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Alternative Pathway Activation of Complement by Shiga Toxin Promotes Exuberant C3a Formation That Triggers Microvascular Thrombosis

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Shiga toxin (Stx)-producing *Escherichia coli* O157:H7 has become a global threat to public health; it is a primary cause of diarrhea-associated hemolytic uremic syndrome (HUS), a disorder of thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure with thrombus occluding renal microcirculation. In this study, we explored whether Stx triggers complement-dependent microvascular thrombosis in vivo and in vitro and in vivo experimental settings of HUS. Stx induced on human microvascular endothelial cell surface the expression of P-selectin, which bound and activated C3 via the alternative pathway, leading to thrombus formation under flow. In the search for mechanisms linking complement activation and thrombosis, we found that exuberant complement activation in response to Stx generated an increased amount of C3a that caused further endothelial P-selectin expression, thrombomodulin (TM) loss, and thrombus formation. In a murine model of HUS obtained by coinjection of Stx2 and LPS and characterized by thrombocytopenia and renal dysfunction, upregulation of glomerular endothelial P-selectin was associated with C3 and fibrinogen deposits, platelet clumps, and reduced TM expression. Treatment with anti–P-selectin Ab limited glomerular C3 accumulation. Factor B-deficient mice after Stx2/LPS exhibited less thrombocytopenia and were protected against glomerular abnormalities and renal function impairment, indicating the involvement of complement activation via the alternative pathway in the glomerular thrombotic process in HUS mice. The functional role of C3a was documented by data showing that glomerular fibrinogen, platelet clumps, and TM loss were markedly decreased in HUS mice receiving C3AR antagonist. These results identify Stx-induced complement activation, via P-selectin, as a key mechanism of C3a-dependent microvascular thrombosis in diarrhea-associated HUS. The Journal of Immunology, 2011, 187: 172–180.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 is a foodborne or waterborne pathogen, responsible for worldwide spread of hemorrhagic colitis complicated by diarrhea-associated hemolytic uremic syndrome (D+HUS), a disorder of thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure that mainly affects infants and small children (1–3). Death or end-stage renal disease occurs in ∼12% of patients with D+HUS, and 25% of survivors demonstrate long-term renal sequelae (4). Over the last two decades, *E. coli* O157:H7 has been the cause of multiple outbreaks, becoming a public health problem in both developed and developing countries (1, 5–7). Apart from supportive therapy, there are presently no specific treatments for D+HUS (8), and strategies including STEC-component vaccines, Stx receptor mimics, and Abs against Stx are still under investigation (9). After STEC ingestion, Stx is transported in the circulation to the capillary bed of target organs, including the kidney (5, 6). Glomerular endothelium that expresses the receptor globotriaosyl ceramide (Gb3)/CD77 (10, 11) is the main target of the toxic effects of Stx1 and 2 (12), which activate a cascade of signals contributing to vascular dysfunction, leukocyte recruitment, and thrombus formation (13–19). We previously reported that Stx promoted von Willebrand factor (VWF)-dependent thrombus growth on microvascular endothelium under flow via upregulation of P-selectin (17). In patients with D+HUS, elevated plasma levels of P-selectin were measured during the acute phase, which reflected increased P-selectin expression by activated endothelial cells and platelets (20). P-selectin overexpressed on activated platelets and transfected Chinese hamster ovary cells had the capacity to activate the complement (C) system by acting as C3b binding protein, thereby generating the anaphylatoxins C3a and C5a and the membrane attack complex (C5b-9) (21). C3a and C5a, through binding to G-protein coupled receptors, elicit inflammatory responses such as cytokine production, adhesion molecule expression, and increased vascular permeability, leading to endothelial cell activation (22–24).

Whether C activation at renal endothelial level may contribute to microangiopathic lesions in D+HUS has never been addressed.

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Abbreviations used in this article: Bf, factor B-deficient; BUN, blood urea nitrogen; C, complement; D+HUS, diarrhea-associated hemolytic uremic syndrome; HMEC, human microvascular endothelial cells; HS, human serum; KIU, kallikrein inhibitor units; pM, picomolar; RU, resonance units; sCR-1, soluble C receptor-1; SPR, surface plasmon resonance; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; TM, thrombomodulin; t-PA, tissue-plasminogen activator; VWF, von Willebrand factor.

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There are anecdotes on reduced C3 and augmented C3b, C3c, and C3d serum levels in patients with active D+HUS (25–27). High plasma levels of Bb and C3b-9 were recently measured in 17 children with D+HUS, indicating C activation via alternative pathway during the onset of D+HUS (28). Another study reported that Stx2 activated C in the fluid phase in vitro (29). However, the use of huge amounts of Stx2, together with the observation of no active Stx in plasma of patients with D+HUS (30), raises concerns about the pathophysiological meaning of these results.

In this study, we investigated in vitro whether Stx activated C via the alternative pathway by promoting binding of C3 on microvascular endothelial P-selectin under flow conditions. The role of C in favoring thrombus formation on endothelial cells in response to Stx was evaluated. A novel mechanism by which C3a generated by Stx-induced C activation impaired endothelial thromboresistance was explored. To validate the in vitro findings, we assessed the causal role of glomerular P-selectin on C3 deposition in a mouse model of HUS obtained by coinjection of Stx2 and LPS. The involvement of C activation via the alternative pathway in the development of microvascular thrombosis was studied applying the HUS model to factor B-deficient (Bf−/−) mice. Finally, the contribution of C3a in the thrombogenic process induced by Stx was assessed by blocking C3a receptor in HUS mice.

Materials and Methods

Endothelial cell culture and maintenance

The human microvascular endothelial cell line of dermal origin (i.e., HMEC-1, from Dr. Edwin Ades and Francis J. Candal [Centers for Disease Control and Prevention] and Dr. Thomas Lawley [Emory University]) (31) was cultured as described (17). Purified Stx1 (provided by Dr. Helge Karch and Dr. Martina Bielaszewska, Institute for Hygiene, University of Munich, Germany) (32) and commercially available Stx1 and Stx2 (Toxin Technology) were used. Human serum (HS) from a pool of four healthy volunteers was used as a source of C. To remove platelet microparticles, serum was filtered with a 0.22-µm filter (Millipore) before each experiment (33). Fifty percent of HS was chosen as the most appropriate nontoxic dose on the basis of preliminary experiments evaluating C3 deposits.

To study the effect of Stxs on C3 accumulation and thrombus formation, HMEC-1 were treated with medium (control), purified Stx1, or commercially available Stx1 or Stx2 (50 picomolar [pM]) for 24 h in static conditions. Next, cells were perfused or not with 50% HS (3 min) followed or not followed by whole blood in a parallel plate flow chamber at a constant flow rate of 1500 s⁻¹ (60 dynes/cm²) for 3 min (Supplemental Fig. 1). Soluble C receptor-1 (sCR-1; 100 µg/ml, CellDex) was incubated for 20 min with HS before perfusion on HMEC-1. To evaluate whether Stx-induced C activation occurred via the alternative pathway, HS was pretreated for 30 min with MgCl₂-EGTA (5 mM; Sigma). Mice injected with Stx2 or LPS alone. Stx2 was chosen because it is active Stx in plasma of patients with D+HUS (30), raises concerns about the pathophysiological meaning of these results.

Platelet adhesion assay under flow conditions

Perfusion of heparinized whole blood (10 UI/ml) obtained from healthy subjects (prelabeled with the fluorescent dye mepacrine, 10 µM) was performed in a flow chamber (17). After 3 min of perfusion, the endothelial cell monolayer was fixed in acetone. Fifteen images per sample of platelet thrombi on endothelial cell surface were acquired by confocal inverted laser microscope, and areas occupied by thrombi were evaluated by using Image J software.

Experimental model of HUS

Male C57BL/6 mice were obtained from Charles River Italia. Bf−/− mice of C57BL/6 genetic background (34) and C57BL/6 wild type mice were a gift from Dr. Marina Botto (Imperial College, London). For this study, Bf−/− mice and control wild type littermates were obtained by heterozygote X heterozygote matings at the Mario Negri Institute. Genotypes were determined by PCR (34). Animal care and treatment were performed in accordance with institutional guidelines in compliance with national and international laws and policies. Animal studies were approved by the institutional animal care and use committees of the Mario Negri Institute (Milan, Italy). HUS was induced in mice (26–28 g body weight) by i.p. injection of Stx2 (200 ng; Toxin Technology) plus LPS (75 µg, O111:B4; Sigma). Mice injected with saline served as controls. In parallel, some mice were injected with Stx2 or LPS alone. Stx2 was chosen because it is associated with HS clinical isolates more often than Stx1 (6) and because of the evidence that Stx2 injected in mice is more toxic and accumulates in the kidney to a greater extent than Stx1 (35).

The dosing protocol was based on previous studies (16, 36). Mice were sacrificed at 3, 6, 24, or 48 h after injection. Blood platelet count and renal function were measured by serum blood urea nitrogen (BUN; Reflotron test, Roche Diagnostics) were assessed. In separate experiments, C57BL/6 mice were given a 50-µg dose i.e. of a function-blocking anti-mouse P-selectin Ab RB40.34 or an isotype-matched control Ab A110.1 (BD Pharmingen), 1 h before Stx2/LPS. A second dose was administered 6 h later; mice were sacrificed 24 h after Stx2/LPS. The dose of RB40.34 was chosen on the basis of previous studies (37). Other experiments included treatment of mice with the C3aR antagonist SB290157 (15 mg/kg i.p.) or an equal volume of saline and dimethyl sulfoxide (four times per day (1 h before and 1, 4, and 8 h after Stx2/LPS injection).

Renal ultrastructural analysis

Rats were killed by a terminal heart puncture. Kidney tissue was fixed in 3% paraformaldehyde and stained with the following Abs: FITC-conjugated anti-human C3c (11.3 µg/ml; Dako), anti-human C3b-9 (27.6 µg/ml; Calbiochem) followed by FITC-conjugated secondary Ab (13.6 µg/ml; Jackson Immunoresearch Laboratories), anti-human P-selectin (20 µg/ml; R&D Systems) followed by Cy3-conjugated secondary Ab (13.6 µg/ml; Jackson Immunoresearch Laboratories), and anti-human TM (1.50; R&D Systems) followed by Cy3-conjugated secondary Ab. Isotype-matched irrelevant Abs were used as a negative control. Coverslips were examined under confocal inverted laser microscopy (LSM 510 Meta; Zeiss, Jena, Germany). Fifteen fields per sample were acquired, and the area of staining was quantified (pixel²/field) using Image J software (National Institutes of Health, Bethesda, MD). For colocalization studies, samples were imaged by confocal microscopy using a z-scan (eight images acquired at 1.5-µm intervals by vertical projection). TM expression was measured by counting cells with moderate or strong intensity staining in 20 fields per sample. Data were expressed as a percentage of fluorescent cells per total cells in each field.
amined using a transmission electron microscope (Morgagni 268D; Philips, Brno, Czech Republic).

**Immunofluorescence analysis of renal tissue**

Periodate lysine paraformaldehyde (PLP)-fixed frozen sections (3 μm) were incubated with FITC-conjugated goat anti-rat fibrinogen Ab (1:40; Nordic Immunology) and with rhodamine WGA lectin (Vector Laboratories) dissolved in DAPI. Fibrinogen (ogen) deposition in glomerular capillary walls was assessed by confocal microscopy and scored from 0 to 3 (0, absent or trace; 1, weak granular, discontinuous staining; 2, strongly staining; 3, occasional obliteration). P-selectin expression was evaluated in frozen sections incubated with anti-mouse P-selectin Ab (3 μg/ml; BD Pharmingen) or with isotype-matched irrelevant Ab, followed by FITC-conjugated secondary Ab (1:150; Sigma-Aldrich). To determine the location of P-selectin in the glomerulus, adjacent sections were incubated with rabbit anti-VWF (1:100; Dako) followed by Cy3-conjugated anti-rabbit Abs (60 μg/ml; Jackson Immunoresearch Laboratories). Negative controls were obtained with secondary Ab alone. C3 deposits were evaluated by staining with FITC-conjugated anti-mouse C3 Ab (10 μg/ml; Cappel). For C9 staining, rabbit anti-rat C9 (1:200; a gift from Paul Morgan, Cardiff, U.K.) and Cy3-conjugated secondary Abs (30 μg/ml; Jackson Immunoresearch Laboratories) were used. Isotype-matched irrelevant Abs were used as negative controls. Glomerular C3 and C9 staining was scored from 0 to 3 (0, no staining or traces [≤5%]; 1, staining in <25% of the glomerular tuft; 2, staining affecting 26 to 50%; 3, staining in >50%). TM was detected in PLP-fixed sections using rabbit anti-TM Ab (1:25; Santa Cruz) followed by Cy3-conjugated goat anti-rabbit IgG (15 μg/ml; Jackson Immunoresearch Laboratories). Glomerular TM expression was estimated using Image J software.

**Statistical analysis**

Results are expressed as means ± SEM. Data were analyzed by the nonparametric Mann–Whitney or Kruskal–Wallis tests, or by ANOVA as appropriate. The *p* values <0.05 were considered statistically significant.

**Results**

**Stx promotes endothelial C3 activation and deposition via the alternative pathway**

To test whether C activation by Stxs involves C3 activation and deposition, we evaluated C3 deposition on endothelial cell surface in response to the toxin. HMEC-1 were incubated with purified Stx1 (50 pM, 24 h) and then perfused in a parallel plate flow chamber (60 dynes/cm², 3 min) with 50% HS as a source of C (Supplemental Fig. 1). Under these conditions, monolayer integrity was preserved (Supplemental Fig. 2A). A marked increase of C3 deposits on Stx1-treated cells was observed with respect to unstimulated cells (Fig. 1A, 1B), indicating that endothelial cells activated by Stx fixed C. Similar results were obtained using non-purified, commercially available Stx1 and Stx2 (Supplemental Fig. 2B). No C5b-9 deposits were detected on Stx-treated HMEC-1 after the short HS perfusion (not shown).

When HS was incubated with the sCR-1—an inhibitor of classical, alternative, and lectin pathways of C activation—endothelial C3 deposits induced by Stx1 were abolished, indicating specificity of the C3 signal (Fig. 1A, 1B). The role of the alternative pathway of C activation was documented by the finding that C3 deposition induced by Stx1 was not modified by inhibiting the classical pathway with MgCl₂-EGTA added to HS before cell perfusion (Fig. 1A, 1B).

**C3 binds with high affinity to P-selectin expressed on Stx-activated endothelial cells**

Because we showed previously that Stx1 induced endothelial expression of P-selectin (17), an adhesive protein that also acts as a C3b receptor on platelets and Chinese hamster ovary cells (21), experiments were performed to evaluate whether P-selectin had a role in C activation and C3 deposition on Stx-activated endothelial cells. First, we documented by immunofluorescence the increase of P-selectin as a granular pattern on the endothelial apical side after exposure to purified Stx1 (Supplemental Fig. 3A). We then evaluated whether P-selectin could interact with C3 on HMEC-1 exposed to Stx and perfused with HS. By confocal microscopy, the majority of C3 deposits colocalized with P-selectin (Fig. 1C). Such a colocalization was visible in a large number of

**FIGURE 1.** Stx1 induces C3 activation and deposition on HMEC-1 via endothelial P-selectin. A, Images of C3 deposition on HMEC-1 exposed or not to Stx1 (24 h) and then perfused at high-shear stress with 50% HS for 3 min. C3 deposition is inhibited by pretreating HS with sCR-1 or with MgCl₂-EGTA before perfusion (original magnification ×200). B, Quantification of C3 deposits. Values (mean ± SEM) are expressed as fold increase versus control + HS = 1 (n = 6 experiments). C, C3 (green) colocalizes with P-selectin (red) resulting in yellow overlap (merge) on HMEC-1 incubated with Stx1 and then perfused with HS. Nuclei are stained with DAPI (blue). D, High-affinity binding of soluble P-selectin (s P-selectin) to C3b by SPR. The left and middle panels show the sensograms of two independent sensors (time course of the SPR signal in RU) obtained by simultaneously injecting three concentrations of soluble P-selectin (s P-selectin) over sensor surfaces immobilizing C3b. The right panel shows the saturation isotherm. E, C3 deposition on Stx-treated HMEC-1 perfused with HS is reduced by anti-P-selectin (anti-P-selectin Ab or PSGL-1). An Ab recognizing cell surface Ag (CD44) is used as irrelevant (irrel). Values (mean ± SEM) are expressed as fold increase versus control + HS = 1 (n = 4 experiments). *p < 0.01 versus control + HS, *p < 0.01 versus Stx1 + irrel Ab + HS, **p < 0.01 versus Stx1 + HS.
Stx-treated endothelial cells. Moreover, binding studies with SPR showed a specific interaction between C3b and P-selectin. Fig. 1D (left and middle panels) shows the sensorgrams (time-course of SPR signal) obtained when flowing different concentrations of soluble P-selectin over C3b immobilized on the sensor chip. A dose-dependent binding signal was detected. A clearly biphasic dissociation curve did not fit the simplest 1:1 interaction. The interaction is more complex, suggesting a conformational change in C3b molecules once bound to P-selectin, or the presence of cooperativity among the multiple binding sites (SCR domains) present on P-selectin, as previously described for binding of C3b to CR1 (38, 39). However, the plot of the equilibrium responses could be fitted by a simple saturation isotherm, allowing the estimation of a Kd value of 77 ± 28 nM (Fig. 1D, right panel).

Next, we documented that P-selectin mediated C3 binding on the surface of Stx-treated HMEC-1. Cell exposure to functional blocking anti-P-selectin Ab, before and during HS perfusion, led to a significant inhibition of C3 deposits in response to Stx1 (Fig. 1E), whereas the addition of irrelevant Ab had no effect. Moreover, a strong decrease of C3 accumulation was observed when PSGL-1, the soluble ligand of P-selectin, was added to HMEC-1 before and during HS perfusion (Fig. 1E).

**Complement activation favors thrombus formation on Stx-treated endothelial cells**

To establish whether C activation and deposition on HMEC-1 could be instrumental to thrombus formation, Stx1-treated cells were exposed to a double perfusion consisting of HS, followed by whole human blood prelabeled with the fluorescent dye mepacrine, or to a single perfusion with blood alone (Supplemental Fig. 1). When Stx1–treated cells were perfused with HS and blood, a more intense deposition of C3 and increased endothelial surface area covered by thrombi were detected, as compared with unstimulated cells (Table I). Notably, after double perfusion, C3 deposits were followed by thrombi detected on the endothelial surface to a significantly greater extent than on Stx1-treated cells subjected to single blood perfusion (Table I). A representative image of organized thrombi formed on the endothelial cell monolayer is shown in Supplemental Fig. 3B. Pretreatment of HS and blood with sCR-1 significantly (p < 0.01) limited platelet thrombi on HMEC-1 exposed to the toxin, indicating a causal link between C3 deposition and thrombus formation (Fig. 2A, 2B).

We next examined whether P-selectin blockade could limit C-dependent thrombus formation on endothelial cells in response to Stx. P-selectin inhibition in Stx1-treated cells exposed to double perfusion of HS and blood resulted in decreased C3 accumulation (Supplemental Fig. 3C), followed by a significant reduction in platelet thrombi (Fig. 2A, 2B). Irrelevant Ab had no effect on Stx-induced C3 deposition (Supplemental Fig. 3C) and thrombus formation (Fig. 2A, 2B). These data indicate that endothelial P-selectin upregulated by Stx is instrumental for C activation and C3-dependent thrombus formation on microvascular endothelium.

C3a generated by Stx-induced C activation impairs endothelial thromboresistance

To discover how C activation triggers thrombus formation on endothelial cells, we studied the effect of the anaphylatoxin C3a—a breakdown product generated during C activation and C3 deposition—on endothelial thromboresistance properties. We focused on TM, a glycoprotein highly expressed on endothelial cell surface, with cytoprotective and antiatherothrombotic effects (40). TM was constitutively expressed on HMEC-1 (percentage of fluorescent cells with moderate and strong intensity: 54.2 ± 4.3 and 6.7 ± 1.1%). Treatment with Stx1 alone slightly reduced TM expression, which further decreased after HS perfusion (Fig. 3A, Supplemental Fig. 3D). Addition of the C3aR antagonist SB290157 on Stx-treated HMEC-1 perfused with HS prevented TM loss (Fig. 3A, Supplemental Fig. 3D). Providing further evidence for a role of this anaphylatoxin, we found a marked reduction of TM expression on the surface of HMEC-1 exposed to exogenous purified C3a (Fig. 3B). Because the cleavage of TM is thought to be dependent by serine protease activity, we examined t-PA, a serine protease present in endothelial Weibel-Palade bodies possibly released upon C3a stimulation (41). Blockade of t-PA activity by treatment of HMEC-1 with the serine protease inhibitor aprotinin prevented TM shedding in response to exogenous C3a (Fig. 3B). Aprotinin alone as a control did not affect the constitutive expression of TM (percentage of fluorescent cells with moderate and strong intensity: aprotinin, 67.7 ± 5.3 and 7.6 ± 0.6 versus control, 62.4 ± 4.9 and 8.3 ± 0.7). Consistently, the addition of t-PA to HMEC-1 significantly (p < 0.01) reduced TM expression on the cell surface (percentage of fluorescent cells with moderate and strong intensity: t-PA, 51.3 ± 2.1 and 1.7 ± 0.3 versus control, 62.2 ± 1.2 and 6.6 ± 0.9). The finding that C3a may favor exocytosis of the Weibel-Palade body’s content was also supported by the increased apical staining of P-selectin on C3a-treated HMEC-1 (Fig. 3C). The effects induced by C3a were critical for thrombus formation, as evidenced by the significant (p < 0.01) reduction of

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<th>Table I. Stx1 induces complement deposition and thrombus formation on HMEC-1 under flow condition</th>
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Area covered by C3 deposits or thrombi is expressed as fold increase over control (single perfusion) imposed as 1.0. Data are mean ± SEM.

a p < 0.01 versus control plus blood perfusion.
b p < 0.01 versus control plus HS plus blood perfusion.
c p < 0.01 versus Stx1 plus blood perfusion.
d p < 0.05 versus Stx1 plus blood perfusion.
The presence of intracapillary glomerular platelet clumps as revealed by transmission electron microscopy (Fig. 4D). Other ultrastructural changes included focal endothelial swelling and polymorphonuclear cell infiltrates.

In animals injected with Stx2 alone, platelet count remained within the normal range, whereas it decreased after LPS (16) (Supplemental Fig. 4A), in accordance with the expression of TLR4 on platelets (43). Each agent caused slight increases in BUN (Supplemental Fig. 4B), mild glomerular endothelial swelling, and low polymorphonuclear cell accumulation (data not shown).

Next, we evaluated the expression of P-selectin in glomeruli of Stx2/LPS-treated mice. Positive staining for P-selectin was observed in glomerular endothelial cells at 3 (Fig. 5A) and 6 h (not shown) after Stx2/LPS injection. The endothelial localization of P-selectin was documented by evidence of labeling of the endothelial marker VWF on the same cells in serial sections (Fig. 5A).

The role of P-selectin in favoring glomerular C deposits in response to Stx2/LPS was documented by finding that treatment with blocking P-selectin Ab effectively limited C3 deposits compared to mice given the irrelevant Ab (Fig. 5C).
C3a derived by C activation, via the alternative pathway, contributes to a glomerular prothrombotic state in HUS mice

To establish the involvement of C activation in the microvascular thrombogenic process, we used mice deficient for factor B, a zymogen that carries the catalytic site of the alternative pathway C3 and C5 convertases (46). After Stx2/LPS treatment, Bf−/− mice exhibited less thrombocytopenia and were protected against renal function impairment compared with wild type littermates (Fig. 6A, 6B). The blockade of the alternative pathway of C activation also resulted in fewer fibrin(ogen) deposits (Fig. 6C; 48 h, score: saline, Bf−/− 0.19 ± 0.04 versus wild type 0.22 ± 0.06; Stx2/LPS, Bf−/− 0.70 ± 0.23 versus wild type 2.15 ± 0.09; p < 0.01). No C3 deposits or platelet clumps were found in the glomeruli of Stx2/LPS-treated Bf−/− mice. Moreover, while glomerular TM expression was reduced by 42.8 ± 7.3% at 48 h in wild type mice given Stx2/LPS compared with saline-treated control mice (Fig. 6D), no changes were observed in Bf−/− mice regarding corresponding controls (Fig. 6D), thereby suggesting the causal role of C activation in the loss of thromboreistance.

The contribution of C3a in the thrombogenic process induced by Stx was evaluated by blocking C3aR in HUS mice. Administration of a C3aR antagonist to Stx2/LPS-treated C57BL/6 mice significantly (p < 0.05) limited glomerular fibrin(ogen) deposition and platelet clumps (not shown) compared to mice receiving vehicle (Fig. 7A). In this study, we also assessed TM expression and found that C3aR antagonist limited (p < 0.01) glomerular TM loss after Stx2/LPS (Fig 7B). A scheme of the mechanisms suggested by the present data is shown in Fig. 8.

Discussion

To our knowledge, this study is the first to demonstrate by multifaceted approaches that Stx triggers microvascular thrombosis via the alternative pathway of C activation and indicates C3a as the key factor in the development of the thrombotic processes in HUS. Previous findings of low serum C3 levels (25, 27) and augmented breakdown C products (27, 28) suggested the involve-

![FIGURE 5](http://www.jimmunol.org/)

![FIGURE 6](http://www.jimmunol.org/)

![FIGURE 7](http://www.jimmunol.org/)
ment of C in D+HUS, although the functional relevance of these abnormalities to the thrombotic process was not addressed. Our data provide evidence that Stx1 promotes C3 activation and deposition on microvascular endothelial cells via the alternative pathway, because no inhibition was found by blocking the classical pathway. A relevant observation is that C3 deposition favors thrombus growth under flow on the surface of Stx-treated HMEC-1, which is prevented by the C inhibitor sCR-1. This mechanism applies also in vivo, because Bf−/− mice with impaired C activation via the alternative pathway were protected from renal prothrombotic processes when injected with Stx2/LPS compared to wild-type mice that showed intraglomerular C3 and fibrin(ogen) deposition, TM loss, and platelet clumps associated with severe thrombocytopenia.

In the search for mechanisms underlying C activation elicited by Stx and ultimately leading to thrombus formation, we have identified P-selectin as an endothelial binding site for C3. As shown previously (17) and documented in this study, upon challenge with Stx the microvascular endothelium expresses P-selectin, an adhesive molecule implicated in VWF-dependent platelet deposition (47). Rather than simply a marker of endothelial activation, P-selectin acts as a specific ligand for C3 through high-affinity binding, based on the evidence that it colocalizes with the majority of C3 deposits on Stx-treated HMEC-1. P-selectin is crucial in C-dependent thrombus formation because functional blocking of P-selectin leads to a marked reduction of C3 accumulation and limits thrombus extension on Stx-treated endothelial cells. The processes by which P-selectin favors C activation on endothelial cells are still poorly defined. It is possible that C3b bound to P-selectin may undergo conformational changes that either prevent its inactivation by C regulatory molecules or favor C3b interaction with factor B, thus leading to assembly of alternative pathway C3 convertase.

We have also provided a novel mechanism through which activated C proteins foster thrombosis on Stx-activated endothelium. In our experimental setting, Stxs promoted C3 activation and deposition that resulted in C3a generation; therefore, we focused on the endothelial effect of this anaphylatoxin. C3a powerfully increased P-selectin expression and reduced TM on the HMEC-1 surface, indicating a major role for C3a as a driving factor in altering endothelial thromboresistance. The proof of this concept rests on data showing that TM loss and thrombus growth on Stx-treated endothelium were markedly reduced by C3aR antagonist. However, we cannot exclude a contribution of C5a in the prothrombotic effects triggered by Stx, considering that C5a shares with C3a various inflammatory activities on endothelial cells (22–24). It is known that TM, a cofactor of thrombin in the activation of protein C, regulates hemostasis and prevents local fibrin formation (40, 48, 49). Other properties of TM include anti-inflammatory and cytoprotective activities (40). It is therefore conceivable that shedding of TM from the endothelial surface can contribute to an increased risk of thrombosis associated with inflammation. In this regard, increased levels of soluble TM have been detected in the plasma of patients with disseminated intravascular coagulation syndrome, pulmonary thromboembolism, and chronic renal disorders (50). Little is known about the mechanisms underlying TM shedding and the proteases involved in its cleavage. Serine proteases were reported to be implicated in thiol-induced proteolytic cleavage of TM in association with damage of endothelial cell membrane (51, 52).

Based on the evidence that t-PA is the serine protease stored in Weibel-Palade bodies of endothelial cells, we show that the inhibition of t-PA by aprotinin markedly limited C3a-dependent TM loss on microvascular endothelial cells. This finding was also supported by the direct effect of exogenously added t-PA on TM shedding in HMEC-1. Our findings shed light on the nexus among endothelium, C, and thrombosis, considering that previous knowledge of the prothrombotic effect of C was restricted to studies on platelets (53). Proteins of the C system, including C3, C5, C6, C7, C8, and C9, significantly enhanced thrombin-induced aggregation and secretion in human platelets. Furthermore, the anaphylatoxin C3a was shown to directly induce aggregation of human platelets and release of serotonin (53). That the mechanisms of thrombosis may be related to C activation has been also suggested by studies in patients with familial or sporadic forms of atypical HUS or paroxysmal nocturnal hemoglobinuria, both characterized by deficiency in C-regulatory molecules, platelet hyperactivity, and thrombosis (33, 54).

The mechanisms proposed in vitro have been validated in a murine model of Stx-associated HUS, which closely mimics human D+HUS. The development of a mouse model able to reproduce the features of human HUS has been problematic, and studies using E. coli O157:H7, purified Stx1 or Stx2 with or without LPS (16, 35, 36, 42, 55, 56), have given controversial results. The role of E. coli LPS has been recognized in the molecular pathogenesis of D+HUS, based on the evidence of serum...
Abs against O157 LPS in children with D+HUS (57). In a baboon model of HUS, LPS increased the toxicity of Stx (58). Consistently, both Stx2 and LPS were required to elicit a HUS-like response in mice (16, 36, 42). Our experiments demonstrate that in mice treated with Stx2/LPS that developed thrombocytopenia and renal failure, enhanced P-selectin expression on glomerular endothelial cells preceded C3 deposits. Accumulation of C3 was associated with fibrinogen deposits, platelet clumps, and TM loss. P-selectin blockade limited glomerular C3 deposits, which argues in favor of a causal relationship between endothelial P-selectin and C deposition. By means of Bf−/− mice that were protected against glomerular alterations and renal function impairment in response to Stx2/LPS, we showed that C activation via the alternative pathway was instrumental to microvascular thrombosis in HUS mice. This study also highlights the critical role of C3a, derived from glomerular C activation, in potentiating glomerular thrombotic processes. This finding is supported by data showing that treatment with a C3aR antagonist markedly reduced fibrinogen and platelet accumulation and limited the loss of TM in the glomeruli of HUS mice.

As suggested earlier (59), our results corroborate concepts that may prove of general significance in the understanding of the causes that trigger thrombosis and vascular occlusions. We previously explored the heterogeneity of endothelial cells based on the variable expression of specific molecules that determined a distinct response to stimuli (17) and demonstrated the link between infections and thrombosis. In this study, we elaborated on those concepts by documenting in a specific setting a phenomenon of interaction between C3 and P-selectin leading to the formation of C3a, which appears to be instrumental in microvascular thrombosis, and may have wider implications for acute occlusive vascular accidents in a broader sense.

In conclusion, as summarized in Fig. 8, we have demonstrated by in vitro and in vivo experiments that Stx, by activating microvascular endothelial cells to express P-selectin, allows C3 deposition through the alternative pathway, favoring thrombus formation. Moreover, we have identified C3a, generated by Stx-induced C activation, as the key factor responsible for further expression of P-selectin, TM loss, and thrombus formation. These findings serve to suggest C inhibitors as possible therapeutic tools in the control of interaction between infections and thrombosis. In this study, we elaborated on those concepts by documenting in a specific setting a phenomenon of interaction between C3 and P-selectin leading to the formation of C3a, which appears to be instrumental in microvascular thrombosis, and may have wider implications for acute occlusive vascular accidents in a broader sense.

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Disclosures
The authors have no financial conflicts of interest.

References


