Examining inflammatory mechanisms and potential cytoprotective therapeutics in animal models of Shiga toxin induced kidney injury

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EXAMINING INFLAMMATORY MECHANISMS AND POTENTIAL CYTOPROTECTIVE THERAPEUTICS IN ANIMAL MODELS OF SHIGA TOXIN INDUCED KIDNEY INJURY

by

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DEDICATION

I would like to dedicate this work to my supportive spouse Kashia, my father Hung Bom, my mother Jeannie, my brother Sean, my sisters Jennifer and Rosalia, and to the rest of my family and friends for all your support and encouragement throughout my life. Without each one of you, none of this would have been possible; I am truly indebted to you all.
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ABSTRACT

Shiga toxin-producing enterohemorrhagic Escherichia coli (EHEC) is an emerging food- and water-borne pathogen, causing approximately 73,000 annual infections in the United States and an estimated 1.5 million infections globally. E. coli O157:H7, the most frequently associated EHEC strain, is primarily transmitted through consumption of contaminated ground beef and produce and leads to hemorrhagic colitis in humans. In 5% to 15% of infected patients, circulating Shiga toxins (Stx1, Stx2) cause hemolytic uremic syndrome (HUS), characterized by the presence of thrombocytopenia, hemolytic anemia, and thrombotic microangiopathy, contributing to acute kidney injury (AKI). Current treatment is supportive and antibiotic therapy is contraindicative as it increases toxin production. Therapeutics for EHEC-induced HUS need to be identified to minimize kidney injury and uncontrolled coagulopathy. Well-characterized animal models of HUS and EHEC infection are available and provide avenues for potential therapeutic discovery. Baboons (Papio) challenged with endotoxin-free Shiga toxins develop full spectrum HUS, and mice infected with Stx2-producing Citrobacter rodentium (Cr Stx2+), a genetically modified enteric mouse pathogen,
develop severe Stx2-mediated kidney injury. Initial studies have shown that soluble thrombomodulin (sTM), an anti-coagulant, is a promising therapeutic in preventing severe kidney injury in pediatric patients. In these studies, we determined whether complement was activated in baboons challenged with Shiga toxins, and evaluated whether intraperitoneal injection of sTM would reduce disease severity from mice infected with Cr Stx2+. D-dimer and cell injury markers (HMGB1, histones) confirmed the presence of coagulopathy and cell injury in Stx challenged baboons. Studies revealed that complement activation is not required for the development of thrombotic microangiopathy and HUS induced by EHEC Shiga toxins in these pre-clinical models. Soluble thrombomodulin treatment in Cr Stx2+ infected mice significantly decreased colonization but did not alter mortality. However, gene expression of kidney injury markers (NGAL, KIM-1) decreased significantly compared to no treatment indicating sTM-associated cytoprotectivity. The C. rodentium mouse model does not develop the coagulopathy seen in HUS patients and sTM treatment may be more effective in the baboon toxemia model. Soluble thrombomodulin is a promising therapeutic for EHEC-induced HUS and should be further evaluated in Stx challenged baboons.
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LIST OF ABBREVIATIONS

A/E.................................................................................... Attaching and effacing
aHUS........................................................................... Atypical hemolytic uremic syndrome
AKI................................................................................ Acute kidney injury
ANOVA........................................................................ Analysis of variance
APC................................................................................ Activated protein C
BUN................................................................................ Blood urea nitrogen
CFU................................................................................ Colony forming unit
C. rodentium..................................................................... Citrobacter rodentium
Cr Stx2+ ........................................................................ Stx2 expressing C. rodentium
Cr WT ............................................................................... Non-Stx2 expressing C. rodentium control
CT...................................................................................... Cycle threshold
DAMP................................................................................ Damage associated molecular pattern
DIC.................................................................................. Disseminated intravascular coagulation
E. coli.............................................................................. Escherichia coli
EHEC................................................................................ Enterohemorrhagic Escherichia coli
EPCR................................................................................ Endothelial protein C receptor
Gb₃..................................................................................... Globotriaosylceramide
HMGB1............................................................................ High mobility group box 1
HUS................................................................................ Hemolytic uremic syndrome
KIM-1.................................................................Kidney injury molecule 1
LPS..............................................................................Lipopolysaccharide
NF-kB .............. Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL..........................................................Neutrophil gelatinase-associated lipocalin
PAR-1................................................Protease activated receptor 1
PCR..............................................................Polymerase chain reaction
RT.................................................................Reverse transcription
sTM ..........................................................Soluble thrombomodulin
Stx1 ..............................................................Shiga toxin 1
Stx2 ..............................................................Shiga toxin 2
TCC ..............................................................Terminal complement complex
TM ..............................................................Thrombomodulin
CHAPTER ONE
INTRODUCTION

1.1 Importance

Shiga toxin-producing enterohemorrhagic *Escherichia coli* (EHEC) is an emerging food- and water-borne pathogen, causing approximately 73,000 annual infections in the United States and an estimated 1.5 million infections globally (Frenzen *et al.*, 2005; Stearns-Kurosawa *et al.*, 2013). Infection with the most common EHEC strain, *E. coli* O157:H7, costs an annual $405 million (in 2003 dollars), including $370 million in premature deaths, $30 million in medical care, and $5 million in lost productivity (Frenzen *et al.*, 2005). It is primarily transmitted to humans through consumption of undercooked ground meat products, raw milk, and contaminated raw vegetables. EHEC infection can cause diarrhea and hemorrhagic colitis in humans. Shiga-like toxins (Stx1 and Stx2) bind to Gb3 expressing endothelial cells, particularly in renal glomeruli, to elicit their ribosome inactivating function (Johannes *et al.*, 2010). Hemolytic uremic syndrome (HUS) is a severe clinical complication that develops in 5% to 15% of infected patients, characterized by hemolytic anemia, thrombocytopenia, and thrombotic microangiopathy, resulting in acute renal failure and death (Mayer *et al.*, 2012).

*Escherichia coli* O157:H7 was first recognized as a pathogen in 1982 during an outbreak of hemorrhagic colitis in 47 patients in Oregon and Michigan (Riliey *et al.*, 1983). It was not until 1993 that *E. coli* O157:H7 was recognized as
an important and threatening pathogen after a multistate outbreak was linked to undercooked ground beef patties sold from a fast-food restaurant chain and hospitalized 501 patients (Bell et al, 1994). From 1982 to 2002, there were 350 outbreaks (≥ 2 cases of same epidemiologic source) reported to the CDC from 49 states, and during that period, 17.4% out of the total 8,598 infected individuals required hospitalization (Bell et al, 1994). Food was the predominant transmission route from 1982 to 2002 and accounted for 61% of outbreak-related cases (Bell et al, 1994).

1.2 Escherichia Coli

*Escherichia coli* is a Gram negative, rod shaped bacteria in the family Enterobacteriaceae. *E. coli* is part of the normal human commensal found in the intestinal tract but introduction to pathogenic serotypes including *E. coli* O157:H7 and O104:H4 can cause severe complications and death (Karch et al, 2012; Mayer et al 2012). Although the reservoir of *E. coli* O104:H4 is not yet known, the key reservoir for *E. coli* O157:H7 are ruminants, particularly cattle (Croxen et al, 2010; Johannes et al, 2010). Infected cattle are asymptomatic and shed EHEC in their feces, which can contaminate bovine products and produce (Cray et al, 1995). Human transmission mainly occurs from consumption of contaminated food and water. Based on *in vitro* and animal model studies, several virulence factors have been identified, the major factor being Shiga-like toxins (Boerlin et
al, 1999; Law, 2001). EHEC have not acquired entero-invasive properties needed for bacteremia and secreted Shiga toxins are responsible for severe organ damage (Taylor, 2008).

1.3 Shiga Toxins

Shiga toxins from EHEC strains were first recognized to be associated with HUS in 1983 (Karmali et al, 1983). Shiga toxins produced by EHEC have similar functional and structural properties to Shiga toxin expressed by Shigella dysenteriae serotype 1 (Calderwood et al, 1987). Stx1 differs by one amino acid to Stx from S. dysenteriae, and shares 58% amino acid identity to EHEC Stx2 (Calderwood et al, 1987). There have been a number of Shiga toxin variants identified, and Stx2 has been epidemiologically linked to increased severity in disease (Boerlin et al, 1999).

Shiga toxins 1 and 2 are multi subunit AB$_5$ protein complexes composed of an enzymatic A subunit non-covalently associated with a receptor-binding pentamer B subunit (Fraser et al, 1994). The pentameric B subunit binds to cell surface globotriaosylceramide (Gb3) receptors found on certain eukaryotic cell types primarily in the two principal target organs, kidney and brain (Trachtman et al, 2012). The primary cell target of Shiga toxin toxicity in humans and non-human primates are the renal glomerular endothelial cells, whereas renal cortical and medullary tubular epithelial cells are targeted in mice (Psotka et al, 2009;
Trachtman *et al*, 2012; Stearns-Kurosawa *et al*, 2013). Following Gb$_3$ receptor binding, Shiga toxin-receptor complexes are endocytosed into the target cell and undergo retrograde transport to the endoplasmic reticulum via the Golgi apparatus (Sandvig *et al*, 1992). The A subunit N-glycosidase removes an adenine base from the 28S ribosomal RNA leading to inhibition of protein synthesis and ER stress (Iordanov *et al*, 1997). Shiga toxins have been shown to induce inflammation in patients and animal models, in addition to the complications seen in the kidneys, intestines, vasculature, and central nervous system (Mayer *et al*, 2012).

### 1.4 Hemolytic Uremic Syndrome

Hemolytic uremic syndrome is defined as the triad of hemolytic anemia, thrombocytopenia, and thrombotic microangiopathy that contributes to acute kidney injury that may progress to acute renal failure and death (Mayer *et al*, 2012). Early symptoms of EHEC infection include watery diarrhea and abdominal cramps that can progress to hemorrhagic colitis. The incubation period range from three to eight days, and most patients recover within ten days. Epidemiology studies have shown that HUS typically develops in approximately 5%-15% of infected patients (Tarr *et al*, 2005). Children, elderly, and the immunocompromised are the most susceptible to complications and death from EHEC infection (Gould *et al*, 2009).
Treatment of HUS is generally supportive and includes managing fluid levels, balancing electrolyte levels, and acute renal replacement therapy (Goldwater et al, 2012). No toxic specific treatments are available yet and antibiotics are usually contraindicated, depending on serotype (Mayer et al, 2012). Wong et al (2012) demonstrated in a multi-state prospective study of 259 US children with HUS from *E. coli* O157:H7 that exposure to antibiotics during the first week of illness tripled the risk of developing HUS. For *E. coli* O157:H7, antimicrobials were shown to induce Stx2 expression (up to 140-fold) due to the location of Stx genes within the antibiotic inducible resident lambdoid prophages (Kimmitt et al, 2000).

### 1.7 Specific Aims and Hypothesis

In these studies, we aim to:

1. **CHAPTER TWO**: Determine whether complement is activated in Stx-challenged non-human primates during the development of HUS and acute renal failure.

   2. **CHAPTER THREE**: Examine the effects of soluble thrombomodulin treatment on mice infected with Stx2-producing *Citrobacter rodentium*.

   We expect these studies to show:
(1) CHAPTER TWO: Development of HUS in non-human primates will present with activated complement similar to clinical observations seen in patients with EHEC induced HUS.

(2) CHAPTER THREE: Treatment with soluble thrombomodulin will provide positive outcomes versus no treatment in mice as it has been shown as a promising therapeutic in patients with EHEC induced HUS.
CHAPTER TWO
QUIESCENT COMPLEMENT IN NONHUMAN PRIMATES DURING E. COLI SHIGA TOXIN-INDUCED HEMOLYTIC UREMIC SYNDROME AND THROMBOTIC MICROANGIOPATHY

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2.1 Key Points

• Complement activation is not required for development of thrombotic microangiopathy and HUS induced by EHEC Shiga toxins in non-human primates
• Complement is an important defense mechanism and benefits or risks of therapeutic inhibition should be studied further for this infection

2.2 Abstract

Enterohemorrhagic Escherichia coli (EHEC) produce ribosome inactivating Shiga toxins (Stx1, Stx2) responsible for development of hemolytic uremic syndrome (HUS) and acute kidney injury (AKI). Some patients show evidence of complement activation during EHEC infection, raising the possibility of therapeutic targeting of complement for relief. Our juvenile non-human primate
(Papio baboons) models of endotoxin-free Stx challenge exhibit full spectrum HUS including thrombocytopenia, hemolytic anemia, and AKI with glomerular thrombotic microangiopathy. There were no significant increases in soluble terminal complement complex (C5b-9) levels after challenge with lethal Stx1 (n=6) or Stx2 (n=5) in plasma samples from T0 to euthanasia at 49.5-128 hrs post-challenge. D-dimer and cell injury markers (HMGB1, histones) confirmed coagulopathy and cell injury. Thus, complement activation is not required for the development of thrombotic microangiopathy and HUS induced by EHEC Shiga toxins in these pre-clinical models, and benefits or risks of complement inhibition should be studied further for this infection.

2.3 Introduction

Shiga toxin-producing enterohemorrhagic Escherichia coli (EHEC) is an emerging food- and water-borne pathogen (Mayer et al, 2012). The E.coli O157:H7 is the most common strain and its ribosome inactivating Shiga toxins (Stx1, Stx2) injure receptor-bearing endothelial cells, particularly in renal glomeruli. Hemolytic uremic syndrome (HUS) is a clinically important complication in 5-15% of these patients, characterized by hemolytic anemia, thrombocytopenia and thrombotic microangiopathy, often resulting in severe acute kidney injury necessitating dialysis. Antibiotics increase HUS risk (Wong et
al, 2012), and this pathogen is the leading cause of acute renal failure in otherwise healthy US children.

The clinical presentation of EHEC-HUS overlaps that of atypical HUS (aHUS), a rare disease induced by genetic abnormalities resulting in unchecked alternative complement pathway activation (Noris et al, 2010). Immuno-inhibition of complement C5 activation in aHUS patients with Eculizumab (Solaris®) reduces levels of inflammatory complement mediators and the terminal complement complex (TCC, soluble C5b-9), and normalizes clinical indicators (Nurnberger et al, 2009). The clinical similarity of these syndromes has led to considerable discussion in the EHEC field about whether Stx activities activate complement, which then becomes a major driving force for HUS development. *In vitro* and murine data support complement activation (Morigi et al, 2011; Noris et al, 2012; Thurman et al, 2009), but *in vitro* data arise from toxin challenges ~500,000 times higher than the 5-20 pg/ml Stx levels observed in infected children (Lopez et al, 2012). Eculizumab was seemingly beneficial in three children with severe EHEC-HUS (Lapeyraque et al, 2011), but apparent efficacy may have been coincident with natural recovery as suggested by already rising platelets and falling LDH levels. Despite these indicators, a direct exploration of whether Stx-induced complement activation is responsible for HUS has not been done. Our nonhuman primate models of endotoxin-free Stx1 and Stx2 challenge present with the full spectrum of human EHEC-HUS including hematology,
physiology, and inflammation responses (Stearns-Kurosawa et al, 2010; Stearns-Kurosawa et al, 2011), with glomerular thrombotic microangiopathy (Stearns-Kurosawa et al, 2013). Here we examined whether complement was activated in the Stx-challenged baboons during the development of HUS and acute renal failure. We quantified D-dimer as a marker of fibrinolysis, as well as cell injury markers HMGB1 and histones.

2.4 Methods

2.4.1 Baboon Samples

Methods and characterization of the nonhuman primate (juvenile Papio baboons, 4~6kg) challenges with lethal Stx1 (100ng/kg) or Stx2 (50ng/kg) are described (Stearns-Kurosawa et al, 2010; Stearns-Kurosawa et al, 2011). All animals developed HUS with thrombotic microangiopathy and progressive loss of renal function. Animal studies were performed under the oversight of the regulatory IACUC and IBC of the Boston University School of Medicine.

2.4.2 ELISA Assays

Baboon EDTA-plasma or urine (Foley catheter; 20 minute timed samples after bladder purge) stored at -80°C were used. Terminal complement complex (TCC) was quantified using the Human Terminal Complement Complex ELISA kit (Hycult Biotech, Plymouth Meeting, PA). D-dimer was quantified using
Asserachrom D-DI kit (Diagnostica Stago, Parsippany, NJ). HMGB1 was quantified using HMGB1 ELISA (IBL International, Hamburg, Germany) and histones by using Cell Death Detection ELISA plus (Roche Inc., Indianapolis, IN). Stored EDTA-plasma from bacteremic baboons challenged intravenously with sub-lethal $5 \times 10^9$ CFU/kg *E. coli* B7 O86a:K61 (SLEC; not toxigenic) (Taylor *et al.*, 2000) or lethal $3 \times 10^9$ CFU/kg *Bacillus anthracis* Sterne strain 34F2 (vaccine strain) (Stearns-Kurosawa *et al.*, 2006) were positive controls. Data were analyzed for differences between groups using Student’s T-test, assuming equal variance.

### 2.5 Results and Discussion

Bacteremia is rare in patients with EHEC infection and the Shiga toxins are widely acknowledged as the primary mediators of organ injury (Sauter *et al.*, 2008; Mallick *et al.*, 2012). Our nonhuman primate models are the only animal models to date that present with full spectrum HUS induced by only Stx challenge. Some differences are observed between the toxins with respect to timing and inflammation (Stearns-Kurosawa *et al.*, 2010) or renal pathology (Stearns-Kurosawa *et al.*, 2013), but the classic triad of hemolytic anemia, thrombocytopenia and thrombotic microangiopathy with acute kidney injury are shared responses after Stx1 or Stx2. Given the success of complement inhibition in aHUS patients, we measured soluble TCC in our Stx-HUS models to
determine whether complement is activated and, if so, when. There were no significant increases in soluble TCC levels in animals after lethal challenge with Stx1 (Fig 2.1A; n=6) or Stx2 (Fig 2.1B; n=5) up until the time of euthanasia at 49.5-128 hours post-challenge. Platelet levels declined (Fig 2.1C,D) and fibrinogen levels were steady or increased (Fig 2.1E,F) as expected during development of HUS. Renal glomerular thrombotic microangiopathy was observed by pathology evaluation after necropsy (Stearns-Kurosawa et al, 2013) and progressively increasing D-dimer levels (Fig 2.1G,H) confirm coagulation activation and subsequent fibrinolysis. The lack of complement activation was surprising, given the known crosstalk between coagulation and complement pathways (Oikonomopoulou et al, 2012) and markers of complement activation in some EHEC-HUS patients (Noris et al, 2012; Thurman et al, 2009). To confirm integrity of the assays with baboons, the well-characterized baboon model of E.coli bacteremia sepsis with disseminated intravascular coagulation (DIC) was evaluated similarly (Silasi-Mansat et al, 2010; Taylor et al, 2000; Taylor et al, 2001). Sub-lethal challenge with this E.coli strain induced complement activation and DIC as judged by thrombocytopenia, fibrinogen consumption, and rapid increases in D-dimer (Fig 2.1A,C,E,G). Complement inhibition after high dose of this E.coli strain in baboons significantly reduces the consumptive coagulopathy and inflammation (Silasi-Mansat et al, 2010). Similarly, soluble TCC in a baboon challenged with i.v. Gram positive attenuated Bacillus anthracis (Stearns-
Kurosawa et al, 2006) peaked at 2,204.99 mAU/ml by 10 hours post-challenge, slowly declining over the next 4 days (Fig 2.1A).

Markers of cell damage also were evaluated in the Stx-challenged baboons to confirm systemic cytotoxic activities. HMGB1 and histones are damage associated molecular patterns (DAMPs), which are released into the circulation by dead or dying cells. They can engage receptors on distant cells and are pro-inflammatory in murine and baboon sepsis models (Xu et al, 2009). Both DAMPs were detected in baboon plasma and urine after Stx challenge (Fig 2.2) with generally earlier rises after Stx1, consistent with earlier increases in inflammation cytokines and chemokines (Stearns-Kurosawa et al, 2010).

Collectively the data show that the complement pathway is not activated to any great extent in this animal model of HUS despite challenge with sufficient Stx to induce coagulation, fibrinolysis, cell injury and ultimately death. Mice infected with Citrobacter rodentium expressing Stx2 develop EHEC-like intestinal adhesion and inflammatory lesions with Stx-induced acute kidney injury, but also do not show evidence of complement activation (Mallick et al, 2012). Yet complement activation is reported in some EHEC-HUS patients. It is not clear whether Eculizumab was effective during the German 2011 outbreak (Menne et al, 2012; Trachtman et al, 2012), but this was an enteroaggregative E.coli O104:H4 strain that acquired both Stx2 expression and unusual virulence in young adults with differing clinical presentation from more typical
enterohemorrhagic strains (Ullrich et al, 2013). Our baboon HUS models are induced by Stx challenge, not an enteric bacterial infection, and bacterial translocation from the intestines is not observed in the baboons (Sursal et al, 2013). Yet there is considerable intestinal injury in EHEC patients that may contribute to complement activation. The colon can be affected with edema, hemorrhage and leukocytosis, consistent with the hemorrhagic colitis that is often seen preceding HUS, but severity may vary widely between patients. While our data do not support a major role for activation of complement during Stx-induced HUS pathogenesis, other host or bacterial virulence factors (Orth et al, 2010) may be important, either alone or in combination with the bacterial toxins. Complement is a fundamental bacterial defense mechanism and further research is warranted to judge therapeutic risks or benefits of modulating this arm of innate immunity in EHEC patients.

2.6 Acknowledgements

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Figure 2.1. Changes in complement and coagulation activation markers.

Stored timed EDTA-plasma samples from baboons challenged with i.v. 100ng/kg Stx1 (▲, left; n=6) or 50ng/kg Stx2 (●, right; n=5) were evaluated by ELISA for levels of (A,B) soluble terminal complement complex (TCC, C5b-9) and D-dimer
After toxin, the thrombocytopenia (C,D) and steady or increasing fibrinogen levels (% change from T0; E,F) are consistent with development of hemolytic uremic syndrome and acute kidney injury in these models. In contrast, bacteremia induced by i.v. challenge with pathogenic *E.coli* (O, A-G) or attenuated *B.anthracis* (dashed ●, A), resulted in rapid and robust rises in complement activation accompanied by increased D-dimer and consumption of platelets and fibrinogen, consistent with disseminated intravascular coagulation. Means are plotted with individual animal values to show variability between animals. Significant differences from T0 (mean of each Stx group): *p<0.05, **p<0.01, ***p<0.001
Figure 2.2. Cell injury markers. Challenge of baboons with lethal Stx1 (▲, left) or Stx2 (●, right) led to increases in plasma and urine levels of HMGB1 (A-D) and histones (E-H). Stx1 led to earlier and higher levels, consistent with a more pro-inflammatory environment after this toxin (Stearns-Kurosawa et al, 2010). Plasma HMGB1 increased modestly after i.v. challenge with pathogenic *E.coli*
(O, A), returning to baseline values within 2 days after this sub-lethal challenge.

Means are plotted with individual animal values to show variability.
CHAPTER THREE
EXAMINING THE EFFECTS OF SOLUBLE THROMBOMODULIN TREATMENT IN A MURINE MODEL OF EHEC INFECTION

3.1 Introduction

Thrombomodulin (TM) is a transmembrane protein expressed on the surface of endothelial cells and plays important roles in both coagulation and inflammation (Weiler et al, 2003). TM is composed of five structural domains: N-terminal C-type lectin-like domain, EGF-like domain, O-glycosylation-rich domain, transmembrane domain, and the short cytoplasmic domain (Ito et al, 2011). The EGF-like domain mediates TM-thrombin binding and is a critical domain for anticoagulant activity (Dittman et al, 1990). The TM-thrombin complex activates protein C to produce activated protein C (APC), which, in the presence of protein S, inactivates factors VIIIa and Va, thereby inhibiting further thrombin formation and shifting to an anti-coagulant environment (Yamakawa et al, 2011).

Thrombomodulin has been shown to play an important role in the attenuation of the inflammatory response through APC-dependent and APC-independent mechanisms. The anti-inflammatory properties of the C-type lectin-like domain of TM are considered APC-independent since this domain is dispensable for APC generation (Ito et al, 2011). TM binds to C3b and factor H and negatively regulates complement by promoting complement factor I-
mediated inactivation of C3 (Delvaeye et al, 2009). TM also inactivates anaphylatoxins C3a and C5a by stimulating activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (Oikonomopoulou et al, 2011). The lectin-like domain also binds to Lewis Y antigen in lipopolysaccharide (LPS) and neutralizes inflammatory responses induced by LPS and Gram-negative bacteria (Shi et al, 2008). Abeyama et al (2005) reported that the lectin-like domain of TM sequesters high mobility box 1 (HMGB1), a molecule with potent cytokine-like activity, and interferes with its pro-inflammatory activities. TM further reduces the pro-inflammatory activity of HMGB1 by enhancing the thrombin-mediated proteolytic degradation of HMGB1 (Ito et al, 2008). One of the major mechanisms that amplifies inflammation is through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation and nuclear translocation from the cytosol (White et al, 2000; Joyce et al, 2002). APC decreases NF-kB activation and nuclear translocation and leads to reduce synthesis of a variety of inflammatory mediators including pro-inflammatory cytokine production (Esmon, 2012). APC has also been shown to exert cytoprotective effects including anti-apoptotic activity and endothelial barrier stabilization (Ito et al, 2011). These cytoprotective effects are mainly mediated by protease activated receptor-1 (PAR-1) and endothelial protein C receptor (EPCR) expressed on the surface of endothelial cells and leukocytes (Griffin et al, 2007).
Recombinant human soluble thrombomodulin (sTM) is currently approved for clinical treatment of disseminated intravascular coagulation (DIC) in Japan. Two recent studies successfully treated pediatric patients who developed EHEC-induced HUS using sTM as a therapeutic (Honda et al, 2013; Kawasaki et al, 2013). Both Honda et al (2013) and Kawasaki et al (2013) observed improved renal function from sTM treatment and reported increased urine output, decreased serum creatinine, decreased proteinuria and hematuria, and normalization of coagulation related markers. In both cases, no adverse effects, including hemorrhaging or liver dysfunction, were observed.

Nonhuman primates that developed Stx2-induced HUS had increased levels of pro-inflammatory cytokines and chemokines in plasma and urine (Stearns-Kurosawa et al, 2010; Stearns-Kurosawa et al, 2013). A recent mouse model of EHEC infection was developed by genetically modifying *Citrobacter rodentium* to express Stx2 (Mallick et al, 2012). *C. rodentium* is a natural mouse pathogen that is related and used to study *E. coli* infection due to the its ability to develop attaching and effacing (A/E) lesions with the intestinal epithelium (Mundy et al, 2005). Although this model does not develop the full requirements to be defined as HUS, infected mice do develop Stx-mediated disease from intestinal colonization (Mallick et al, 2012). The Stx2-producing *C. rodentium* provides a model for EHEC colonization and toxin-mediated disease in mice and better mimics the colonization observed in humans (Mallick et al, 2012).
Our lab has adopted the Stx2-producing *C. rodentium* model to identify potential therapeutics to reduce mortality and disease severity. Soluble thrombomodulin is one possible therapeutic that has shown promising results in pediatric patients with EHEC-induced HUS (Honda *et al.*, 2013; Kawasaki *et al.*, 2013). In this study, we examined the effects of sTM treatment on mice infected with *C. rodentium* expressing Stx2 by examining survival, weight loss, colonization, BUN, and acute kidney injury (AKI) markers NGAL and KIM-1.

3.2 Material and Methods

3.2.1 Murine Model of EHEC Infection

Six-week old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were challenged with *Citrobacter rodentium* that expresses a Stx2 phage (Cr Stx2+) or non-toxin expressing control phage (Cr WT) (Mallick *et al.*, 2012). Bacteria were grown in LB broth containing chloramphenicol (10 ug/ml; Cr Stx2+) or both chloramphenicol and kanamycin (25 ug/ml; Cr WT) to an OD$_{600}$ of 0.75-1.0. This OD$_{600}$ range corresponds to approximately 1x10$^9$ CFU/ml according to an OD$_{600}$ versus CFU standard curve. Bacteria were centrifuged and resuspended in sterile saline. Mice were gavaged with approximately 1x10$^9$ CFU in 100 ul of sterile saline. Inoculum concentration was determined by serial dilution plating on appropriate restrictive antibiotic LB agar plates.
Weight was monitored daily and blood was collected by facial vein bleed in 35mM of EDTA periodically. Samples were centrifuged and plasma stored at -80°C until assay. Mice were euthanized upon reaching euthanasia criteria (loss of ≥15% body weight or behavioral changes). *C. rodentium* fecal shedding was determined by serial dilution plating of fecal slurries (10%w/v in PBS) on LB agar plates with appropriate restrictive antibiotics. Plates were incubated inverted overnight at 32°C and bacterial colonies were counted the following day. Organs harvested at necropsy were stored in either RNAlater (Ambion, Austin, TX) and stored at -80°C or in 10% neutral buffered formalin for downstream tissue processing. Plasma blood urea nitrogen (BUN) concentration was determined using the QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA).

*C. rodentium* strains were generously provided by Dr. John M. Leong (Department of Molecular Biology and Microbiology, Tufts University Medical Center, Boston, MA). Animals were housed and used in accordance with approved IACUC and IBC protocols from Boston University School of Medicine.

### 3.2.2 Soluble Thrombomodulin

For recombinant human soluble thrombomodulin (sTM; Asahi Kasei Pharma, Tokyo, Japan) experiments, mice were challenged as described above and given daily IP injections of sTM (6 ug/g) until 9 days post infection. The
recombinant sTM is purified from transformed Chinese hamster ovary cells and is composed of the extracellular domain of TM (Gomi et al, 1990).

### 3.2.3 RNA Isolation from Kidneys

Tissue samples stored at -80°C in RNAlater were thawed on ice. Tissue pieces <20mg in weight were added to Buffer RLT (Qiagen, Hilden, Germany) and 3.5% 2-merceptoethanol. Tissue was lysed using a 5mm stainless steel bead in the Tissue Lyser II for 4 minutes at 25Hz (Qiagen). Total RNA was isolated from tissue lysate using the RNeasy Plus Mini Kit (Qiagen) and QIAcube (Qiagen) following the manufacturer’s instructions. RNA concentration was quantified by a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA).

### 3.2.4 Reverse Transcription

Total RNA was converted to cDNA using Quantifast Reverse Transcription Kit (Qiagen) and Thermocycler (Applied Biosystems, Beverly, MA) following the manufacturer’s instructions. A total RNA concentration of 250 ng was used for each reaction.

### 3.2.5 Quantitative PCR for NGAL and KIM-1
Amplification of cDNA was performed in a Step One Plus qPCR machine (Applied Biosystems) using a Quantifast SYBR Green PCR Kit (Qiagen) according to manufacturer’s instructions. Primers sets (Integrated DNA Technologies, Coralville, IA) for murine neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and hypoxanthine guanine phosphoribosyl transferase (HPRT) were used at a final concentration of 1μMol/liter and are listed in Table 3.1. Obtained cycle threshold (CT) values were normalized as follows: \[ \frac{(2^{\text{CT gene}})}{(2^{\text{CT HPRT}})} / \text{total RNA in RT reaction} \].

### 3.2.6 Statistical Analysis

Analysis of variance (ANOVA) tests followed by Tukey post tests were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) to compare data collected from Cr Stx2+ & sTM and Cr WT to either Cr Stx2+ or all groups at different time points for multiple outcomes. This software was used to determine significant differences among the groups of mice, if present.
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<tr>
<th>Target</th>
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<tr>
<td></td>
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3.3 Results and Discussion

*Citrobacter rodentium* lysogenized with the Shiga toxin 2 gene carrying phage produces mucus-activatable Stx, provides a model that features prototypic A/E lesions during intestinal colonization, Stx-mediated weight loss and death, renal dysfunction, and renal tubular injury similar to murine toxin injection models (Mallick *et al*, 2012). Mice infected with approximately 1x10⁹CFU/100ul of Cr Stx2+ corresponded with mortality or severe disease warranting euthanasia between 6 to 9 days after infection (Fig 3.1A). Control mice infected with non-toxin producing *C. rodentium* (Cr WT) all survived and were euthanized on day 14 for sample collection (Fig 3.1A). Treatment with sTM on mice infected with Cr Stx2+ did not alter overall survival outcome, but did delay death a few days (Fig 3.1A). The Stx2-producing *C. rodentium* model does not feature thrombocytopenia and anemia seen in patients with HUS and most likely does not benefit from the anti-coagulant properties associated with survival in patients with EHEC-HUS (Mallick *et al*, 2012; Honda *et al*, 2013). Typical infection of mice with Cr Stx2+ saw slight rise in weight between days 1 to 4 and declined significantly by days 6 to 9 compared to Cr WT (Fig 3.1B). Mice infected with Cr Stx2+ lost > 15% of their starting weight by 8 days after infection, whereas sTM treatment mice lost >15% body weight by day 12 (Fig 3.1B). Although sTM mice declined significantly by day 4 compared to no treatment Cr Stx2+ mice, the weight loss was gradual and was significantly higher on Cr Stx2+ endpoint days
8 and 9 (Fig 3.1B). Interestingly, sTM treatment did significantly decrease fecal shedding of Cr Stx2+, with similar kinetics to Cr WT, compared to no treatment Cr Stx2+ infected mice (Fig 3.1C). Neutralizing antibodies to Stx2 have been shown to reduce colonization in mice infected with *E. coli* O157:H7 (Mohawk *et al*, 2010). Although sTM does not have Stx2 neutralizing capabilities, the anti-inflammatory and cytoprotective properties may contribute to decreased bacterial burden in the intestines and extend survival.

Soluble thrombomodulin improved kidney function in pediatric patients with EHEC-HUS so we examined three markers of renal dysfunction. Treatment with sTM did not decrease plasma levels of BUN compared to no treatment and both Cr Stx2+ and Cr Stx2+ with sTM were significantly higher than Cr WT (Fig 3.2A). Humans and non-human primates express Gb₃ on renal glomerular endothelial cells and are the primary target from Stx2 injury (Stearns-Kurosawa *et al*, 2013). Mice express Gb₃ on renal epithelial cells in the proximal tubules, distal tubules, and collecting duct so thrombocytopenia and anemia does not manifest, as they are associated with endothelial injury from Stx2 (Tesh *et al*, 1993; Rutjes *et al*, 2002; Psotka *et al*, 2009). Both NGAL and KIM-1 are markers for renal injury in the proximal tubules of human and mice (Ichimura *et al*, 1998; Mishra *et al*, 2003). Treatment with sTM in Cr Stx2+ infected mice significantly decreased mRNA level expression of NGAL and KIM-1 in kidneys harvested at euthanasia compared to no treatment (Fig 3.2B,C). As expected, Cr WT infected
mice showed no sign of renal pathology. Anti-Stx2 antibody rescues mice injected with lethal doses of Stx2 and improves serum BUN levels (Sauter et al, 2008). Soluble thrombomodulin’s cytoprotective properties decreased renal NGAL and KIM-1 expression but was not able to decrease BUN and prevent kidney dysfunction caused by Stx2.

Although sTM was not able to prevent mortality and kidney injury based on BUN levels, it did delay weight loss and death, decrease NGAL and KIM-1 gene expression, and decrease intestinal colonization of Cr Stx2+. Soluble thrombomodulin has been shown to be a promising therapeutic in human HUS patients and would be worth exploring in the non-human primate baboon toxemia model of HUS. Baboons (Papio) injected with 50ng/kg of Stx2 develop full spectrum HUS including thrombocytopenia, hemolytic anemia, and AKI with glomerular endothelial injury (Stearns-Kurosawa et al, 2010). The effects of sTM may be more significant in this model due to the coagulopathy and injury to the renal glomerular endothelium. Soluble thrombomodulin treatment in Cr Stx2+ infected mice requires further characterizing but is a promising therapeutic for the treatment of EHEC induced HUS in human patients.
Figure 3.1. Treatment with sTM in *C. rodentium* (Cr Stx2+) infected mice delays mortality and decreases colonization. Mice were infected with...
approximately $1 \times 10^9$ CFU/100ul with either Cr WT (●; n=10), Cr Stx2+ (■; n=14), or Cr Stx2+ & sTM (▲; n=10). (A) Percent survival of groups of 6-week-old C57BL/6 mice infected with either Cr WT, Cr Stx2+, or Cr Stx2+ with sTM treatment. *** P<0.001 compared to Cr Stx2+ by 2-way ANOVA followed by Tukey post tests. (B) Body weight during infection of 6-week-old mice, expressed as percent change from day 0. Mean values and standard deviation are shown. * P<0.05, ** P<0.01, **** P<0.0001 compared to Cr Stx2+ by 2-way ANOVA followed by Tukey post tests. (C) Colonization of 6-week-old mice determined by viable stool counts. Mean values and standard deviation are shown. ** P<0.01, **** P<0.0001 compared to Cr Stx2+ by 2-way ANOVA followed by Tukey post tests.
A

Relative KIM-1 expression/ug RNA

Cr WT
Cr Stx2+
Cr Stx2+ & sTM

Relative NGAL expression/ug RNA

B

BUN (mg/dl)

****
***
**

0
20
40
60
80
100
120

C

Relative KIM-1 expression/ug RNA

0
20
40
60
80
100
120

0
20
40
60
80
100
120

Cr WT
Cr Stx2+
Cr Stx2+ & sTM
Figure 3.2. Treatment with sTM decreases kidney injury marker expression but does not alter plasma BUN levels. Kidney injury was examined in mice infected with Cr WT (●; n=10), Cr Stx2+ (■; n=10), or Cr Stx2+ & sTM (▲; n=8). (A) BUN of 6-week-old mice, each represented as a single data point, from EDTA-plasma samples at day of necropsy. *** P<0.001, **** P<0.0001 compared with all other groups by 1-way ANOVA followed by Tukey post tests. (B) and (C) Measurement of AKI marker gene transcripts in kidneys. Each animal represents a single data point. Kidney tissues were processed to obtain total RNA for the generation of cDNA and qPCR was performed for the indicated gene as described in Materials and Methods. * P<0.05, ** P<0.01, *** P<0.001 compared with all other groups by 1-way ANOVA followed by Tukey post tests.
CHAPTER FOUR

SUMMARY AND CONCLUSION

In these studies, we have determined that (1) complement activation is not required for the development of thrombotic microangiopathy and HUS induced by EHEC Shiga toxins, and benefits or risks of therapeutic complement inhibition should be carefully considered before applying in human EHEC patients; and (2) soluble thrombomodulin treatment in mice infected with Stx2-producing C. rodentium reduced renal cellular injury, but was not sufficient to alter mortality. Both animal models feature the severe kidney injury caused by Shiga toxins and highlight key pathology seen in human patients. Soluble thrombomodulin has already been identified as a promising therapeutic in human patients with HUS but should further be characterized in animal models of HUS and EHEC infection to determine the pharmacodynamics and potential adverse side effects with treatment.
## LIST OF JOURNAL ABBREVIATIONS

<table>
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Taylor, F.B., et al. Staging of the pathophysiologic responses of the primate microvasculature to *Escherichia coli* and endotoxin: examination of the


CURRICULUM VITAE

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EDUCATION

Master of Arts in Pathology (2014)
Pathology and Laboratory Medicine, Boston University School of Medicine,
Boston, MA
Master’s Thesis: Examining Inflammatory Mechanisms and Potential
Cytoprotective Therapeutics in Animal Models of Shiga Toxin Induced Kidney
Injury (supervisors: Drs. Deborah Stearns-Kurosawa and Shinichiro
Kurosawa)

Bachelor of Science in Biology, Minor: Chemistry (2012)
School of Biological Sciences, University of Northern Colorado, Greeley, CO
Ronald E. McNair Scholar’s Thesis: Cloning of Immunoglobulin cDNAs from
the Jamaican Fruit Bat (Artibeus jamaicensis) (supervisors: Drs. Ann C.
Hawkinson and Tony Schountz)

RESEARCH EXPERIENCES

Master’s Thesis, December 2012 – Present
Pathology and Laboratory Medicine, Boston University School of Medicine,
Boston, MA
PIs: Drs. Deborah Stearns-Kurosawa and Shinichiro Kurosawa
• Explored the role of complement in the development of Escherichia coli
  Shiga toxin induced hemolytic uremic syndrome in non-human primates
• Established a Shiga toxin mouse model using genetically modified
  Citrobacter rodentium expressing Shiga toxin 2
• Examined potential therapeutics to prevent and/or alleviate kidney injury in
  our Shiga toxin animal models

Master’s Rotation, September 2012 – December 2012
Pathology and Laboratory Medicine, Boston University School of Medicine,
Boston, MA
PI: Dr. Jacqueline Sharon
• Identified epitopes for a viable and efficient vaccine for Francisella tularensis
• Tested antibody production to F. tularensis antigens LPS and GroEL by ELISA

Master’s Rotation, June 2012 – September 2012
Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA
PIs: Drs. Deborah Stearns-Kurosawa and Shinichiro Kurosawa
• Challenged Vero cells with Shiga toxins 1 or 2 to determine cell viability
• Studied the proteolytic processing of TVP peptide that leads to cell permeability and/or rescue activity from Shiga toxins 1 and 2

Research Assistant, August 2010 – May 2012
School of Biological Sciences, University of Northern Colorado, Greeley, CO
PIs: Drs. Tony Schountz and Ann C. Hawkinson
• Characterized Jamaican fruit bat (Artibeus jamaicensis) immunoglobulins
• Cloned, isolated, and sequenced A. jamaicensis IgA, IgM, and IgG genes
• Worked on the expression of bat immunoglobulins and Tacaribe virus nucleocapsid

Research Technician, January 2009 – May 2010
School of Biological Sciences, University of Northern Colorado, Greeley, CO
PI: Dr. Gregory DeKrey
• Maintained proper laboratory function and service
• Aided fellow undergraduate and graduate students in active projects
• Have experience in handling and experimenting on rodents

RESEARCH INTERESTS
Infectious disease, pathogen-host interactions, immunology, pathology

PUBLICATIONS

PROFESSIONAL PRESENTATIONS

June 2013. Benjamin Lee, Chad Mayer, Caitlin Leibowitz, Deborah Stearns-Kurosawa and Shinichiro Kurosawa. Complement is not activated in nonhuman primates during development of hemolytic uremic syndrome and thrombotic microangiopathy induced by E. coli Shiga toxins. XXIV Congress of the International Society of Thrombosis and Haemostasis. Amsterdam, Netherlands. (Talk)


*Dr. Schountz directed me to maintain the same title in various poster and oral presentations. New data was added to subsequent presentations.


TEACHING AND MENTORING EXPERIENCES

Foster Care Mentor, August 2013 – Present
Cambridge Family & Children’s Services, Cambridge, MA
• Provided one on one mentoring to an adolescent in the Intensive Foster Care program

Teaching Assistant, August 2011 – May 2012
School of Biological Sciences, University of Northern Colorado, Greeley, CO
• Assisted instructor with preparation and instruction of undergraduate laboratories including general biology for majors, advanced human anatomy and physiology and microbiology

Biology Tutor, August 2011 – May 2012
School of Biological Sciences, University of Northern Colorado, Greeley, CO
• Provided individual and group tutorial assistance to students in general biology and advanced human anatomy and physiology

**CHE Student Mentor**, August 2011 – May 2012
Center for Human Enrichment, University of Northern Colorado, Greeley, CO
• Provided tutoring and support to freshman and sophomore first-generation undergraduate students

**WORK EXPERIENCES**

**Laboratory Assistant/Business Support**, November 2010 – August 2011
Summit Pathology Labs, Loveland, CO
• Assessed specimen quality, made any necessary corrections and prepared specimens for grossing and processing
• Accessioned patient information using Ligolab software
• Immunostained tissue specimens for pathologists
• Provided customer service to patients, physicians, and office personnel to ensure proper medical treatment

**Program Assistant**, August 2008 – May 2011
Asian Pacific American Student Services, University of Northern Colorado, Greeley, CO
• Completed clerical duties for office personnel
• Provided support and resources for students, staff and community
• Programmed educational, social and outreach programs to bring awareness of Asian and Pacific Islander cultures

**CLINICAL AND VOLUNTEER EXPERIENCES**
• **Department of Pathology Observations**, Boston Medical Center, 2013
• **Emergency Department Volunteer**, East Boston Neighborhood Health Center, 2013
• **Success in Biology Group Discussion for Freshmen**, University of Northern Colorado, 2011
• **24/7 Kids Respite Night**, First Congregational Church, 2009 – 2011
• **Quiet at Night Rounds/Patient Transport/Pediatrics**, Northern Colorado Medical Center, 2009 – 2011
• **Relay for Life**, University of Northern Colorado 2009 – 2010
• **Habitat for Humanity**, 2008 – 2011
EXTRA-CURRICULAR AND LEADERSHIP EXPERIENCES

Beta Beta Beta National Biological Honor Society, 2010 – 2012
University of Northern, Greeley, CO
• Secretary and Treasurer 2011 – 2012

Student Senate, 2010 – 2012
University of Northern, Greeley, CO
• College of Natural and Health Sciences Representative, 2010 – 2012

College of Natural and Health Sciences Student Council, 2010 – 2012
University of Northern, Greeley, CO
• President, 2010 – 2012

Biological Student Association, 2009 – 2011
University of Northern, Greeley, CO
• Vice President, 2010 – 2011

Lu’au Committee, 2010 – 2011
University of Northern, Greeley, CO
• Country Store/Children’s Corner Chair, 2010 – 2011

Ha’aheo ‘O Hawai’i Club, 2008 – 2011
University of Northern, Greeley, CO
• Vice President, 2010 – 2011
• Treasurer, 2009 – 2010

HONORS, AWARDS, AND GRANTS

• Robert and Ludie Dickeson Presidential Prize for Leadership Award, 2012
• UNC Research Day School of Biological Sciences Award, First Place, 2012
• UNC Research Excellence Award Finalist for Undergraduate Poster Presentation, First Place, 2012
• Beta Beta Beta National Biological Honor Society Frank G. Brooks Award for Excellence in Student Research, Third Place, 2012
• UNC Fall Undergraduate Research Symposium Finalist, 2011
• BIOTA Supply Fund Grant, 2011
• BIOTA Travel Grant, 2011
• Annual Biomedical Research Conference for Minority Students Travel Grant, 2011
• Rocky Mountain Branch American Society for Microbiology Undergraduate Oral Presentation, Third Place, 2011
• Rocky Mountain Branch American Society for Microbiology Travel Award, 2011
• Beta Beta Beta National Biological Honor Society Frank G. Brooks Award for Excellence in Student Research, First Place, 2011
• UNC Summer Undergraduate Research Stipend, 2011
• Beta Beta Beta National Biological Honor Society Research Grant, 2010 – 2012
• UNC Center for Human Enrichment Academic Honors, 2008 – 2011
• UNC Cultural Center Academic Achievement Award, 2008 – 2011
• UNC National Undergraduate Scholarship, 2008 – 2011
• BIOTA Scholars Program Scholarship, 2008 – 2010

PROFESSIONAL AFFILIATIONS
• American Society for Microbiology, 2011 – 2013
• Beta Beta Beta National Biological Honor Society, 2010 – Present
• Ronald E. McNair Scholar’s Program, 2010 – Present