thrombin was present that was abrogated by treatment with APC and exacerbated by treatment with histones. Significance was determined using 1-way ANOVA with a Dunnett post-test. *p<0.05, ***p<0.001
CHAPTER NINE: SUMMARY AND DISCUSSION

DAMPs Are Released by Stx2-Induced Tissue Injury

Here we demonstrate the novel finding of a significant rise in plasma levels of HMGB1 and extracellular histones in two murine models of Stx2-induced kidney injury. The first model uses Stx2 that has been purified of contaminating LPS and leads to significant rises in extracellular histones on day 3 and HMGB1 as early as day 2. The second model, which uses a mouse pathogen engineered to express Stx2, also demonstrates elevations in DAMPs, and the control mice that are infected with non-toxin-expressing bacteria do not demonstrate any significant increases in plasma DAMPs. These data show that Stx2 is necessary and sufficient to elevate DAMPs in mice, as demonstrated by the fact that mice colonized with the pathogen alone demonstrated no rising DAMPs, even though \textit{C. rodentium} has been shown to produce A/E lesions that characterize infection by EHEC in humans (31).

Shiga Toxins and DAMPs Promote a Prothrombotic Endothelium

Both HMGB1 and histones have been implicated in the pathogenesis of sepsis and inhibition of either has shown to be beneficial in models of LPS-induced pro-inflammatory states that demonstrate endothelial dysfunction (19, 23). We
hypothesized that the presence of these (and presumably other) DAMPs in the plasma of patients experiencing D+HUS are at least partly responsible for the endothelial dysfunction that drives so much of the morbidity and mortality. The D+HUS that a subset of EHEC-infected patients develop is currently wholly attributed to the bacterially produced Shiga toxins’ direct effects on the endothelium. It has been previously demonstrated that Shiga toxins cause a swelling and detachment of endothelial cells (35) which could potentially expose the endothelium to factors able to prompt coagulation. There has, however, been great difficulty in detecting Shiga toxins in the plasma of patients that demonstrate D+HUS, with detectable Shiga toxin stopping days before the development of the syndrome (36), and it is hard to explain how such low to undetectable levels of Stx could lead to such exaggerated pathology and endothelial dysfunction. Here we have shown that DAMPs can cause a dysfunctional endothelial environment by decreasing the surface expression of EPCR and TM, while increasing the surface expression of PAR1 on human endothelial cells. All of these changes promote a pro-thrombotic and barrier disruptive environment. Lowered EPCR and TM could possibly lead to decreased activation of zymogen protein C in patients, a risk factor for death during sepsis (37), and something demonstrated here in vitro; increased PAR1 is prothrombotic in part due to its increased induction of tissue factor and VWF when stimulated by thrombin (38, 39).

Using multiple approaches, we demonstrate that EPCR is significantly decreased on the cell surface of both human aortic and renal glomerular endothelial
cells treated with Stx and extracellular histones. In addition, histones raise PAR1 levels on macrovascular endothelium, while both Stx1 and Stx2 lower PAR1. Finally, both Stx1 and Stx2 lower TM on HAEC, while histones lower TM on HRGEC.

**DAMPs Inhibit Activation of Protein C, a Preventable Effect**

To investigate if the changes in protein C pathway components lead to a functional difference in the activation of protein C by the endothelial cells we performed protein C activation assays using both HAEC and HRGEC. We demonstrate that the changes seen in the protein C pathway translate to a significant reduction in the endothelium's ability to activate protein C when Stx1 or extracellular histones are used to challenge HAEC, or when histones are used to challenge HRGEC. In addition, we demonstrated in both HAEC and HRGEC that this inhibition of protein C activation is partially reversible with anti-histone antibody co-treatment. The functional decline in protein C activation is an event which could help explain the larger context in which tissue damage and the initial pathology seen in patients could reach a tipping point and progress to HUS.

**Histones Exacerbate Thrombin Effects and Block APC**

Although endothelial barrier function is a contribution to sepsis pathology that is not well understood (40), it can have a large bearing on outcomes. Thus we investigated how Stx and DAMPs affected endothelial barrier function using an
electrical impedance assay. We had seen that although Stx caused a significant decrease in surface expression of the thrombin receptor (PAR1), extracellular histones caused a striking increase in its expression, which, coupled with the opposite shift in EPCR, we hypothesized to lead to a more barrier disruptive effect from thrombin. With less EPCR available to be occupied by its ligand, and more PAR1 available for signaling, the decreased ratio of EPCR:PAR1 could conceivably create an environment where thrombin has more receptor to act through and less of its action is switched to barrier-protective by occupied EPCR.

Indeed, we saw that pretreatment of a human aortic endothelial monolayer with histones greatly delays the ability of the endothelial barrier to recover to baseline after thrombin challenge, and that pre-incubation with histones also inhibited the barrier-protective effects of activated protein C. PAR1 can function in both barrier protective and disruptive capacities, depending on which protease cleaves it, with APC generating a different tethered ligand than thrombin (41, 42), and whether EPCR is occupied by a ligand. Transactivation of the S1P1 receptor by APC/EPCR activation of PAR1 leads to Rac1 activation and barrier protectiveness, but thrombin transactivation of S1P3 through PAR1 leads to RhoA-mediated barrier disruption (43-45).

DAMPs have been shown to increase thrombin generation (46, 47) and so the cycle of Shiga toxin-induced DAMPs leading to increased thrombin and a decreased EPCR:PAR1 ratio could rationally be hypothesized to lead to negative effects on endothelial barrier function by tipping the balance towards harmful S1P3 signaling.
Indeed, increased microvascular permeability directly due to histones has been observed in vivo using FITC-dextran in mice injected with histones intrascrotally (48). We observed a similar response in experiments utilizing electrical cell impedance sensing with human aortic endothelial cells that had been pre-treated with histones before thrombin exposure.

**DAMP Sources in Tissue**

That DAMPs are produced by the action of Shiga toxins on tissue is to the best of our knowledge a novel finding. Our mouse models do not demonstrate the thrombotic microangiopathy of other models and human patients, so we did not expect the same level of tissue damage seen in higher organisms, e.g. non-human primate models. During EHEC infection, it is possible that the initial insult of Shiga toxins to the kidney leads to DAMP production, worsening damage in the kidney and setting the stage for the microangiopathy that drives HUS and could further DAMP production.

A surprising finding is that levels of plasma HMGB1 in both models decreased after an initial significant rise instead of continuing to rise with disease severity, but we hypothesize that this is possibly a result of excessive urination due to renal epithelial damage. Shiga toxin has been shown to specifically target the renal tubule epithelial cells in mice (49), and it is possible that most of the HMGB1 produced was excreted in the kidney as urination became more excessive in the
mouse models. Investigating the urine of mice challenged with Stx2+ *C. rodentium* for evidence of HMGB1 and extracellular histones would be helpful in shedding light on whether the decrease in systemic levels is due to increased renal loss of HMGB1. Our novel finding that Stx and DAMPs can affect the amount of EPCR or PAR1 available on the endothelial surface helps to explain the mechanism by which endothelial dysfunction occurs and tells more about the context in which thrombotic microangiopathy in HUS might take place. Furthermore these studies have implications for all models where DAMPs are produced. A cycle of tissue damage and endothelial dysfunction that is started by Shiga toxins but propagated by the DAMPs that result is one hypothesis for how relatively small amounts of Stx can precipitate such severe complications in patients, but any insult leading to DAMP production would conceivably have similar effects. Anti-DAMP therapies should be considered, especially in conditions such as sepsis, where there are no currently approved therapies.

**DAMPs are Markers, not Drivers of Tissue Injury in Stx2 Mouse Models**

In our experiments we introduced the anti-histone antibody BWA3, which is derived from an autoimmune mouse model and targets H4 and H2a (30). We performed experiments in both mouse models where we gave a bolus dose of BWA3 prior to challenge and in both cases observed significant decreases in plasma levels of histones and HMGB1, although in neither case was the outcome altered for the
model. These results point to DAMPs being a marker of tissue injury in these models rather than a driver of disease. However, this result is not wholly unexpected, as models that have demonstrated changes in outcome measures when BWA3 was used were models that were driven mainly by endothelial injury, and our models are both models of tubular epithelial injury as mice do not express high levels of Gb3 on their glomerular endothelium (50), and do not demonstrate the thrombotic microangiopathy and thrombocytopenia of HUS with the Shiga toxins alone. However, the experiments with human endothelial cells raise the possibility that BWA3 might have more of an effect on outcomes in models of kidney injury that are driven by Stx-induced endothelial damage, and show that anti-DAMP therapies could have promise in the higher-organism models such as non-human primate, that more closely mimic human pathology.

Limitations of the Present Work

One limitation of the present work is that full-spectrum HUS cannot be generated reproducibly in mouse models of Stx without the addition of LPS (34, 51). Non-human primate models exhibit full-spectrum HUS with thrombocytopenia, hemolytic anemia, thrombotic microangiopathy, and renal failure, but non-human primates are an impractical tool for studying mechanisms and genetic manipulations. While our in vitro work points to interesting changes in endothelial surface proteins involved in barrier and anticoagulant function, the endothelium in
mouse kidneys is not the prime target of Shiga toxins, and so therapies such as the one described here that rescue protein C activation would not be presumed as effective in mitigating pathogenesis.

Another limitation of the work outlined in this manuscript is a lack of cellular mechanisms contributing to the changes observed in endothelial surface antigens, specifically a mechanism linking Shiga toxins and extracellular histones to the regulation of EPCR and PAR1. Previous research has shown that matrix metalloproteinases and MAPK pathways are involved in the shedding of EPCR (52, 53). As previous investigators have shown that Shiga toxins can activate some of the same MAPK pathways involved (10) it is possible that this surface decrease is due to shedding by MMPs or possible to the inhibition of translation that is a hallmark of the toxins. The question as to how extracellular histones, which are known to act through toll-like receptors, could induce the expression of PAR1 has not been directly investigated to the best of our knowledge. Stimulation of dendritic cells with LPS, a canonical TLR4 agonist, showed upregulation of PAR1 and PAR3, though not PAR2 or PAR4 (54). It is possible that histones acting through their TLR4 receptor might induce PAR1 through a similar mechanism, though we do not confirm this here. There are also reports of LPS upregulating PAR1 in rat astrocytes (55) at the transcription level, and perhaps this is the case for histones as well, although further research is needed to confirm this or another mechanism. This increased understanding of the context in which thrombotic microangiopathy takes place may help us apply already available therapies in novel ways.
Future Directions

First, analysis of non-human primate tissues using immunohistochemistry to look at levels of endothelial protein C pathway proteins would be a fundamental step in understanding how anticoagulant mechanisms might change in the presence of full-spectrum HUS in an animal model.

Second, anti-DAMP therapies such as the BWA3 antibody or similar ones could be used in the non-human primate model to assess whether targeting DAMPs proves effective in correcting any of a variety of outcome measures. This would add valuable information as to what level a role DAMPs might play in the animal model of Stx-induced HUS.

Finally, the in vitro work looking at mechanism connecting DAMP receptors and the surface receptor expression in the protein C pathway should be examined more closely to see what links DAMPs and Shiga toxins to changing levels of EPCR, PAR1, and thrombomodulin. Understanding in what ways DAMPs might play a role and how they might accomplish their ends could provide hope in a disease that causes such physical and economic devastation and for which we are currently without therapy.
List of Journal Abbreviations

Am J Pathol............................................................... The American Journal of Pathology
Biochem J.............................................................. Biochemical Journal
Cellular Microbiol.................................................... Cellular Microbiology
Infect Immun.......................................................... Infection and Immunity
J Am Soc Nephrol ................................................. Journal of the American Society of Nephrology
J Immunol .............................................................. The Journal of Immunology
Kidney Int .............................................................. Kidney International
Leukoc Biol............................................................ Journal of Leukocyte Biology
Nat Med ................................................................. Nature Medicine
Nat Rev Immunology............................................... Nature Reviews Immunology
Pediatr Nephrol ....................................................... Pediatric Nephrology
Proc Natl Acad Sci USA............................................. Proceedings of the National Academy of Sciences of the United States of America
Thromb Haemost ..................................................... Journal of Thrombosis and Haemostasis
BIBLIOGRAPHY


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**FIRST AUTHOR PUBLICATIONS**

PMID: 23202315  
“Shiga toxins and the pathophysiology of hemolytic uremic syndrome in humans and animals” Mayer, CM et al. Toxins. 2012

**OTHER PUBLICATIONS**

PMID: 23733336  
“Quiescent complement in nonhuman primates during *E. coli* Shiga toxin-induced hemolytic uremic syndrome and thrombotic microangiopathy” Blood. 2013

**AWARDS AND FELLOWSHIPS**

2012-2014  
**Cardiovascular Training Program Fellowship (T32) Boston, MA**  
Competitive fellowship supporting Ph.D. candidates involved in cardiovascular research

2013  
**Diversity Travel Award, National M.D., Ph.D. Conf. Keystone, CO**
This award is intended to defray travel costs for minority candidates to attend the annual national M.D., Ph.D. student conference in Keystone.

2009-Pres.  **BUSM Fellowship Award**  Boston, MA

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