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Chapter 11

HOST-DEFENSE IN A MURINE ESCHERICHIA COLI PERITONITIS MODEL IS NOT INFLUENCED BY FUNCTIONAL THROMBOMODULIN DEFICIENCY


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Submitted for publication
Summary

Based on clinical observations in human gram-negative sepsis, we postulated that a prothrombotic state might lead to impaired host defense and enhanced development of sepsis in mice infected intraperitoneally with Gram-negative bacteria. To investigate this we inoculated Tmp<sup>−/−</sup> mice, that have functionally impaired thrombomodulin, and wildtype littermates intraperitoneally with E. coli at doses of 5x10<sup>4</sup>, 5x10<sup>5</sup>, and 1x10<sup>5</sup> colony forming units (CFU). The endpoints assessed were: signs of illness, bacterial counts, cytokine profiles in plasma, histological evidence for inflammation, and thrombosis. Coagulation activation was evaluated by the measurement of thrombin-antithrombin (TAT) complex levels in plasma. The results showed comparable responses in mutant and wildtype littermates. The conclusion from this experiment is that in the present model of gram-negative peritonitis, impairment of the protein C pathway by dysfunctional thrombomodulin does not lead to impaired host defense.
**Introduction**

Thrombomodulin (TM) is a transmembrane glycoprotein of the luminal endothelium with the capability of serving as a specific receptor for thrombin. In complex with thrombin, TM accelerates the activation of protein C (PC). Activated protein C (APC), proteolytically degrades coagulation factors Va and VIIIa, thereby reducing thrombin formation. In addition, thrombin loses its procoagulant properties upon binding to TM, including further activation of factors V and VIII, inactivation of protein S (PS) and fibrinogen cleavage. Impairment of this anticoagulant pathway through deficiencies in its components (PC, PS, and TM) results in enhanced coagulation activation, increased fibrin production, and an increased risk of venous thrombosis.

Thrombin is known to exert pro- and anti-inflammatory effects and might play a key role in the crosstalk between innate immunity and coagulation. These effects of thrombin may become important in infectious disease, when the coagulation cascade is initiated. Modulation of thrombin production may therefore influence the outcome of infectious disease. Indeed, Gram-negative sepsis is associated with acquired deficiencies of coagulation inhibitors PC, PS, and antithrombin (AT); low levels of these anticoagulant proteins predict poor disease outcome. Moreover, recent clinical and experimental studies show that supplementation of PC, PS and AT reduces mortality from Gram-negative sepsis.

On these data we based our working hypothesis, that a defective anticoagulant state leads to impaired host defense and enhanced development of sepsis in mice infected intraperitoneally with Gram-negative bacteria. To test this hypothesis, a mouse model presenting with a prothrombotic state due to a functional deficiency of TM (TM$^{pro/pr}$) was chosen. In the present study we find no difference in host defense between TM$^{pro/pr}$ mice and WT littermates, which indicates that in these mice alterations in thrombin generation do not contribute to bacterial host defense.

**Material and methods**

*Mice*

The generation of mice lacking the capability of thrombomodulin binding to thrombin by a targeted point mutation in the TM gene (TM$^{pro/pr}$) was described elsewhere. These mice have a severely impaired capacity to generate APC but are viable and without overt spontaneous phenotype. Animals were bred and maintained in a 12-h dark and light cycle room in our animal facility department. The experiments were approved by the Institution Review Board for Animal Experiments of the Academic Medical Center and performed according to the guidelines of the American Physiological Society and the Dutch Law for Animal Experiments.
In the present experiments mutant TM<sup>pr</sup>/pr mice were compared to wildtype sex and age matched littermates that were born from a cross between F2 heterozygotes. The reasons for using littermates were the following. The mice that we imported into our facility as a gift from Dr R.D. Rosenberg (MIT, Boston, USA) were homozygous direct descendants from an F1 cross, and thus genetically 50% C57Bl/6 and 50% 129Sv. In a pilot experiment comparing these homozygotes with commercially available C57Bl/6 mice (Harlan, The Netherlands) we observed striking susceptibility differences in a peritonitis model with E coli (not shown). In particular the TM mice seemed much more resistant to E coli than wildtype C57Bl/6. Moreover, the mice responded very differently to an intraperitoneal injection with LPS, but in this case the mutant mice did much worse. Given the mixed genetic background these results raised the question, whether background per se influences the outcome of peritonitis. Therefore, we resorted to using littermates, which in addition ensures identical health status between the animals that participate in an experiment.

**Intraperitoneal bacterial challenge model.**

Peritonitis was induced by Gram-negative E.coli O18:K1:H7, Bort, a pathogenic smooth, encapsulated strain presenting the O18 (LPS) serotype and the capsular K1 antigen<sup>13</sup> (provided by Dr P. Abraham). After growing in LB medium to mid-log phase, bacteria were washed and adjusted spectrophotometrically to an optical density (OD) of 1.0 (at 650 nm), corresponding to a bacterial concentration of approximately 2.2 x 10<sup>8</sup> CFU/mL. From this concentration, inoculates of 5x10<sup>3</sup>, 5x10<sup>4</sup>, and 1x10<sup>5</sup> CFU/0.5 mL were prepared by dilution with physiological saline. In an initial experiment, a dose of 1 x 10<sup>5</sup> CFU of E. coli O18:K1:H7/Bort, was injected intraperitoneally (i.p) in male TM<sup>pr</sup>/pr mice and WT littermates (n = 4 in each group), causing lethal sepsis within 36 hours in both groups. Based on the assumption that differences in host defense between the two groups might only be detectable when using a sublethal bacterial challenge, a tenfold lower dose, 5 x 10<sup>4</sup> CFU, was i.p injected in 7 TM<sup>pr</sup>/pr and 7 WT mice (all males). Animals were sacrificed 24 hours later showing severe signs of illness in both groups. In parallel, four mice of each group were injected 5 x 10<sup>4</sup> CFU and sacrificed at 4 hours, in order to document early cytokine responses, that might explain potential differences in host defense. Finally, a relatively low dose of 5 x 10<sup>3</sup> CFU E. coli/mouse was administered in 10 male and 10 female TM<sup>pr</sup>/pr and WT littermates, which were sacrificed 24 hours following bacterial challenge.

**Preparation and analysis of peritoneal lavage fluid, blood, and liver tissue homogenates.**

Blood and peritoneal fluid (PF) were collected at different time intervals after bacterial challenge for cytokine measurement, granulocyte influx counts, and bacterial cultures. At least 1 mL of PF was obtained after i.p injection of 3 mL of physiological saline. Citrated blood was obtained by
cannulation of the inferior caval vein. For cytokine measurements, plasma and PF were stored at -70°C until assay. Bacterial counts were determined by plating 10-fold dilutions of blood, PF and homogenized liver tissue samples on blood agar and incubating overnight at 37°C.

Cytokine assays.
Concentrations of tumor necrosis factor alpha (TNFα), interleukin-6, and -10 (IL-6, IL-10) in blood and peritoneal fluid were measured by specific ELISA kits (R&D Systems, ITK Diagnostics BV, Uithoorn, the Netherlands) according to the instructions of the manufacturer.

Thrombin-Antithrombin-Complexes.
Rabbit immunization New Zealand White rabbits were immunized with mouse thrombin (Sigma, St.Louis, MO, USA) and rat antithrombin (AT) (Sigma) and boosted at nine and fifteen weeks after the first immunization with thrombin and AT in combination with Freund's Incomplete Adjuvant (Difco, 1475 Athens Hwy Grayson, GA 30017 USA). During the first three months test samples of approximately 5 ml blood were obtained regularly and thereafter samples of approximately 50 ml were obtained monthly from each rabbit.

Sandwich TAT-complex ELISA MaxiSorp plates were coated overnight at 4°C with purified antithrombin antibodies from the immunized rabbit. The plates were washed and incubated with 50 µL standard or plasma samples diluted in PBS/Tween/FCS. After washing, the plates were incubated with 100 µL purified DIG-conjugated rabbit anti-AT, rewashed, and reincubated with HRP-100 mL conjugated sheep F(ab)2 anti-DIG fragments (Roche Diagnostics) diluted in PBS/Tween. For quantitation, the OPD-method was applied with 1 M H₂SO₄ for termination of the reaction. The OD was determined at 490 and 650 nm. A standard for the mouse TAT ELISA was made from two-fold serial dilutions of mouse serum (Sigma) in PBS/Tween/FCS buffer. To test the linearity of the standard diluted in buffer, mouse serum was also serial diluted in mouse plasma (Sigma) or pooled human plasma. The concentration of TAT complexes in serum diluted in buffer of approximately 6000 ng/ml was determined by the human TAT assay (Enzygnost TAT, Dade Behring, Marburg, Germany). The standard curve was pipetted on each plate from a freshly thawed aliquot in two-fold serial dilutions starting with a 100-fold dilution.

Granulocyte influx in peritoneal fluid.
Peritoneal fluid was centrifuged at 1400 g for 5 minutes, the supernatant was kept and frozen for cytokine measurements. The pellet was resuspended in 200 µl of PBS and white blood cells were counted under a focal light microscope. The pellet was then diluted to white blood cell concentrations of 10^5 cells/mL for a cytospin. The cell smears were stained by hematoxylin/eosin (Wright, MSG, Grogg, Bern) for cell count analysis under a focal light
microscope. The number of macrophages, lymphocytes and granulocytes were expressed in percentage, counting 100 cells per smear.

Tissue thrombosis and inflammation.
Immediately before sacrifice, the animals were heparinised with 400 IU through a tail vein in order to avoid postmortem thrombus formation. Liver, spleen, and lungs were collected following peritoneal lavage and blood collection. Lungs were perfused with PBS before resection. After removing small portions from the tissues for homogenization, the organs were fixed in 10 % buffered formalin and embedded in paraffin. Four μm sections were stained with haematoxylin and eosin. Tissue analysis was performed by one independent and experienced pathologist who was unaware of the status of the mice. For the evaluation of histological differences in liver and lungs a four point semi-quantitative scale was used. Using this score, the sections were evaluated for the presence of fibrin, thrombosis, necrosis, inflammation, neutrophil influx, and edema. For each mouse quantitation was performed in 5 different microscopical fields, and the mean of the different counts was calculated.

Data analysis.
All values are expressed as mean ± SE. Statistical analysis of differences between groups was performed using the Mann-Whitney test. Data were considered significantly different when p < 0.05.

Results

Bacterial outgrowth.
Bacterial outgrowth in TMpro/+/pr and WT littermates, inoculated with \(5 \times 10^4\) CFU/mouse (high dose), was high (WT: PF \(1.7 \times 10^4\) CFU/mL; blood \(9.8 \times 10^4\) CFU/mL; liver \(1.8 \times 10^5\) CFU/mL; TMpro/+/pr: PF \(4.1 \times 10^3\) CFU/mL; blood \(1.1 \times 10^5\) CFU/mL; liver \(1.4 \times 10^7\) CFU/mL) at 24 hours and did not differ in PF, blood, and liver between groups (Figure 1). This finding was consistent with the poor condition of the animals, showing clear signs of sepsis in both groups. At 4 hours the animals showed no signs of illness and bacterial outgrowth was equal in both groups (Figure 2).

Male TMpro/+/pr and WT mice, inoculated with \(5 \times 10^3\) CFU/mouse (low dose), presented only minor signs of illness, while females did not become ill at all. Bacterial counts in PF, blood, and liver revealed moderate bacterial growth in males, while most of the females had cleared all bacteria (Figure 3,4).
Figure 1. Bacterial outgrowth in peritoneal fluid, blood, and liver in male TMpro/pro (TM) and WT mice (dose $5 \times 10^9$ CFU E.coli), 24 hours after inoculation.

Figure 2. Bacterial outgrowth in peritoneal fluid, blood, and liver in male TMpro/pro (TM) and WT mice (dose $5 \times 10^9$ CFU E.coli), 4 hours after inoculation.

Figure 3. Bacterial outgrowth in peritoneal fluid, blood, and liver in TMpro/pro (TM) and WT male mice (dose $5 \times 10^9$ CFU E.coli), at 24 hours.

Figure 4. Bacterial outgrowth in peritoneal fluid, blood, and liver in female TMpro/pro (TM) and WT mice (dose $5 \times 10^9$ CFU E.coli), 24 hours after inoculation.
Cytokine response in blood and peritoneal fluid.

Overall, in all animals IL-6, TNFα, and IL-10 concentrations in plasma corresponded to the amount of bacteria found in the same individual, reflecting LPS challenge on cytokine expression elicited by the presence of Gram-negative bacteria. Mice inoculated with $5 \times 10^4$ CFU/mL showed higher plasmatic IL-6 levels in WT than in TM<sup>pm/ pm</sup> mice ($1.3 \times 10^2$ ng/ml for WT, 52 ng/ml for TM<sup>pm/ pm</sup>, Figure 5), showing a tendency towards a stronger cytokine response in WT animals ($p = 0.09$). At 4 hours, IL-6 levels were higher in TM<sup>pm/ pm</sup> than in WT mice ($1.5 \pm 0.5$ ng/mL, $0.5 \pm 0.3$ ng/mL, respectively, Figure 6), but not statistically significant. TNFα response, which was much weaker at 24 hours than IL-6, showed a tendency towards higher levels for WT animals as well ($231 \pm 0.5$ and $186 \pm 0.3$ pg/ml, respectively).

In those animals injected with $5 \times 10^1$ CFU, who did not show any signs (females) or slight signs (males) of illness with low bacterial counts, cytokine concentrations were proportionally lower, demonstrating a clear dose-dependent relationship between bacterial challenge and inflammatory response (Figures 7-9). IL-10 at 24 hours had only detectable levels in two animals of both groups with mean values of $23.4 \pm 29$ (WT) and $32 \pm 40$ pg/ml (TM<sup>pm/ pm</sup>), while the other individuals had non-detectable levels. All results are consistent with the observation, that females are less sensitive to bacterial challenge than males.
Figure 5. IL-6 levels in plasma of male TM<sup>pm/pm</sup> and WT mice (dose 5 x 10<sup>6</sup> CFU E.coli) at 24 hours

Figure 6. IL-6 concentration in plasma of male TM<sup>pm/pm</sup> and WT mice (5 x 10<sup>6</sup> CFU E.coli) at 4 hours

Figure 7. IL-6 concentration in plasma of male TM<sup>pm/pm</sup> and WT mice (5 x 10<sup>6</sup> CFU E.coli) at 24 hours

Figure 8. IL-6 concentration in plasma of female TM<sup>pm/pm</sup> and WT mice (5 x 10<sup>6</sup> CFU E.coli) at 24 hours
Figure 9. TNF-α levels in citrated plasma of WT and TM<sup>+/−</sup> male mice at 24 (A) and 4 (B) hours after inoculation of 5 x 10<sup>4</sup> CFU and in male (C) and female (D) mice 24 hours after 5 x 10<sup>4</sup> CFU.
Thrombin-Antithrombin-Complexes.

TAT levels in plasma did not reveal differences in activation of coagulation between groups. Mean plasmatic values of TAT complexes in ng/mL for WT and TM^{pro/pro} high dose (10^4 CFU/mL) and low dose (10^3 CFU/mL) in males and females, are shown below in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>24 hours</th>
<th>High dose (10^4 CFU) (ng/mL)</th>
<th>Low dose (10^3 CFU) (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT males</td>
<td></td>
<td>0.99 ± 0.72</td>
<td>0.60 ± 0.5</td>
</tr>
<tr>
<td>TM^{pro/pro} males</td>
<td></td>
<td>1.15 ± 0.68</td>
<td>0.51 ± 0.3</td>
</tr>
<tr>
<td>WT females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM^{pro/pro} females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td></td>
<td>1.43 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>WT males</td>
<td></td>
<td>0.79 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>TM^{pro/pro} males</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. TAT levels were measured in citrated plasma and did not differ between groups.

Histological analysis of liver and lungs

In the liver, sharply demarcated necrotic areas located chiefly around thrombosed vessels were observed in both groups. The degree of inflammation was also comparable. Beside fibrin deposition and few thrombi in small vessels, the pathological findings in the lungs consisted predominantly of interstitial granulocytic inflammation. (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Fibrin Deposition</th>
<th>Necrosis</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thrombosis</td>
<td></td>
<td>Granulocyte Influx</td>
</tr>
<tr>
<td>Liver</td>
<td>TM/-</td>
<td>WT</td>
<td>TM/-</td>
</tr>
<tr>
<td>Lungs</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2. Histological analysis of liver and lungs showed comparable degrees of inflammation and thrombosis formation.
Granulocyte influx in peritoneal fluid.

The means (and SE, Table 3) for the white blood cell counts and differentiation are shown in table 3. Granulocyte influx counts. At 24 hours, in both groups, wildtype and Tmpro/pro, there was an increase of granulocytes compared to the earlier timepoint (4 hours) after bacterial inoculation. Again, there was no significant difference detectable between groups.

<table>
<thead>
<tr>
<th></th>
<th>TM 24 h</th>
<th>WT 24 h</th>
<th>TM 4h</th>
<th>WT 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC x 10⁶/mL</td>
<td>10.3 ± 4.8</td>
<td>13.5 ± 2.7</td>
<td>0.8 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>88</td>
<td>93</td>
<td>75</td>
<td>78</td>
</tr>
<tr>
<td>Macrophages %</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>10</td>
<td>6</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>

*Table 3.* Mean values and SE for total white blood cell counts and percentage of granulocyte and macrophages for Tmpro/pro and WT mice 24 hours and 4 hours following high dose bacterial challenge (5 x 10⁴ CFU).

Discussion

The present study was performed in order to investigate whether a functional TM deficiency influences host defense in a murine model of E. coli peritonitis. According to the present data, there is no evidence to support this hypothesis.

Led by a pilot experiment we took great care to ensure that genetic background differences, or differences in health status between the animals, would influence the results. This proved to be a well-chosen approach. In the pilot experiment we did a head-on comparison between homozygous TM⁺/⁻ mice with commercial littermates. In that experiment striking differences were observed between the mutant and wildtype mice. With a dose of 10⁵ CFU E. coli wildtype mice became severely septic, whereas TM⁺⁺/⁺⁺ mice almost all had cleared the bacteria after 24 hours. As is evident from the presented data this result could not be reproduced in the littermate experiment, which underlines the importance of using littermates in evaluating disease susceptibility in genetically modified mice.

It might be too early to conclude that the TM⁺⁺/⁺⁺ have a normal susceptibility to every infectious disease. The reason is that TM expression is particularly high in some organs, e.g. the lungs, compared to other organs. The quantity of TM in the vasculature of the peritoneum is not
known but if low, this might lead to comparable local levels of thrombin between mutant and wildtype mice. In other models of infection, e.g. pneumonia, local levels of thrombin might well be very different, and disease susceptibility may vary between mutant and normal mice.

It is also too early to conclude that a procoagulant state as such does not influence susceptibility to peritonitis. The reason for this is that TM not only functions to trigger the protein C pathway, but at the same time is crucial for the activation of TAFI (thrombin activatable fibrinolysis inhibitor). When TM cannot bind to thrombin, as in the present murine model, TAFI is not or less well activated, leading to increased lysis. Thus, the prothrombotic state, which was mainly characterized as diminished protein C activation, might in fact be compensated by increased thrombolysis. Other prothrombotic mice, like mice carrying the factor V Leiden mutation, should not show this defect in TAFI generation and could very well behave very differently in the same peritonitis model.

Acknowledgments

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References


