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Lack of the Lectin-like Domain of Thrombomodulin Worsens Shiga Toxin-Associated Hemolytic Uremic Syndrome in Mice

Carlamaria Zoja,* Monica Locatelli,* Chiara Pagani,* Daniela Corna,* Cristina Zanchi,* Berend Isermann,† Giuseppe Remuzzi,* Edward M. Conway,‡ and Marina Noris†

Shiga toxin (Stx)-producing _Escherichia coli_ is a primary cause of diarrhea-associated hemolytic uremic syndrome (HUS), a disorder of thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure. The pathophysiology of renal microvascular thrombosis in Stx-HUS is still ill-defined. Based on evidence that abnormalities in thrombomodulin (TM), an anticoagulant endothelial glycoprotein that modulates complement and inflammation, predispose to atypical HUS, we assessed whether impaired TM function may adversely affect evolution of Stx-HUS. Disease was induced by coinjection of Stx2/LPS in wild-type mice (TM<sup>wt/wt</sup>) and mice that lack the lectin-like domain of TM (TM<sup>LeD/LeD</sup>), which is critical for its anti-inflammatory and cytoprotective properties. After Stx2/LPS, TM<sup>wt/wt</sup> mice exhibited more severe thrombocytopenia and renal dysfunction than TM<sup>LeD/LeD</sup> mice. Lack of lectin-like domain of TM resulted in a stronger inflammatory reaction after Stx2/LPS with more neutrophils and monocytes/macrophages infiltrating the kidney, associated with PECAM-1 and chemokine upregulation. After Stx2/LPS, intraglomerular fibrin(ogen) deposits were detected earlier in TM<sup>LeD/LeD</sup> than in TM<sup>wt/wt</sup> mice. More abundant fibrin(ogen) deposits were also found in brain and lungs. Under basal conditions, TM<sup>LeD/LeD</sup> mice exhibited excess glomerular C3 deposits, indicating impaired complement regulation in the kidney that could lead to local accumulation of proinflammatory products. TM<sup>LeD/LeD</sup> mice with HUS had a higher mortality rate than TM<sup>wt/wt</sup> mice. If applicable to humans, these findings raise the possibility that genetic or acquired TM defects might have an impact on the severity of microangiopathic lesions after exposure to Stx-producing _E. coli_ infections and raise the potential for using soluble TM in the treatment of Stx-HUS. 

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The online version of this article contains supplemental material.

Abbreviations used in this article: BUN, blood urea nitrogen; D+HUS, diarrhea-associated hemolytic uremic syndrome; HPF, high-power fields; HUS, hemolytic uremic syndrome; PMN, polymorphonuclear cell; STEC, Shiga toxin-producing _Escherichia coli_; Stx, Shiga toxin; TM, thrombomodulin; TM<sup>LeD/LeD</sup>; mice that lack the lectin-like domain of TM; TM<sup>wt/wt</sup>, wild-type mice.

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*Address correspondence and reprint requests to Dr. Carlamaria Zoja, Mario Negri Institute for Pharmacological Research, Centro Anna Maria Astori, Bergamo, Italy. E-mail address: carlamaria.zoja@marionegri.it

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Thrombomodulin (TM) is a transmembrane, endothelial glycoprotein receptor for thrombin, best known for its function as a cofactor in the protein C anticoagulant pathway (16). Beyond its role in coagulation, TM has properties that impact on fibrinolysis, complement activation, inflammation, and cell proliferation (17–19). Full-length TM comprises five structural domains, including a C-type lectin-like NH2-terminal module (Supplemental Fig. 1). Using transgenic mice that lack the lectin-like domain of TM (TM<sub>ΔLD</sub>), it was shown that TM through its lectin-like domain interferes with inflammation by suppressing neutrophil adhesion to endothelial cells and dampening complement activation (20, 21). Mutations that impair the function of TM have been identified in patients with atypical HUS, a form not linked to STEC infection (19). Three of the six identified missense mutations were in the lectin-like domain of TM. In the same study, cultured cells expressing TM variants, including those involving the lectin-like domain, had diminished capacity to modulate complement activation as compared with cells transfected with wild-type TM, implying that TM mutations associated with atypical HUS cause defective complement regulation. There is in vitro evidence that TM expression was decreased in human glomerular microvascular endothelial cells exposed to Stx2 after presensitization with inflammatory mediators (22). More recently, in a mouse model of HUS induced by coinjection of Stx2 and LPS, reduced glomerular TM expression was observed in association with fibrin(ogen) and platelet deposition (23).

The earlier findings prompted us to investigate whether impaired TM function may adversely affect the evolution of Stx-associated HUS. To this end, we compared the course and severity of the disease in TM<sub>ΔLD</sub> mice as compared with wild-type mice (TM<sub>wt/wt</sub>) in response to systemic administration of Stx2/LPS.

Materials and Methods

Experimental design

Male Swiss/129sv/ev mice (TM<sub>wt/wt</sub>) and age-matched Swiss/129sv/ev TM<sub>ΔLD</sub> mice were generated by homologous recombination in embryonic stem cells as previously described (20) and bred in the animal facility of the Vesalius Research Center, University of Leuven, Leuven, Belgium, in accordance with the local institutional animal ethics committee. Mice were then maintained at Mario Negri Institute in conformity with the institutional guidelines that are in compliance with national and international laws and policy (23). All animal studies were approved by the Institutional Animal Care and Use Committees of Mario Negri Institute, Milan, Italy. HUS was induced in TM<sub>wt/wt</sub> and TM<sub>ΔLD</sub> mice (28–32 g body wt) by i.p. injection of Stx2 (200 ng/mouse; Toxin Technology, Sarasota, FL) plus LPS (75 μg/mouse, O111:B4; Sigma, St. Louis, MO) (23, 24). Mice were sacrificed at 3, 6, 24, or 48 h after Stx2/LPS injection. Groups of TM<sub>wt/wt</sub> and TM<sub>ΔLD</sub> mice were injected with saline and served as controls. For survival studies, TM<sub>wt/wt</sub> (n = 14) and TM<sub>ΔLD</sub> (n = 17) mice treated with Stx2/LPS were used. In additional experiments, TM<sub>wt/wt</sub> (n = 16) and TM<sub>ΔLD</sub> (n = 24) mice were treated with saline or low doses of Stx2 (10, 20 ng/mouse) plus LPS (75 μg/mouse), and sacrificed 24 or 48 h later. Blood platelet count and renal function measured by serum blood urea nitrogen (BUN, Reflotron test; Roche Diagnostic Corporation, Indianapolis, IN) were assessed. Kidneys were removed for immunohistochemical analysis and/or real-time PCR experiments, and brain and lungs for the evaluation of fibrin(ogen) deposits.

Immunohistochemical analysis

Kidney, brain, and lung tissues were immersed overnight in periodate lysine paraformaldehyde fixative at 4°C and then were transferred to 30% sucrose and frozen in OCT compound. Fibrin(ogen) staining was revealed by FITC-conjugated goat anti-rat fibrinogen Ab (1:40; Nordic Immunology) using an inverted confocal laser microscope (LSM 510 meta; Zeiss, Jena, Germany). Nuclei and cell membranes were stained with DAPI (blue) and rhodamine wheat germ agglutinin-lectin (red; Vector Laboratories), respectively. Negative controls were obtained using FITC-conjugated polyclonal goat Ab (1:40; Nordic Immunology) on adjacent sections. Fibrin (ogen) expression was estimated using ImageJ software. C3 deposits were evaluated in aceto--fixed frozen sections (3 μm) by staining with FITC-conjugated goat anti-mouse C3 Ab (10 μg/ml; Cappel). Glomerular C3 staining was scored from 0 to 3: 0, no staining or traces (<5%); 1, staining in <25% of the glomerular tuft; 2, staining affecting 25–50%; 3, staining >50%. Polymorphonuclear cells (PMNs) were revealed in renal OCT-frozen sections by monoclonal rat anti-mouse Ly-6B.2 alloantigen Ab (1:400; AbD Serotec). PMNs were counted for each glomerulus and in randomly selected high-power fields (HPF) of interstitial areas (×400). Ab against a cytoplasmic Ag present in mouse monocytes/macrophages (F4/80, 2.5 μg/ml; Caltag Laboratories) was used for the detection of mono-nuclear cells by immunoperoxidase technique in Duboscq-Brazil–fixed and paraffin-embedded sections. F4/80<sup>+</sup> cells were counted in randomly selected HPF (×400) of interstitial areas. PECAM-1 was detected in OCT-frozen sections using rat anti-mouse Ab (1:50, BD Biosciences) followed by FITC-conjugated goat anti-rat IgG (1:150; Sigma). Negative controls were obtained using rat IgG2a isotype control (1:1600; BD Biosciences) on adjacent sections. Glomerular and peritubular expression was estimated by ImageJ software. MIP-2/CXCL2 expression was evaluated in aceto-fixed frozen sections by staining with goat anti-mouse MIP-2 Ab (1:250; Santa Cruz). MIP-2 expression was estimated by ImageJ software. MCP-1/CCL2 expression was detected by goat polyclonal anti–MCP-1 Ab (1:100; Santa Cruz) using avidin/biotin complex immunoperoxidase on paraffin-embedded tissue. Negative controls were obtained by omitting the primary Ab on adjacent sections. MCP-1 immunostaining in the cortical tubulo-interstitium was expressed as number of positive tubuli/HPF (×400).

Quantitative real-time PCR

Total RNA was isolated from kidney tissue using TRIzol reagent (Invitrogen). Purified RNA (2 μg) was reverse transcribed. Amplification was performed on 7300 Real-Time PCR System (Life Technologies) using TaqMan Universal PCR Master Mix and investigator TaqMan assay (Genbank accession number; M00436450_m1 for mouse MIP2/CXCL2, Mm99999056_m1 for mouse MCP-1, and a mouse β-actin endogenous control (VIC/MGB probe) as reference gene. The ΔΔCt method was used to calculate relative changes in expression of target genes in respect to a calibrator sample (untreated TM<sub>wt/wt</sub>).

Statistical analysis

Results are expressed as mean ± SE. Data were analyzed by the non-parametric Mann–Whitney or Kruskal–Wallis tests or by ANOVA where appropriate. Survival curves were analyzed by the log-rank test. Statistical significance level was defined as p < 0.05.

Results

Thrombocytopenia and renal function impairment after Stx2/LPS are more severe in TM<sub>ΔLD</sub> mice

Under baseline conditions, TM<sub>wt/wt</sub> and TM<sub>ΔLD</sub> mice had normal platelet counts and renal function (Fig 1A, 1B). A decrease in the platelet count was observed in TM<sub>wt/wt</sub> mice treated with Stx2 (200 ng/mouse) plus LPS (75 μg/mouse) (23) at 24 and 48 h compared with baseline (45 and 63% reduction, respectively; Fig. 1A). However, the thrombocytopenia was accentuated in mice that lack the lectin-like domain (54 and 77% reduction at 24 and 48 h versus basal), with a significant (p < 0.05) difference in platelet count between TM<sub>ΔLD</sub> and TM<sub>wt/wt</sub> mice. Renal function was impaired in TM<sub>wt/wt</sub> mice treated with Stx2/LPS, as indicated by increased serum BUN levels (Fig. 1B). After exposure to Stx2/LPS, renal function was worse in TM<sub>ΔLD</sub> mice, represented by significantly higher BUN levels as compared with TM<sub>wt/wt</sub> mice (24 h, p < 0.01; 48 h, p < 0.05; Fig. 1B).

The highest susceptibility of TM<sub>ΔLD</sub> mice to develop renal function impairment was observed also when consistently lower doses of Stx2 were given (Table I). Thus, whereas in TM<sub>wt/wt</sub> mice only a mild increase in BUN levels was observed after 20 ng Stx2, TM<sub>ΔLD</sub> mice showed a significant increase in serum BUN levels at 24 (p < 0.01) and 48 h (p < 0.05) with respect to baseline. Notably, in TM<sub>ΔLD</sub> mice, BUN levels were significantly higher than those measured in the TM<sub>wt/wt</sub> mice (24 h, p < 0.05). A trend toward increase was observed in TM<sub>ΔLD</sub> mice after 10 ng Stx2, which, however, did not reach statistical sig-
FIGURE 1. Platelet count and renal function in TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice treated with Stx2 plus LPS. (A) TM\textsuperscript{LeD/LeD} mice exhibited more severe thrombocytopenia compared with TM\textsuperscript{wt/wt} mice. (B) Lack of the lectin-like domain of TM worsened the decline of renal function, assessed as BUN levels. Data are mean ± SE (n = 25 mice/group at time 0; n = 20 mice/group at 24 h; n = 11 mice/group at 48 h).

Significance (Table I). Platelet count was reduced by 42 ± 4.4% in TM\textsuperscript{LeD/LeD} mice after 20 ng Stx2/LPS as compared with 22 ± 2.5% reduction in TM\textsuperscript{wt/wt} mice at 24 h (p < 0.05).

Glomerular and interstitial inflammatory reaction in TM\textsuperscript{LeD/LeD} mice after Stx2/LPS

We quantified PMN and monocyte/macrophage infiltration into the kidneys of TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice before and after Stx2/LPS treatment. Before Stx2, there was no genotype-dependent difference in the number of glomerular or interstitial PMNs, as quantified after immunofluorescence staining of histologic sections (Fig. 2A, 2B). However, after Stx2/LPS, the number of PMNs in the glomeruli and interstitium of TM\textsuperscript{LeD/LeD} mice was significantly increased as compared with TM\textsuperscript{wt/wt} mice (Fig. 2A, 2B). Accumulation of F4/80\textsuperscript{+} monocytes/macrophages in the renal interstitium of TM\textsuperscript{LeD/LeD} mice was significantly increased as compared with TM\textsuperscript{wt/wt} mice (Fig. 2A, 2B). Accumulation of F4/80\textsuperscript{+} monocytes/macrophages in the renal interstitium of TM\textsuperscript{LeD/LeD} mice was 2-fold higher under basal conditions in TM\textsuperscript{LeD/LeD} mice as compared with TM\textsuperscript{wt/wt} mice (p < 0.05). Similar to the findings with PMNs, Stx2/LPS injection induced greater accumulation of monocytes/macrophages in the renal interstitium of TM\textsuperscript{LeD/LeD} versus TM\textsuperscript{wt/wt} mice (24 h, p < 0.05; Fig. 2C).

We evaluated the expression of PECAM-1, a multifunctional adhesion and signaling molecule involved in transendothelial migration of neutrophils and monocytes (25–27). PECAM-1 was constitutively expressed by glomerular endothelial cells and peritubular capillaries in the kidneys of both TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice, although the staining was more intense and diffuse in TM\textsuperscript{LeD/LeD} mice (p < 0.05; Fig. 3A). After Stx2/LPS treatment, the lack of the lectin-like domain of TM resulted in a further increase in expression of PECAM-1 compared with TM\textsuperscript{wt/wt} mice (p < 0.05; Fig. 3A). No signal was observed when renal sections were stained with isotype primary Ab control (Supplemental Fig. 2A).

Next, we investigated the expression of chemokines allowing neutrophil and macrophage recruitment in the Stx2/LPS model, specifically, MIP-2/CXCL2 and MCP-1/CCL2 (28, 29). Chemokine mRNA was evaluated in renal tissue using quantitative real-time PCR. After Stx2/LPS injection, 176- and 264-fold increases in MIP2/CXCL2 mRNA were observed at 6 h in TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice, respectively, as compared with untreated mice (Fig. 4A). MCP-1 mRNA expression increased by 103-fold in the kidney of TM\textsuperscript{wt/wt} mice treated with Stx2/LPS with respect to control mice. In TM\textsuperscript{LeD/LeD} mice, MCP-1 levels were 230-fold higher than untreated mice (p < 0.05 versus TM\textsuperscript{wt/wt}; Fig. 4B).

As shown in Fig. 3B, MIP-2 staining was minimal in the kidneys of untreated TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice. MIP-2 staining at periglomerular and tubular levels was observed within 5 h after Stx2/LPS administration in both groups of mice, with more intensity in TM\textsuperscript{LeD/LeD} mice (p < 0.05). Constitutive expression of MCP-1 was detected in the proximal tubuli of TM\textsuperscript{LeD/LeD} mice (Fig. 3C). After challenge with Stx2/LPS, MCP-1 staining was evident in TM\textsuperscript{wt/wt} mice at 6 h, but it was more abundant in TM\textsuperscript{LeD/LeD} mice (p < 0.01).

Lack of the lectin-like domain of TM favors glomerular prothrombotic state after Stx2/LPS

Under baseline conditions, kidneys from TM\textsuperscript{wt/wt} and TM\textsuperscript{LeD/LeD} mice exhibited minimal or undetectable amounts of fibrin(ogen) deposition in the glomerular capillaries (Fig. 5A). At 3 h after Stx2/LPS injection, there were only traces of fibrin(ogen) in TM\textsuperscript{wt/wt} kidneys, whereas staining became more prominent starting at 6 h (Fig. 5A). In contrast, in TM\textsuperscript{LeD/LeD} mice, the deposition of fibrin(ogen) in response to Stx2/LPS became readily evident at the early time point of 3 h and was more abundant than in TM\textsuperscript{wt/wt} mice at 6 h. As shown by quantification analysis (Fig. 5A), a significant difference in fibrin(ogen) deposition between the two groups of mice persisted at 24 and 48 h. No signal was found when renal sections were stained with control Ab (Supplemental Fig. 2B).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Baseline (mg/dl)</th>
<th>24 h (mg/dl)</th>
<th>48 h (mg/dl)</th>
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<tr>
<td>TM\textsuperscript{wt/wt} mouse</td>
<td>19.05 ± 1.01</td>
<td>28.54 ± 5.81</td>
<td>30.53 ± 7.08</td>
</tr>
<tr>
<td>Stx2 (20 ng)/LPS</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Stx2 (10 ng)/LPS</td>
<td>19.62 ± 1.51</td>
<td>24.70 ± 4.10</td>
<td>29.15 ± 3.35</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 3)</td>
<td></td>
</tr>
<tr>
<td>TM\textsuperscript{LeD/LeD} mouse</td>
<td>19.21 ± 1.03</td>
<td>45.97 ± 8.20** **</td>
<td>38.52 ± 7.32***</td>
</tr>
<tr>
<td>Stx2 (20 ng)/LPS</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Stx2 (10 ng)/LPS</td>
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<td>34.46 ± 13.41</td>
<td>32.76 ± 11.49</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
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</table>

*p < 0.01 versus control; **p < 0.05 versus TM\textsuperscript{wt/wt} at 24 h; ***p < 0.05 versus TM\textsuperscript{LeD/LeD} at 48 h.
Histologic sections of kidneys from untreated TM wt/wt mice that were immunostained for C3 revealed staining along Bowman’s capsule (23, 30) and minimal focal staining in the glomeruli (score: 0.45 ± 0.09; Fig. 5B). Under the same basal conditions, there was notably more C3 deposition within the glomeruli of TM LeD/LeD mice (p < 0.01). The findings are consistent with spontaneous complement activation in the TM LeD/LeD kidneys, which could favor the development of a glomerular pro-thrombotic state after HUS induction. At 24 h after Stx2/LPS injection, glomerular C3 staining was significantly (p < 0.01) increased in TM wt/wt mice, as compared with TM wt/wt mice treated with saline. TM LeD/LeD mice also responded to Stx2/LPS with an increase in glomerular C3 deposits (p < 0.05 versus saline), but the amount of C3 staining was not different from that observed in Stx2/LPS-treated TM wt/wt mice (Fig. 5B). When mice were exposed at a lower dose of Stx2 (20 ng) plus LPS, we could appreciate at 24 h a slight increase in C3 deposition only in TM LeD/LeD mice, which, however, did not reach a statistical significance as compared with glomerular C3 deposits under basal condition (Supplemental Fig. 3).

FIGURE 2. TM LeD/LeD mice had a stronger kidney inflammatory reaction after Stx2/LPS than TM wt/wt mice. (A) Accumulation of PMNs in the glomeruli (original magnification ×630) and (B) in the renal interstitium (original magnification ×400); (C) infiltrates of F4/80+ monocytes/macrophages in the renal interstitium of TM wt/wt and TM LeD/LeD mice treated with saline or Stx2 plus LPS (original magnification ×400). PMNs were revealed by immunofluorescence, and counted for each glomerulus and in randomly selected HPF of interstitial areas (×400). F4/80+ cells were counted in randomly selected HPF (×400) of interstitial areas. Data are mean ± SE of n = 7–9 mice/group. *p < 0.05, **p < 0.01 versus TM wt/wt mice.

FIGURE 3. Renal expression of PECAM-1, MIP-2, and MCP-1 is higher in TM LeD/LeD mice than TM wt/wt mice in response to Stx2/LPS. Representative images and quantification of (A) PECAM-1, (B) MIP-2, and (C) MCP-1 staining in the kidney of TM wt/wt and TM LeD/LeD mice treated with saline or Stx2/LPS (n = 6 mice/group). Original magnification ×400. Data are mean ± SE. *p < 0.05, **p < 0.01 versus TM wt/wt mice.
Fibrin(ogen) deposits in brain and lungs of TMLeD/LeD mice treated with Stx2/LPS

Extrarenal manifestations of diarrhea-associated HUS (D+HUS) are increasingly being recognized, with the most common organ affected being the brain (31–33). Indeed, neurologic involvement in HUS may be an important determinant of morbidity and mortality. Pulmonary involvement, although less common, has also been reported in HUS (34). In both of these organs, histopathologic studies from fatal cases of HUS have revealed the presence of microthrombi (32, 35). We therefore examined the brain and lungs of Stx2/LPS-treated TM wt/wt and TM LeD/LeD mice for fibrin(ogen) staining. Representative images and quantification data are shown in Fig. 6. Consistent with the notion that the lectin-like domain of TM reduces the sensitivity of mice to the thrombotic microangiopathy associated with D+HUS, there was more abundant fibrin(ogen) deposition in the brain (Fig. 6A) and lungs (Fig. 6B) of TMLeD/LeD mice compared with TMwt/wt mice, as evaluated 24 and 48 h after Stx2/LPS injection.

Lack of the lectin-like domain of TM increases mortality in a murine model of HUS

The induction of HUS by Stx2/LPS resulted in the death of >50% of TMLeD/LeD mice within 54 h, whereas only 20% of TMwt/wt mice died during the same period (Fig. 7A). At 120 h, when all the TMLeD/LeD mice had died, 29% of TMwt/wt mice were still alive (Fig. 7A). The difference in survival between the two groups was statistically significant (p < 0.01), confirming the enhanced sensitivity of the TMLeD/LeD mice to Stx2/LPS-HUS associated mortality. Moreover, although both TMwt/wt and TMLeD/LeD mice progressively lost weight after injection of the Stx2/LPS (Fig. 7B), the reduction was significantly greater in the TMLeD/LeD mice.

Discussion

This study demonstrates that lack of the lectin-like domain of TM in mice increases their sensitivity to manifesting the phenotypic changes in a model of Stx-HUS. Thus, after exposure to Stx2/LPS, TMLeD/LeD mice develop more severe thrombocytopenia and renal function impairment, earlier and more abundant glomerular fibrin (ogen) deposits, and increased mortality. The findings are consistent with the important role that TM plays as an anticoagulant and anti-inflammatory molecule in the microvasculature, particularly in the setting of Stx-mediated injury. We have already shown that TM mutations in humans increase the risk for development of...
atypical HUS (19) (i.e., not Stx-associated). If our current findings are applicable to humans, it is reasonable to consider that genetic or acquired functional defects in TM may also contribute to an adverse outcome during the course of Stx-associated HUS.

TM is a 557-aa endothelial glycoprotein that is anchored to the cell by a short cytoplasmic tail and a single transmembrane domain (36). A stretch of six epidermal growth factor-like repeats support thrombin-mediated generation of activated protein C, which has anticoagulant and anti-inflammatory properties, and generation of activated thrombin activatable fibrinolysis inhibitor that also inactivates complement-derived anaphylatoxins C3a and C5a. Farthest from the membrane is the lectin-like domain of TM that has distinct anti-inflammatory properties, interfering with neutrophil and monocyte adhesion to endothelial cells, suppressing activation of MAPK and NF-κB pathways, interfering with complement activation (20, 21), and blocking the activity of the proinflammatory cytokine high mobility group box 1 (37). TM LeD/LeD mice accumulated more neutrophils in the lung, had higher serum cytokine levels, and had reduced survival (20) in response to endotoxia. Similarly, after myocardial ischemia/reperfusion, TM LeD/LeD mice developed larger infarcts than TM wt/wt, associated with more intense neutrophil extravasation within the ischemic regions (20). Finally, TM LeD/LeD mice had enhanced sensitivity to develop inflammatory arthritis that was associated with augmented monocyte/macrophage accumulation into joints (21). Administration of recombinant lectin-like domain of TM protected TM LeD/LeD mice against these insults (20, 38). Consistently, in this study, we found that after Stx2/LPS treatment, TM LeD/LeD mice exhibited a stronger inflammatory reaction in the kidney than the TM wt/wt, with a higher number of neutrophils infiltrating the glomeruli and renal interstitium. Interstitial accumulation of monocytes/macrophages was also more abundant. There is considerable evidence that neutrophils and monocytes play an active role in the pathogenesis of Stx-associated HUS, and that their interactions with activated endothelial cells serve to amplify microvascular injury in the kidney (15). During the acute phase of D+HUS, neutrophils are activated, become more adhesive, and damage the endothelium by producing α1-antitrypsin–complexed elastase (39, 40). Kidney biopsies from D+HUS children showed mononuclear and PMNs within the glomeruli, along the zone of microvascular injury (41, 42). In vitro, Stx promoted leukocyte adhesion and transmigration across glomerular endothelial cells via upregulation of adhesion molecules and chemokines (24, 43). In TM LeD/LeD mice, the increased accumulation of leukocytes in the kidneys after Stx2/LPS injection was associated with overexpression of PECAM-1, a transmembrane glycoprotein belonging to the Ig superfamily of adhesion molecules with functional roles in leukocyte transendothelial cell migration (25, 26). PECAM-1 is expressed at high density at the lateral borders of endothelial cells and at a lower density on the surface of hematopoietic and immune cells, including neutrophils, monocytes, and platelets (27). Endothelial PECAM-1 facilitates leukocyte transmigration through homophilic interactions with leukocyte PECAM-1 and heterophilic interactions with neutrophil CD177 (25, 26). Studies with blocking Abs or inactivation of the PECAM-1 gene indicate that the role of this protein in modulating leukocyte infiltration in inflamed tissues strongly depends on the

FIGURE 6. In brain and lung of TM LeD/LeD mice, more prominent fibrinogen staining was detected after Stx2/LPS injection compared with TM wt/wt mice. Representative images and quantification of fibrinogen staining in (A) brain and (B) lung of TM wt/wt and TM LeD/LeD mice treated with saline or Stx2/LPS (24, 48 h). Fibrinogen staining was detected by confocal microscopy. Original magnification ×630 (A) and ×400 (B). Data are mean ± SE of n = 6 mice/each group. *p < 0.05, **p < 0.01 versus TM wt/wt mice.

FIGURE 7. Influence of the lack of lectin-like domain of TM on survival and body weight of HUS mice. (A) Survival and (B) weight loss of TM wt/wt (n = 14) and TM LeD/LeD (n = 17) mice treated with Stx2/LPS. Lack of the lectin-like TM domain increased mortality of HUS mice. Data are mean ± SE. *p < 0.01 versus TM wt/wt (log-rank test). After Stx2 plus LPS injection, the percentage of total body weight loss was greater in TM LeD/LeD than TM wt/wt mice. Data are mean ± SE. †p < 0.05, ‡p < 0.01 versus corresponding time 0; *p < 0.05 versus TM wt/wt.
stimulus and the experimental model used (26, 27). There is evidence showing that in a mouse model of HUS (28, 29), chemokines, including MIP-2 and MCP-1, are critical effectors of Stx-associated renal inflammation. In this study, we found that these chemokines were expressed at more extent in the kidneys of TM LeD/LeD than TM wt/wt mice after Stx2/LPS, indicating that they may contribute to the exuberant accumulation of PMNs and monocyte/macrophages in TM LeD/LeD mice.

We recently documented that complement activation via the alternative pathway plays a major role in mediating Stx-induced microvascular injury and thrombosis (23). In vitro experiments, we found that Stx induced C3 activation and deposition on the surface of human microvascular endothelial cells, which was, in turn, instrumental in thrombus formation. Stx2/LPS treatment in mice was associated with intense C3 deposits in the glomeruli, and factor B-deficient mice that cannot activate the alternative pathway of complement were protected from thrombocytopenia and renal function impairment after Stx2/LPS (23). TM has been shown to negatively regulate C3 activation via the alternative pathway in vitro by accelerating factor I-mediated inactivation of C3b in the presence of the cofactor CFH (19). The TM variants found in patients with atypical HUS and affecting the lectin-like domain had diminished capacity to inactivate C3b (19), indicating that this domain is important for TM-complement regulatory properties. In line with these findings, we previously demonstrated that there is excessive C3 deposition in the articular joints of TM LeD/LeD mice under basal conditions as compared with TM wt/wt mice (21). Our data that TM LeD/LeD mice also have more glomerular C3 deposits under basal conditions would indicate impaired complement regulation in these mice and supports a role for the lectin-like domain of TM in preventing spontaneous complement activation in several organs, including the kidney. Enhanced baseline complement activation in TM LeD/LeD mice may contribute to the more rapid onset and increased severity of disease after Stx2/LPS, by facilitating accumulation of proinflammatory products, such as C3b and C5b-9, and of the anaphylatoxins C3a/C5a that mediate the recruitment of inflammatory cells. Of note, we found no difference in glomerular C3 deposits between TM LeD/LeD and TM wt/wt mice after Stx2/LPS injection, which is in line with our previous observation that at day 6 after collagen Ab-induced arthritis, when clinical signs were worse in the TM LeD/LeD mice, complement deposits were similarly increased in TM LeD/LeD and TM wt/wt mice (21). The earlier observations would suggest that maximal complement stimulation induced by Stx2/LPS (23) or by collagen Abs (44, 45) overcomes the complement regulatory activity of the lectin-like domain of TM.

In conclusion, we have shown that after Stx2/LPS induction of HUS, TM LeD/LeD mice as compared with TM wt/wt mice exhibit more severe and earlier onset disease, with a shortened survival time. The accelerated renal microvascular injury in the TM LeD/LeD mice cells may reasonably be attributed to increased accumulation of inflammatory cells and overactivation of complement in the renal glomeruli. The Stx2/LPS-treated TM LeD/LeD mice also developed changes associated with thrombotic microangiopathic lesions in the brain and lung. These findings are consistent with those in humans with D+HUS and the fact that the Stx receptor Gb3 is expressed in the brain and lungs (46, 47), and help to explain the reduced survival of the TM LeD/LeD mice. Our findings are rationale for determining whether TM gene variants in humans are associated with increased risk for the development of HUS upon STEC infection. This report also raises the possibility that recombinant soluble TM might have therapeutic efficacy in HUS. Indeed, in a phase 3 clinical trial for disseminated intravascular coagulation (48), recombinant soluble TM has been shown to be efficacious.

There have also been reports of benefit for patients with thrombotic microangiopathies associated with thrombotic thrombocytopenic purpura (49), systemic lupus erythematosus (50), and transplant-related graft-versus-host disease (51). Further study will demonstrate whether soluble TM can protect against HUS.

Disclosures
E.M.C. holds a patent for the use of the lectin-like domain of TM as an antiinflammatory agent. The other authors have no financial conflicts of interest.

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Supplemental Information

Supplemental Fig. 1. Structure of thrombomodulin (TM). TM is represented with its 5 structural domains including the cytoplasmic tail and a transmembrane domain, a serine/threonine-rich region with an attached chondroitin sulphate (CS) moiety, 6 EGF-like repeats, and the N-terminal lectin-like domain.
Supplemental Fig. 2. Representative images of (A) PECAM-1 staining in the kidney of TM^{LeD/LeD} mice at 48 h after Stx2/LPS injection. PECAM-1 was detected in OCT-frozen sections using rat anti-mouse Ab (1:50, BD Biosciences) followed by FITC-conjugated goat anti-rat IgG (1:150, Sigma). Negative control was obtained using rat IgG2a isotype control (1:1600, BD Biosciences) on adjacent section. (B) Fibrin(ogen) deposits in the kidney of TM^{LeD/LeD} mice at 48 h after Stx2/LPS injection. Fibrin(ogen) staining was revealed by FITC-conjugated goat anti-rat fibrin(ogen) Ab (1:40; Nordic Immunology). Negative control was obtained using FITC-conjugated polyclonal goat Ab (1:40; Nordic Immunology) on adjacent section.
Supplemental Fig. 3. Representative images and quantification of C3 deposits in glomeruli of TM<sup>wt/wt</sup> and TM<sup>LeD/LeD</sup> mice treated with saline (n=3-4 mice/group) or Stx2 at the dose of 20 ng/mouse plus LPS (n=4-5 mice/group), as evaluated at 24 h. Magnification, X630. Data are mean±SE. * p < 0.05 versus TM<sup>wt/wt</sup> mice.