Quiescent complement in nonhuman primates during *E. coli* Shiga toxin-induced hemolytic uremic syndrome and thrombotic microangiopathy

Benjamin C. Lee, Chad L. Mayer, Caitlin S. Leibowitz, DJ Stearns-Kurosawa*, Shinichiro Kurosawa*

Department of Pathology and Laboratory Medicine, Boston University School of Medicine

Short Title: Quiescent complement in Stx-HUS

Scientific Category: Thrombosis and Haemostasis

*Corresponding Authors:

Shinichiro Kurosawa, MD, PhD
Tel: 617-414-7091
Email: kurosawa@bu.edu

DJ Stearns-Kurosawa, PhD
Tel: 617-414-7092
Email: dstearns@bu.edu

Department of Pathology and Laboratory Medicine
Boston University School of Medicine
670 Albany Street
Boston, MA 02118
Fax: 617-414-7073
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

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.
**Introduction**

Shiga toxin-producing enterohemorrhagic *Escherichia coli* (EHEC) is an emerging food-and water-borne pathogen.\(^1\) 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,\(^2\) 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.\(^3\) 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.\(^4\) 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,\(^5,7\) but *in vitro* data arise from toxin challenges ~500,000 times higher than the 5-20 pg/ml Stx levels observed in infected children.\(^8\) Eculizumab was seemingly beneficial in three children with severe EHEC-HUS,\(^9\) 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,\textsuperscript{10,11} with glomerular thrombotic microangiopathy.\textsuperscript{12} 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.

**Methods**

**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.\textsuperscript{10,11} 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.

**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 $5\times10^9$ CFU/kg *E. coli* B7 O86a:K61 (SLEC; not toxigenic)\textsuperscript{13} or lethal $3\times10^9$ CFU/kg *Bacillus anthracis* Sterne strain 34F2 (vaccine strain)\textsuperscript{14} were positive controls. Data were analyzed for differences between groups using Student’s T-test, assuming equal variance.
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.\textsuperscript{15,16} 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\textsuperscript{10} or renal pathology\textsuperscript{12}, 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 1A; n=6) or Stx2 (Fig 1B; n=5) up until the time of euthanasia at 49.5-128 hours post-challenge. Platelet levels declined (Fig1 C,D) and fibrinogen levels were steady or increased (Fig1 E,F) as expected during development of HUS. Renal glomerular thrombotic microangiopathy was observed by pathology evaluation after necropsy\textsuperscript{12} and progressively increasing D-dimer levels (Fig1 G,H) confirm coagulation activation and subsequent fibrinolysis. The lack of complement activation was surprising, given the known crosstalk between coagulation and complement pathways\textsuperscript{17} and markers of complement activation in some EHEC-HUS patients.\textsuperscript{6,7} To confirm integrity of the assays with baboons, the well-characterized baboon model of \textit{E.coli} bacteremia sepsis with disseminated intravascular coagulation (DIC)\textsuperscript{13,18,19} was evaluated similarly. Sub-lethal challenge with this \textit{E.coli} strain induced complement activation and DIC as judged by thrombocytopenia, fibrinogen consumption, and rapid increases in D-dimer (Fig1 A,C,E,G). Complement inhibition after high dose of this \textit{E.coli} strain in baboons significantly reduces the consumptive coagulopathy and
inflammation. Similarly, soluble TCC in a baboon challenged with i.v. Gram positive attenuated *Bacillus anthracis* peaked at 2,204.99 mAU/ml by 10 hours post-challenge, slowly declining over the next 4 days (Fig1 A).

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. Both DAMPs were detected in baboon plasma and urine after Stx challenge (Fig2) with generally earlier rises after Stx1, consistent with earlier increases in inflammation cytokines and chemokines.

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. Yet complement activation is reported in some EHEC-HUS patients. It is not clear whether Eculizumab was effective during the German 2011 outbreak, 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. 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. 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 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.

Acknowledgements
The authors gratefully acknowledge Valta Freeman, Scott Freeman and Diann Debord for technical assistance, and Lyndianne Joseph for administrative assistance. This work was supported by NIH/NIAID grants RO1 AI058107, UO1 AI1075386, and U19 AI062629 (to S.K.); R42AI062095 (S. Opal, subcontract to SK). Animals were obtained from Baboon Research Resources, supported by NIH P40OD010988 (G.White).

Authorship Contributions
BL performed the immunoassays; SK and DSK conceived the hypothesis and designed the experiments; all authors contributed to data interpretation and writing the manuscript; and all authors approved the final manuscript.

Disclosures Conflicts of Interest
The authors declare no conflicts of interest
References


Figure Legends

Figure 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 (G,H). 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 (○, 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. 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. Plasma HMGB1 increased modestly after i.v. challenge with pathogenic E.coli (○, A), returning to baseline values within 2 days after this sub-lethal challenge. Means are plotted with individual animal values to show variability.
Figure 1

Stx1

Stx2

A

B

C

D

E

F

G

H

Plasma TCC (mAU/ml)

Plasma TCC (mAU/ml)

Platelet (E-3/10^9/mm^3)

Platelet (E-3/10^9/mm^3)

Fibrinogen (% baseline)

Fibrinogen (% baseline)

D-dimer (ng/ml)

D-dimer (ng/ml)
Figure 2

Stx1

A

Plasma HMGB1 (ng/ml)

Time (hrs)

0 24 48 72 96

B

Plasma HMGB1 (ng/ml)

Time (hrs)

0 24 48 72 96

C

Urine HMGB1 (ng/ml)

Time (hrs)

0 24 48 72 96

D

Urine HMGB1 (ng/ml)

Time (hrs)

0 24 48 72 96

E

Plasma Histone (U/ml)

Time (hrs)

0 24 48 72 96

F

Plasma Histone (U/ml)

Time (hrs)

0 24 48 72 96

G

Urine Histone (U/ml)

Time (hrs)

0 24 48 72 96

H

Urine Histone (U/ml)

Time (hrs)

0 24 48 72 96
Quiescent complement in nonhuman primates during *E. coli* Shiga toxin-induced hemolytic uremic syndrome and thrombotic microangiopathy

Benjamin C. Lee, Chad L. Mayer, Caitlin S. Leibowitz, D. J. Stearns-Kurosawa and Shinichiro Kurosawa