Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Mice lacking the lectin-like domain of thrombomodulin are protected against melioidosis

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ABSTRACT

Objective
Thrombomodulin (TM) is a multidomain receptor primarily expressed by vascular endothelium. The lectin-like domain of TM has anti-inflammatory properties. In this study we investigated the role of the TM lectin-like domain in the host response to Gram-negative sepsis caused by *Burkholderia (B.) pseudomallei*, a “Tier 1” biothreat agent and the causative agent of melioidosis, a common form of community-acquired sepsis in Southeast Asia.

Design and Setting
Animal study, University research laboratory.

Subjects and Interventions
Wild-type (WT) mice and mice lacking the lectin-like domain of TM (TMLeD/LeD mice). Mice were intranasally infected with live *B. pseudomallei* and sacrificed after 24, 48 or 72 hours for harvesting of lungs, liver, spleen and blood. Additionally, survival studies were performed.

Measurements and Main Results
Following exposure to *B. pseudomallei*, TMLeD/LeD mice showed a survival advantage, accompanied by decreased bacterial loads in the blood, lungs, liver and spleen. While lung histopathology did not differ between groups, TMLeD/LeD mice displayed strongly attenuated systemic inflammation, as reflected by lower plasma cytokine levels, maintenance of normal kidney and liver function, histologic evidence of reduced organ damage and damage to the spleen.

Conclusions
This study reveals for the first time, a detrimental role for the TM lectin-like domain in the host response to sepsis caused by a clinically relevant Gram-negative pathogen.
INTRODUCTION

Thrombomodulin (TM, CD141) is a multidomain type-I transmembrane glycoprotein that is expressed on the surface of vascular endothelial and various hematopoietic cells. TM is located strategically and hereby has interactions with thrombin, protein C (PC), thrombin activatable fibrinolysis inhibitor (TAFI), complement factors, Lewis Y antigen, a cell-surface tetrasaccharide involved in endothelial cell-cell interactions and high-mobility group box 1 (HMGB1). Via these interactions TM modulates inflammation, coagulant and fibrinolysis and cell proliferation.

TM consists of five structural domains: the N-terminal C-type lectin-like domain, six epidermal growth factor (EGF)-like repeats (EGF-domain), a serine/threonine-rich domain, a transmembrane domain and a cytoplasmic domain. The EGF-domain is required for thrombin-mediated generation of activated PC (APC), which has anticoagulant and cytoprotective functions, and thrombin-mediated generation of activated TAFI (TAFI), which has both anti-fibrinolytic and complement factor (C)3a- and C5a-degrading properties. In contrast to the EGF-domain, the lectin-like domain lacks anti-coagulant and anti-fibrinolytic properties. Instead, this domain of TM, with homology to C-type lectins, plays a major role in the regulation of inflammation, complement activation, cell adhesion and cell proliferation. As compared to wild-type (WT) mice, mice lacking the TM lectin-like domain (TMLeD/LeD mice) exhibited reduced survival after a systemic lipopolysaccharide (LPS) challenge and increased accumulation of polymorphonuclear neutrophils in their lungs. These effects may be caused by specific interaction of the lectin-like domain of TM with Lewis Y antigen of LPS. The lectin-like domain of TM also displayed anti-inflammatory properties in models of myocardial ischemia-reperfusion injury, acute inflammatory arthritis, shiga-toxin induced hemolytic uremic syndrome (HUS), diabetic glomerulopathy and allergic lung inflammation. Recently, administration of recombinant lectin-like domain of TM was shown to be protective in Klebsiella (K.) pneumonia-induced sepsis. In contrast to the preceding compelling observations, lack of the lectin-like domain of TM was beneficial in the host response to pneumonia caused by the Gram-positive bacterium Streptococcus (S.) pneumoniae. Indeed, infected TMLeD/LeD mice had a significant survival advantage, lower bacterial loads and less pulmonary inflammation as compared to WT mice.

In spite of the protection afforded by soluble TM lectin-like domain in K. pneumonia induced sepsis, our understanding of the role of the lectin-like domain of TM during Gram-negative sepsis is limited. This is of particular interest, given that there are reportedly direct interactions between the TM lectin-like domain and LPS, the proinflammatory component of the Gram-negative cell wall. Moreover, delineating the mechanisms by which the lectin-like domain of TM regulates the host response to various pathogens may lead to novel therapies. In the present study we sought to characterize the role of the lectin-like domain of TM during melioidosis, an infectious disease caused by the Gram-negative bacillus Burkholderia (B.) pseudomallei. Melioidosis is characterized by a wide array of systemic symptoms and signs with fever and pain and involvement of essentially any organ,
but primarily the lungs, skin and joints. In Southeast Asia and Northern Australia where it is endemic and an important cause of community acquired sepsis, infection with melioidosis may be associated with mortalities up to 40% despite appropriate antibiotic therapy. Indeed, B. pseudomallei was recently classified as a ‘Tier 1’ disease agent considered to be an exceptional threat to security. Interestingly, previous data showed marked upregulation of TM in culture-proven melioidosis patients when compared to healthy controls, prompting us to evaluate whether this association has pathophysiologic relevance. In this study, we investigated the role of the lectin-like domain of TM by comparing WT mice with TMLeD/LeD mice after intranasal challenge with B. pseudomallei.

MATERIALS AND METHODS

Mice
Pathogen-free 10-week old male wild type (WT) C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands). TMLeD/LeD mice were generated as described and backcrossed at least eight times to a C57BL/6 genetic background. Deficiency of the TM lectin-like domain was confirmed by polymerase chain reaction (PCR) of lysed toes of newborn TMLeD/LeD mice. TMLeD/LeD mice express normal antigenic levels of TM and thrombin-mediated activation of PC is intact and unaffected by the lack of the lectin-like domain. In addition, activation of TAFI is also unaffected as for this process not the lectin-like domain but the EGF-like repeats of TM are involved. Mice were maintained at the animal care facility of the Academic Medical Center (University of Amsterdam), according to national guidelines with free access to food and water. The Committee on Use and Care of Animals of the University of Amsterdam approved all experiments.

Experimental infection and determination of bacterial growth
Experimental melioidosis was induced by intranasal inoculation with B. pseudomallei strain 1026b (300 or 750 colony forming units (CFU)/50µL 0.9%NaCl) as previously described. These doses represent an approximate 65% (LD65) and 100% (LD100) lethal dose respectively. For survival experiments, mice were checked every 4-6 hours until death occurred, or until a maximum of 15 days. Sample harvesting and processing and determination of bacterial growth were performed as described. For details, see the Supplementary Material.

Assays
Lung homogenates were diluted in lysis buffer, sterilized stored at -20°C until analysis. Citrated blood was centrifuged at 664xg, plasma was snap frozen at -80°C. Before storage, all samples were filtered through a 22 µm filter (Millipore, Billerica, MA). Assays for cytokines, chemokines, thrombin-antithrombin (TATc), plasminogen-activator inhibitor 1 (PAI-1), tissue-type plasminogen activator (tPA), Western blotting on fibrin degradation products, lactate dehydrogenase
(LDH), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), both markers for hepatocellular injury and urea, a marker for renal failure, are described in the Supplementary Material, as are methods for Western blots, cell counts and flow cytometry.

**Histology and immunohistology**
Paraffin-embedded 4 µm tissue sections were stained with haematoxylin and eosin (H&E) and analyzed for inflammation and tissue damage as described. Granulocyte stainings using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) were performed. For details see the Supplementary Material.

**Statistical analysis**
Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation or as medians with interquartile ranges. All variables were rank-transformed prior to analysis. Comparisons between groups were conducted using overall non-parametric ANOVA with modeled effects for strain, time and their interaction (if appropriate), followed by post-hoc Mann-Whitney U tests at the individual time points. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using SPSS (SPSS Inc, version 18, Armonk, NY). P-values less than 0.05 were considered statistically significant.

**RESULTS**

**Lack of the lectin-like domain of TM is associated with reduced Gram-negative bacterial growth and dissemination**
Our model of lung sepsis is associated with marked bacterial proliferation in the lungs, as well as in the blood, spleen and liver. In order to establish whether the lectin-like domain of TM affects bacterial growth and dissemination, WT and TM LeD/LeD mice were infected with 750 CFU of *B. pseudomallei* and sacrificed after 24, 48 and 72 hours to determine bacterial loads in lungs (the primary site of infection), liver, spleen and blood. At 48 hours, TM LeD/LeD mice had lower bacterial loads in the lungs and liver (both *P* < 0.05, Figure 1A,C), while after 72 hours bacterial loads were lower in all TM LeD/LeD organs, as compared to those from WT mice (*P* < 0.01 for lung, Figure 1A; *P* < 0.01 for blood, liver and spleen, Figure 1B-D). These data demonstrate that the lectin-like domain of TM plays a role in overcoming antibacterial defense mechanisms during Gram-negative lung sepsis.
Our murine model of melioidosis is associated with severe lung damage and both WT and TMLeD/LeD mice infected with *B. pseudomallei* displayed inflammatory infiltrates in the lungs (Figure 2). There were, however, no significant differences in lung inflammation between WT and TMLeD/LeD mice (Figure 2A-C). We additionally analysed neutrophil recruitment to lung tissue, as it is known that neutrophils play an important role in the host defense during melioidosis. Immunostaining of histologic sections for Ly-6G revealed neutrophil infiltrates in the lungs of WT and TMLeD/LeD mice that increased over time during the course of the infection. However, there were no significant differences in Ly6G-staining between WT and TMLeD/LeD mice (Figure 2D-F). Accordingly, levels of myeloperoxidase (MPO), a marker for total neutrophil content, in lung homogenates were also similar in WT and TMLeD/LeD mice (Figure 2G).

**TMLeD/LeD and WT mice exhibit similar lung histopathology and pulmonary neutrophil recruitment**

Infection with *B. pseudomallei* is associated with a prominent inflammatory response with elevations in cytokines. We measured levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6,
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IL-12p70, interferon (IFN)-γ and monocyte-chemoattractant protein (MCP)-1 in lung homogenates at various times following infection (Table 1). Although we could not detect differences in cytokine levels at 24 hours, there was a time-dependent progressive difference at 48 and 72 hrs. Thus, 48 hours post-infection, IL-6, IFN-γ and MCP-1 were significantly lower in lung homogenates of TMLeD/LeD mice compared to WT mice (\(P < 0.01\), 0.001 and 0.01 respectively). At 72 hours, TNF-α, IL-12p70, IL-6, IFN-γ and MCP-1 were lower in TMLeD/LeD mice (\(P < 0.01\), 0.001, 0.01, 0.001 and 0.01 respectively).

Lack of the lectin-like domain of TM is associated with a less severe proinflammatory response in BALF

Pneumonia may lead to alveolar damage and subsequent alveolar leakage and release of pro-inflammatory factors. We therefore assessed the role of the TM lectin-like domain on CFU and inflammatory cytokines in BALF 72 hours post-inoculation when bacterial growth (Figure 1) and lung homogenate cytokine levels (Table 1) were most elevated. As shown in Figure 3, TMLeD/LeD mice had significantly lower bacterial loads in BALF as compared to WT mice (\(P < 0.001\); Figure 3A). This was associated with lower total protein and total cell counts in BALF (\(P < 0.05\) and < 0.001 respectively for the difference between WT and TMLeD/LeD mice; Figure 3B-C). The reduced...
number of cells was mainly due to fewer neutrophils and a decreased amount of macrophages in BALF of TMLeD/LeD mice when compared to WT mice (both \( P < 0.001 \); Figure 3D–E). Since TM may interact with components of the complement system\(^5, 6\), we also measured C3a and C5a levels in BALF. C3a levels were significantly lower in the BALF of TMLeD/LeD mice as compared to WT mice (\( P < 0.01 \); Figure 3F). In contrast, BALF C5a levels were not detectably different (data not shown). The lectin-like domain of TM also reportedly binds to HMGB1\(^9\) and prevents the latter’s interaction with the receptor for advanced glycation endproducts (RAGE), thereby dampening HMGB1’s pro-inflammatory effects \(^9\). We therefore semi-quantitatively measured HMGB1 levels in BALF of WT and TMLeD/LeD mice by Western blot. Although BALF levels of HMGB1 in uninfected WT and TMLeD/LeD mice were similar (Figure 3G), HMGB1 levels in infected TMLeD/LeD mice were significantly lower when compared to WT mice (\( P < 0.05 \); Figure 3G–H).

**TMLeD/LeD** mice exhibit reduced systemic coagulation activation after infection with *B. pseudomallei*

We have previously shown that murine melioidosis is associated with inflammation and coagulation activation, which is most evident at later time points, such as after 48 and 72 hours of infection\(^23-25\). Although the lectin-like domain of TM is not known to directly regulate coagulation or fibrinolysis\(^2, 11\), we considered whether lack of the lectin-like domain of TM would influence coagulation and fibrinolysis in our model of murine melioidosis. Levels of TATc, a marker of coagulation activation, were similar in the lung homogenates of WT and TMLeD/LeD mice 48 and 72 hrs post-

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**Table 1.** Cytokine concentrations in lung homogenates of WT and TMLeD/LeD mice during murine melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>201 (135-365)</td>
<td>238 (136-306)</td>
<td>1167 (603-1446)</td>
</tr>
<tr>
<td>IL-6</td>
<td>732 (570-1432)</td>
<td>448 (350-638)</td>
<td>1154 (588-2026)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>9.6 (6.0-16)</td>
<td>16 (7.1-20)</td>
<td>16 (12-24)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>11 (8.8-18)</td>
<td>13 (9.3-16)</td>
<td>15 (14-21)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2429 (2093-2470)</td>
<td>2661 (2304-3160)</td>
<td>4019 (2699-4818)</td>
</tr>
</tbody>
</table>

**IFN-γ** interferon-γ, **IL** interleukin, **MCP-1** monocyte-chemoattractant protein-1, **TNF-α** tumor necrosis factor-α. **\( P < 0.01 \)** and ***\( P < 0.001 \)** for WT versus TMLeD/LeD mice (Mann-Whitney U test).
The lectin-like domain of thrombomodulin in melioidosis infection (Figure 4A). At the same time, however, in plasma TATc levels were significantly lower in TM<sup>LeD/LeD</sup> mice (<i>P</i> < 0.05; Figure 4B), suggesting lower generation of thrombin in these mice. Levels of PAI-1 did not differ between both groups at any of the time points (Figure 4C), whereas, surprisingly, levels of tPA were increased in TM<sup>LeD/LeD</sup> mice in comparison to WT mice, 48 hours after infection (<i>P</i> < 0.01; Figure 4D). Finally, levels of fibrin degradation products (including fragment X and D-dimer) were not significantly different between WT and TM<sup>LeD/LeD</sup> mice (Figure 4E-H) at any of the time points, indicating that activation of fibrinolysis was equal in both groups.

**Lack of the lectin-like domain of TM is associated with attenuated systemic inflammation and organ injury**

Since TM<sup>LeD/LeD</sup> mice respond to the Gram-negative infection with less bacterial dissemination to blood, liver and spleen, we considered whether this would result in attenuated systemic inflammation...
Figure 4. TM$^{40Lc4D}$ mice exhibit reduced systemic coagulation activation after infection with *B. pseudomallei*. After induction of melioidosis, WT and TM$^{40Lc4D}$ mice exhibit coagulation activation in lung homogenates (A) and plasma (B), as quantified by levels of TATc. Mice were infected intranasally with 750 CFU of *B. pseudomallei* to induce melioidosis and sacrificed after 24, 48 and 72 hours. No significant differences between WT and TM$^{40Lc4D}$ mice were seen in lung homogenates, whereas in plasma after 72 hours, TATc levels were significantly lower in TM$^{40Lc4D}$ mice. Additionally, levels of PAI-1 (C) and tPA (D) were measured. Western blotting on fibrin(ogen) degradation products including fragment X (240-260 kD) and D-dimer (200 kD) in lung homogenates 24, 48 and 72 hours after infection (E-I). After semi-quantitative analysis of the blots comparative levels of fragment X (E) and D-dimer (F), two main fibrin degradation products, were seen in lung homogenates in WT and TM$^{40Lc4D}$ mice, 24 (G), 48 (H) and 72 hours (I) after infection. FrX Fragment X, Dd D-dimer, C positive control sample (fully clotted mouse plasma, uninfected, dilution 1:50). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey boxes represent WT mice, white boxes represent TM$^{40Lc4D}$ mice. $n = 8$ mice per group for each time point (except for HMGB1 blots: $n = 5$ per group). *$P < 0.05$ for the difference between WT and TM$^{40Lc4D}$ mice (Mann-Whitney U test).
The lectin-like domain of thrombomodulin in melioidosis

Indeed, at 72 hrs after infection, significantly decreased levels of pro-inflammatory cytokines were measured in plasma of TMLeD/LeD mice in comparison to WT mice; at 24 and 48 hours no differences between groups were detected (Table 2). Moreover, at 72 hrs post-infection, plasma levels of LDH, urea, ASAT and ALAT were all significantly elevated in WT mice (Figure 5A-D). In striking contrast, at 72 hrs, mice lacking the TM lectin-like domain had minimally elevated levels of these markers of tissue damage, and all were significantly lower than in WT mice (*P < 0.05, **P < 0.01 and ***P < 0.001 for WT versus TMLeD/LeD mice (Mann-Whitney U test).

**Table 2. Cytokine concentrations in plasma of WT and TMLeD/LeD mice during murine melioidosis.**

<table>
<thead>
<tr>
<th></th>
<th>pg/mL</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
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<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td>WT</td>
<td>TMLeD/LeD</td>
<td>WT</td>
</tr>
<tr>
<td>&lt; 3.0</td>
<td>5.3</td>
<td>(3.7-6.9)</td>
<td>10</td>
<td>(7.2-14)</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td>33</td>
<td>(28-52)</td>
<td>77</td>
</tr>
<tr>
<td><strong>IL-12p70</strong></td>
<td></td>
<td>4.4</td>
<td>(2.1-195)</td>
<td>9.9</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td>10</td>
<td>(7.8-20)</td>
<td>39</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td>183</td>
<td>(139-263)</td>
<td>299</td>
</tr>
</tbody>
</table>

Plasma cytokine levels after intranasal infection with 750 CFU of *B. pseudomallei*. Wild type (WT) mice are compared with mice deficient for the lectin-like domain of thrombomodulin (TMLeD/LeD mice). Mice were sacrificed 24, 48 and 72h after infection. Data are expressed as median (interquartile ranges) of n = 8 mice per group per time point. IFN-γ interferon-γ, IL interleukin, MCP-1 monocyte-chemoattractant protein-1, TNF-α tumor necrosis factor-α. *P < 0.05, **P < 0.01 and ***P < 0.001 for WT versus TMLeD/LeD mice (Mann-Whitney U test).

and organ injury. Indeed, at 72 hrs after infection, significantly decreased levels of pro-inflammatory cytokines were measured in plasma of TMLeD/LeD mice in comparison to WT mice; at 24 and 48 hours no differences between groups were detected (Table 2). Moreover, at 72 hrs post-infection, plasma levels of LDH, urea, ASAT and ALAT were all significantly elevated in WT mice (Figure 5A-D). In striking contrast, at 72 hrs, mice lacking the TM lectin-like domain had minimally elevated levels of these markers of tissue damage, and all were significantly lower than in WT mice (P < 0.01 for LDH, urea, ASAT and ALAT; Figure 5A-D). In addition, histopathological scores of damage and inflammation of spleens were lower in infected TMLeD/LeD mice when compared to WT mice, 72 hours after inoculation (P < 0.01; Figure 5E-G). Altogether, these data indicate that in response to infection with *B. pseudomallei*, systemic proinflammatory cytokine levels are dampened and protection from tissue damage is afforded when the lectin-like domain of TM is lacking.

**TMLeD/LeD mice display a survival advantage during murine melioidosis**

To determine the impact of the lectin-like domain of TM during severe Gram-negative lung sepsis caused by *B. pseudomallei* we infected WT mice with 750 CFU *B. pseudomallei* (i.e., the dose used in the experiments described above) and performed a survival study. Whereas all WT mice died, 2 out of 16 TMLeD/LeD mice (13%) survived (*P = 0.2; Figure 6A). Arguing that this dose might have been too overwhelming to detect a significant difference between the groups, we inoculated mice with a lower (300 CFU) dose of *B. pseudomallei* and found a decreased mortality of TMLeD/LeD mice when compared to WT mice: 5 out of 15 WT mice (33%) survived versus 11 out of 13 TMLeD/LeD mice (85%) (*P < 0.01; Figure 6B). These results indicate that lack of the lectin-like domain of TM is important for protection against mortality in this model.
Chapter 11

DISCUSSION

TM is a multidomain transmembrane glycoprotein involved in multiple physiologically important anti-inflammatory, anti-coagulant and anti-fibrinolytic mechanisms\(^2, 29\). While the function of the lectin-like domain of TM has been studied in models of sterile inflammation\(^6-8, 11, 13, 14\) and Gram-positive infection\(^17\), the role of this TM domain in Gram-negative infection has hitherto not been reported. The lectin-like domain of TM has been implicated as an important mediator of inflammation in several in vivo models\(^11\). After intraperitoneal challenge with LPS, TM\(^{Ld/D:Ld/D}\) mice displayed reduced survival, accumulation of more neutrophils in their lungs and greater elevation of serum cytokine levels\(^11\). TM\(^{Ld/D:Ld/D}\) mice also exhibited a more severe phenotype in comparison to WT

Figure 5. Lack of the lectin-like domain of TM is associated with attenuated systemic inflammation and organ injury. At 72 hours after infection with 750 CFU of \(B.\) *pseudomallei*, TM\(^{Ld/D:Ld/D}\) mice, as compared to WT mice, had lower plasma LDH levels (A), lower urea levels (B) and less hepatocellular damage as evidenced by lower ASAT (C) and ALAT (D). At 72 hrs post-infection, TM\(^{Ld/D:Ld/D}\) mice also had less severe damage of the spleen as quantified by a scoring system (see Supplementary Methods) of the histopathology (E). Representative photographs of the spleen at 72 hours post-inoculation of WT (F) and TM\(^{Ld/D:Ld/D}\) mice (G) (H&E staining x100). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey boxes represent WT mice, white boxes represent TM\(^{Ld/D:Ld/D}\) mice. \(n = 8\) mice per group for each time point. **\(P < 0.01\) for the difference between WT and TM\(^{Ld/D:Ld/D}\) mice (Mann-Whitney \(U\) test).
The lectin-like domain of thrombomodulin in melioidosis mice in response to experimentally-induced murine arthritis, ischemia-reperfusion lung injury, shiga toxin-associated HUS and diabetic glomerulopathy. In contrast, our laboratory recently reported that TMLeD/LeD mice have improved survival and diminished bacterial growth and dissemination during Gram-positive pneumonia caused by S. pneumoniae. Interestingly, administration of recombinant soluble TM lectin-like domain resulted in enhanced bacterial clearance and dramatically improved survival after intraperitoneal infection with the Gram-negative pathogen Klebsiella pneumoniae. Soluble TM lectin-like domain at least in part acts as a decoy receptor, which could explain similar beneficial effects of administration of recombinant soluble lectin-like domain during Klebsiella sepsis and of deficiency of the TM lectin-like domain as described here. One possible mechanism by which the (soluble) TM lectin-like domain could influence host defense during Gram-negative infection is by interacting with LPS. One could speculate that in the case of a gradually expanding bacterial burden at the primary site of infection, such as in the current model, the diminished inhibition of LPS due to the absence of the TM lectin-like domain might result in an attenuated early innate immune response to Gram-negative infection. On the other hand, during late stage infection, uncontrolled LPS activity might exaggerate tissue injury. We here show that such a damaging effect of an absent TM lectin-like domain does not occur during lethal Gram-negative lung sepsis, i.e. there was minimal evidence of tissue injury in the TMLeD/LeD mice, presumably due to the much lower bacterial loads. Future research should establish whether S. pneumoniae and/or B. pseudomallei directly interact with TM.

HMGB1, released from necrotic cells or secreted from inflammatory cells after cytokine stimulation, is a potent pro-inflammatory mediator, mediated by its binding to RAGE and/or TLR4. The lectin-like domain of TM is capable of sequestering HMGB1, thereby dampening the pro-inflammatory properties of HMGB1. We hypothesized that lack of the lectin-like domain

Figure 6. TMLeD/LeD mice display a survival advantage during murine melioidosis. WT (black dots) and TMLeD/LeD mice (open triangles) were infected with 750 (LD100; A) or 300 (LD65; B) CFU of B. pseudomallei and mortality was assessed every 6 hours. After infection with 750 CFU all WT mice died whereas 2 out of 16 TMLeD/LeD mice (13%) remained alive. After an LD65 5 out of 15 WT mice (33%) survived versus 11 out of 13 TMLeD/LeD mice (85%). Comparison between groups was done by using Kaplan-Meier analysis followed by log rank tests. LD100/65: dosage causing 100/65% lethality.
would result in increased HMGB1 levels, which on the one hand could exert antibacterial effects and on the other hand could exaggerate collateral organ damage. However, HMGB1 levels in BALF of TM^{LeD/LcD} mice were lower than in BALF of WT mice, most likely as a consequence of the lower bacterial loads in the former group, inducing a less severe pro-inflammatory response.

The complement system is a major component of innate immunity by formation of cell-killing membrane attack complexes, that are formed upon activation of (among others) complement factors C3a, C3b and C5b. In previous studies, TM^{LeD/LcD} mice were shown to exhibit enhanced complement activation that was manifest by increased C3 deposition when compared to WT mice. In diabetic TM^{LeD/LcD} mice, glomerular C3 deposition was increased when compared to non-diabetic TM^{LeD/LcD} mice and to diabetic WT mice. These observations were consistent with a role for the lectin-like domain of TM in dampening activation. In the model of experimentally induced arthritis, C3 deposition increased to such an extent that no differences could be measured between WT and TM^{LeD/LcD} mice, while in the model of shiga toxin-associated HUS, there was a slight, albeit not statistically significant increase in C3 deposition. In contrast, we demonstrated markedly decreased C3a levels in BALF of TM^{LeD/LcD} mice infected with *B. pseudomallei* when compared to WT mice, presumably consistent with the overall dampened state of inflammation in these mice. It remains to be established whether the previously described basal augmented complement activation in TM^{LeD/LcD} mice contributes to the attenuated growth of *B. pseudomallei* in these animals, resulting in lower bacterial loads and as a consequence thereof diminished inflammation and mortality. Thus far only limited data exists on the role of complement in the host defense against *B. pseudomallei*.

Neutrophils play an important role in host defense against *B. pseudomallei* infection. TM^{LeD/LcD} mice displayed increased neutrophil recruitment to lungs upon LPS nebulization and during ischemia/reperfusion induced lung injury. In theory, enhanced neutrophil influx to the primary site of infection could have resulted in an improved antibacterial defense during experimental melioidosis. However, lung neutrophil counts were similar between TM^{LeD/LcD} and WT mice at all time points.

In contrast to the TM EGF-rich domain, which is responsible for PC and TAFI activation, the lectin-like domain of TM does not affect activation of pro- or anticoagulant factors. TM^{LeD/LcD} mice have unaltered plasma levels of PC and APC, even after intraperitoneal LPS challenge, indicating that deficiency of the lectin-like domain of TM does not influence inflammation-induced coagulation activation. In accordance, we found similarly enhanced pulmonary coagulation activation, as reflected by lung TATc levels, in TM^{LeD/LcD} and WT mice. Plasma TATc levels, however, were lower in TM^{LeD/LcD} mice when compared to WT mice at 72 hours post infection. This may be a reflection of the decreased bacterial loads and, consequently, the less severe pro-inflammatory response in the TM^{LeD/LcD} mice. Along the same line, during this late stage of infection, shortly
before the first WT mice started to succumb, TM^{LeD/LeD} mice had much lower plasma cytokine levels and less evidence of organ damage, as reflected by reduced plasma levels of urea, transaminases and LDH, and supported histologically by less damage to the spleen. Altogether, the attenuated systemic inflammatory response and the mitigated multi-organ failure in TM^{LeD/LeD} mice most likely played a major role in their reduced mortality. Notably, TM^{LeD/LeD} mice demonstrated elevated lung tPA levels at 48 hours while PAI-1 concentrations were similar to those measured in WT mice. The TM^{LeD/LeD} mutation has not been reported to influence fibrinolysis. In accordance, TM^{LeD/LeD} mice did not show altered levels of fibrin degradation products in their lungs, indicating that overall fibrinolytic activity was not altered in these animals. Further research is warranted to obtain insight into the possible indirect effects of the TM^{LeD/LeD} mutation on tPA release and fibrinolysis during severe infection. In conclusion, we have shown that mice that lack the lectin-like domain of TM are protected against Gram-negative lung sepsis caused by *B. pseudomallei* and that this is associated with inhibition of bacterial growth, reduced release of pro-inflammatory cytokines, and ultimately by inhibition of multiple organ failure. Recent clinical studies provided preliminary evidence that administration of full-length soluble TM may be of benefit to patients with severe sepsis[^37][^38]. While the present results cannot be extrapolated easily to human sepsis, our findings provide novel insights into the mechanisms that regulate the inflammatory response to Gram-negative infection.

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REFERENCES

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Experimental infection and determination of bacterial growth
Twenty-four, 48 and 72 hours after infection, mice were sacrificed under intraperitoneal anesthesia containing ketamin (Eurovet Animal Health, Bladel, The Netherlands) and medetomidin (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands). Blood was drawn into syringes containing sodium citrate 3.2% (4:1 vol/vol). Lungs, liver and spleen were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Bilateral bronchoalveolar lavage fluid (BALF) was obtained by exposing the trachea through a midline incision followed by canulation with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland) and instilling and retrieving of two 0.5 mL aliquots of sterile phosphate buffered saline (PBS). CFU were determined from serial dilutions of organ homogenates, blood and BALF that were plated on blood agar plates and incubated at 37°C 5% CO₂ for 20 h before colonies were counted.

Assays
Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN), incubated at 4°C for 30 min and centrifuged at 1730xg at 4°C for 10 min. Supernatants were sterilized using 0.22 µm pore-size filters (Millipore, Billerica, MA) and stored at -20°C until analysis. Interleukin (IL)-6, IL-12p70, interferon (IFN)-γ, monocyte-chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations (lower limits of detection: 5, 2, 2, 20, 3 pg/mL respectively1, 2, upper limits of detection 10000 pg/mL for all cytokines; reference ranges for lung homogenates of uninfected WT mice1: IL-6 6.3 pg/mL, IL12p70 not detectable, IFN-γ not detectable, MCP-1 180 pg/mL and TNF-α 8.3 pg/mL; reference ranges for plasma of uninfected WT mice: all below detection limits). Myeloperoxidase (MPO; HyCult Biotechnology, Uden, The Netherlands), thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany), plasminogen activator inhibitor-1 (PAI-1; Molecular Innovations, Novi, MI) and tissue-type plasminogen activator (tPA; Molecular Innovations, Novi, MI) were measured with commercially available ELISA kits. For TATc the lower limit of detection was 8.8 ng/mL, reference ranges for lung homogenates and plasma of uninfected WT mice were: 16.6 ng/mL and ‘below detection limits’ respectively. C3a and C5a in lung homogenates and BALF were quantified by ELISA as described3. Protein levels in BALF were measured using a Bradford-based protein assay (Bio-Rad Laboratories, Hercules, CA). Lactate dehydrogenase (LDH), aspartate aminotranspherase (ASAT), alanine aminotranspherase (ALAT) and urea were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers’ instructions.
Western blot on HMGB1
HMGB1-expression was visualized by Sodium Dodecyl Sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE). BALF samples were mixed with SDS sample buffer containing 6% β-mercaptoethanol in a 2:1 ratio. A positive control was prepared by lysing 10⁶ RAW cells with SDS sample buffer containing 2% 2-mercaptoethanol. All samples were denatured for 5 minutes at 95°C. Fifteen microliters of each sample was separated on a 15% polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Pharmacia, Piscataway, NJ). After blocking with 5% nonfat dry milk proteins (Nutricia, Zoetermeer, The Netherlands) in 0.1% Tween-20 phosphate buffered saline (PBS-T), the membranes were incubated overnight at 4°C with a rabbit-antiHMGB1 polyclonal antibody (1 µg/mL, Abcam, Cambridge, UK) in 1% nonfat dry milk in PBS-T. Then, the membrane was incubated with HRP-labeled goat-anti-rabbit polyclonal IgG secondary antibody (Cell Signalling Technology, Danvers, MA) in 1% bovine serum albumin (BSA) and developed using Lumilight plus Western Blotting substrate (Roche, Mijdrecht, The Netherlands). Positive bands were detected using a LAS3000 Luminescent image Analyzer dark box (Fujifilm, Tokyo, Japan) and were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD).

Western Blot on fibrin degradation products
Fibrin degradation products (including fragment X and D-dimer) were visualized by SDS-PAGE as described previously⁴-⁵. In brief, after dilution with SDS sample buffer lung homogenate samples were heated for 5 minutes at 95°C, run on 6% SDS-PAGE gels under non-reducing conditions and subsequently transferred to a blotting membrane. Following blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween-80 (TBS-T), the membrane was incubated overnight in 1:1000 polyclonal goat-anti-mouse-fibrinogen (Kordia, Leiden, the Netherlands) in 1% skimmed milk (Sigma-Aldrich). Then, the membrane was incubated in 1:3000 polyclonal donkey-anti-goat IgG-HRP secondary antibody (Abcam) in 1% BSA and imaged on a LAS3000 dark box (Fujiﬁlm, Tokyo, Japan). Anti-fibrin(ogen) reactive bands of fibrin degradation products (including fragment-X and D-dimer) were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD). Densities were expressed as -fold increase compared to the mean density of the WT-group.

Cell counts and flow cytometry
Total counts of paraformaldehyde (4%)-fixed BALF cells were measured using a Coulter Counter (Beckman Coulter Inc. Brea, CA). Neutrophil counts were determined by FACS analysis (FACSCalibur, Becton Dickson, San Jose, CA) using directly labeled antibodies against Gr-1 (Gr-1 FITC; BD Pharomingen, San Diego, CA) and F4/80 (F4/80 APC; AbD Serotec, Oxford, UK). Neutrophilic granulocytes were defined according to their scatter pattern and Gr-1 positivity, macrophages according to their scatter-pattern and F4/80 positivity. All antibodies were used in concentrations recommended by the manufacturer.
Histology and immunohistology

All slides were coded and scored by a pathologist blinded for the experimental groups. Lung tissues were scored for the following parameters: interstitial inflammation, necrosis, endothelialitis, bronchitis, edema, pleuritis, presence of thrombi and percentage of lung surface with pneumonia. Spleen tissues were scored for splenitis, necrosis and presence of thrombi. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition). The total histopathological score was expressed as the sum of the scores of the individual parameters, with a maximum of 24 for lungs and 12 for spleen. For granulocyte staining, slides were deparaffinized and rehydrated using standard procedures. Endogenous peroxidase activity was quenched by a solution of 0.3% H2O2 in Methanol. Slides were then digested by a solution of pepsin 0.025% (Sigma-Aldrich, St. Louis, MO) in 0.1 M HCl. After being rinsed, the sections were incubated in Ultra V Block (Thermo Scientific, Fremont, CA) and then exposed to FITC-labeled anti-mouse Gr-1 monoclonal antibody (BD PharMingen, San Diego, CA). After washes, slides were incubated with a rabbit anti-FITC antibody (Nuclilab, Ede, the Netherlands) followed by further incubation with Brightvision horseradish peroxidase anti Rabbit IgG (Immunologic, Duiven, the Netherlands), rinsed again and developed using Bright DAB (Immunologic, Duiven, the Netherlands). The sections were counterstained with methyl green (Sigma Aldrich, St. Louis, MO), hydrated and mounted in Pertex (Histolab, Gothenburg, Sweden). The total tissue area of these Ly-6G-stained slides was scanned with a slide scanner (Olympus dotSlide, Tokyo, Japan) and the obtained scans were exported in TIFF format for digital image analysis. The digital images were analyzed with ImageJ (version 2006.02.01, National Institutes of Health, Bethesda, MD) and the immunopositive (Ly6G+) area was expressed as the percentage of the total lung surface area.

REFERENCES


