The lectin-like domain of thrombomodulin hampers host defense in pneumococcal pneumonia

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Running head: Thrombomodulin in pneumococcal pneumonia

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Abstract

Background: The lectin-like domain of thrombomodulin (TM) plays an important regulatory role in sterile inflammatory conditions but its role in severe gram-positive infectious disease is unknown. *Streptococcus (S.) pneumoniae* is the most common cause of community-acquired pneumonia. The aim of this study was to determine the role of the lectin-like domain of TM in murine pneumococcal pneumonia.

Methods: Wild-type (WT) and TM^{LeD/LeD} mice (lacking the lectin-like domain of TM) were infected intranasally with viable *S. pneumoniae* and either observed in a survival study or euthanized 6, 24 or 48 hours after infection.

Results: TM^{LeD/LeD} mice had a markedly better survival in pneumococcal pneumonia as compared to WT mice. At 48 hours post-infection with *S. pneumoniae*, TM^{LeD/LeD} mice had lower bacterial loads in blood and liver, and exhibited less pulmonary inflammation as evidenced by less lung histopathology, less neutrophil influx and lower cytokine and chemokine levels. Plasma levels of pro-inflammatory cytokines were also reduced in TM^{LeD/LeD} mice after exposure to the infection.

Conclusions: Deletion of the lectin-like domain of TM improves the host defense in pneumococcal pneumonia. The lectin-like domain of TM may have a differential role in response to gram-positive or gram-negative bacteria.

Word count abstract: 193

Keywords: coagulation, inflammation, sepsis, *Streptococcus pneumoniae*
**Introduction**

*Streptococcus (S.) pneumoniae* is the leading cause of community-acquired pneumonia [1]. Annually, approximately 570,000 cases of pneumococcal pneumonia occur in the United States, accounting for ~175,000 hospitalizations [2]. When associated with sepsis, mortality rates of pneumococcal pneumonia can exceed 20% [3]. Worldwide, *S. pneumoniae* is responsible for an estimated 10 million deaths per year [4]. Together with the increasing incidence of antibiotic resistance to this pathogen [1], there is an urgent need to expand our knowledge of host defense mechanisms that influence the outcome of pneumococcal pneumonia and sepsis.

Thrombomodulin (TM, CD141) is a transmembrane multidomain glycoprotein receptor that is expressed predominantly on vascular endothelial cells, but also on monocytes, neutrophils, osteoblasts, synovial cells and dendritic cells [5-7]. TM exhibits several distinct properties in coagulation, fibrinolysis, innate immunity and inflammation that are based largely on its distinct structural domains. The central domain of TM is comprised of six EGF-like repeats, three of which provide critical cofactor activity for thrombin-mediated generation of activated protein C (APC) and of activated thrombin activatable fibrinolysis inhibitor (TAFIa). Protein C activation by thrombin-TM is further augmented by the endothelial protein C receptor (EPCR) [5-8]. APC suppresses further generation of thrombin by cleaving and inactivating coagulation cofactors Va and VIIIa. APC also has profound anti-inflammatory properties [8]. The plasma carboxypeptidase B, TAFIa, inhibits fibrinolysis by modifying fibrinogen, rendering the fibrin resistant to plasminogen binding, and limiting plasmin generation [9]. TAFIa also inactivates the complement fragments and anaphylatoxins C3a and C5a, thereby dampening the innate immune response [10]. In addition to its pivotal role in activating PC and TAFI [8;9], TM also has direct anti-inflammatory properties [5-7] that are mediated at least in part by its N-terminal C-type lectin-like domain. This structure interferes
with neutrophil adhesion, complement activation and cytokine generation [13-15], and mice lacking the lectin-like domain of TM (TM$^{\text{LeD/LeD}}$ mice) exhibit increased sensitivity to tissue injury in models of endotoxemia, lung and myocardial ischemia-reperfusion, and inflammatory arthritis [13-15].

Although alterations in regulation of the coaguation system have been studied extensively in pneumonia and sepsis (reviewed in [8]), there is limited knowledge of the role of TM in the host response to bacterial infection. Since TM is highly expressed in the lung - particularly in the alveolar capillary endothelial cells [16;17] - we have been evaluating its participation in the response to respiratory tract infections. Mice with a point mutation in the gene encoding TM that significantly reduces its capacity to generate APC and lowers TM antigen levels, surprisingly did not exhibit alterations in the pulmonary response to endotoxin or to respiratory pathogens, including *S. pneumoniae* [18]. Using TM$^{\text{LeD/LeD}}$ mice and their counterpart wild-type (WT) mice, we sought to determine the *in vivo* role of the lectin-like domain of TM in the response to pneumococcal pneumonia.

**Materials and methods**

**Animals**

TM$^{\text{LeD/LeD}}$ mice were generated as described [13] and backcrossed eight times to a C57BL/6 genetic background. TM$^{\text{LeD/LeD}}$ mice express normal antigenic levels of TM, and activation of PC is intact and unaffected by the deletion of the lectin-like domain [13]. C57BL/6 WT mice were purchased from Charles River (Maastricht, the Netherlands). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam.
Experimental infection and sampling

Pneumonia was induced by intranasal inoculation with ~ 5 x 10^4 colony forming units (CFU) of S. pneumoniae serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) as described [18;19]. Mice were observed for one week (n = 14 per group), after which survivors were killed or were euthanized at predefined time points (n = 8 per group per time point). Citrated plasma was prepared from blood drawn from the inferior vena cava. Organ homogenates were prepared as described [18;19]. In brief, the left lung and approximately half of the spleen and liver were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospect Products, Bartlesville, UK). Bacterial outgrowth in organ homogenates was determined as described [18;19]. For further measurements in lung tissue, lung homogenates were diluted 1:2 with lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl_2, 2 mM CaCl_2, 1% (v/v) Triton X-100, pH 7.4) with protease inhibitor mix added (AEBSF (4-(2-aminoethyl)benzenesulfonylfluoride), EDTA-NA_2, Pepstatin and Leupeptin, all purchased from MP Biomedical) and incubated for 30 minutes on ice, followed by centrifugation at 680 g for 10 minutes. Supernatants were stored at -20°C until analysis.

In a separate series of experiments, the trachea was exposed and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland) after which bronchoalveolar lavage (BAL) was performed by instilling and retrieving two 0.5 ml aliquots of saline (n = 8 per group per time point). Cell counts were determined for each BAL fluid (BALF) sample in a hemocytometer (Beckman Coulter, Fullerton, CA). Differential cell counts were performed on cytospins stained with Giemsa-stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Histology and immunohistochemistry
The right lung was fixed in 10% formalin/PBS at room temperature for 24 hours and subsequently embedded in paraffin. Sections of 5 µm thickness were cut. Granulocyte staining was performed using fluorescein isothiocyanate–labeled anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA) as described [20]. Ly-6G stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzlar, Germany). Ten random pictures were taken per slide. Stained areas were analyzed with Image Pro Plus (Media Cybernetics, Bethesda, MD) and expressed as a percentage of the total surface area. Also, slides were stained with hematoxylin and eosin (H&E) and analyzed by a pathologist who was blinded to the identity of the different groups. To score lung inflammation and damage, the entire lung section was analyzed with respect to the following parameters: bronchitis, interstitial inflammation, oedema, endothelialitis, pleuritis and thrombus formation. Each parameter was graded on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe). The total histopathological score was expressed as the sum of the scores for the different parameters.

**Assays**

Myeloperoxidase (MPO; HyCult Biotechnology, Uden, the Netherlands) was measured by ELISA. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, IL-12p70, IFN-γ and IL-10 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA). Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured by ELISA (R&D systems, Minneapolis, MN). Thrombin-antithrombin complexes (TATc) were measured by ELISA (Siemens Healthcare Diagnostics, Marburg, Germany).

**Statistical analysis**

Data are expressed either as box-and-whisker diagrams depicting the smallest observation, lower
quartile, median, upper quartile and largest observation, as medians with interquartile ranges or as Kaplan Meier plots. Differences between groups were determined with Mann-Whitney U test or log rank test where appropriate. Analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). P-values of less than 0.05 were considered statistically significant.

Results

**TM^{LeD/LeD} mice exhibit a survival advantage after pneumococcal infection**

To determine whether the lectin-like domain of TM alters survival in response to lethal doses of *S. pneumoniae*, TM^{LeD/LeD} and WT mice were inoculated intranasally with the bacteria and monitored for one week (Figure 1). TM^{LeD/LeD} mice were significantly protected from the lethal infection as compared to WT mice (P < 0.05). TM^{LeD/LeD} mice started dying later than WT mice. 5 of 14 TM^{LeD/LeD} mice were alive at the end of the study, whereas only 1 of 14 WT mice survived.

**TM^{LeD/LeD} mice show diminished dissemination of the infection**

To determine whether the difference in survival between TM^{LeD/LeD} and WT mice could be attributed to a difference in antibacterial defense, we measured bacterial outgrowth 6, 24 and 48 hours post-infection in lungs, blood and distant organs (spleen, liver) (Figure 2). At 6 hours, TM^{LeD/LeD} mice had slightly lower bacterial loads in their lungs than WT mice (P < 0.05, Figure 2A). However, at later time points, there were no detectable differences in lung bacterial burdens in the different genotype mice (Figure 2A). Whereas cultures in blood and distant organs were almost invariably negative during the first 24 hours after infection (Figure 2B-D), at 48 hours the majority of mice displayed systemic infection. Notably, at this time point, TM^{LeD/LeD} mice had significantly lower bacterial counts in blood (Figure 2B) and liver (Figure 2D) as compared to WT mice (both P <
0.05) whereas pneumococcal loads in spleen (Figure 2C) were not significantly different between groups.

**Lungs of TM<sup>LeD/LeD</sup> mice are relatively resistant to pro-inflammatory effects of *S. pneumoniae***

Pneumococcal pneumonia was associated with pulmonary inflammation and damage as evidenced by influx of neutrophils into the lungs and the occurrence of bronchitis, interstitial inflammation, oedema and endothelialitis. The absence of the lectin-like domain of TM in mice results in enhanced neutrophil accumulation in the lungs subsequent to endotoxin inhalation [13]. To assess the role of the lectin-like domain of TM on neutrophil influx into the pulmonary compartment in response to pneumococcal pneumonia, we performed Ly-6G staining on lung sections and measured MPO levels in lung homogenates at 24 and 48 hours post-infection. While there were no differences at 24 hours (data not shown), Ly-6G positivity at 48 hours was significantly lower in the lungs of TM<sup>LeD/LeD</sup> mice than of WT mice, indicating reduced neutrophil accumulation (Figure 3A to C). MPO levels were also significantly lower in lung homogenates of TM<sup>LeD/LeD</sup> mice at 48 hours (Figure 3D). In line with these findings, mean histopathological scores of lung sections at the same time point were lower in TM<sup>LeD/LeD</sup> mice (Figure 4A to C). To investigate whether differences in lung tissue neutrophil influx resulted in differences in cell influx in the bronchoalveolar compartment, we determined total cell counts, neutrophil numbers and MPO in BALF at 6, 24 and 48 hours after induction of pneumococcal pneumonia. There were no differences in total cell counts and neutrophil numbers between TM<sup>LeD/LeD</sup> and WT mice at any of the time points studied (Table 1). Moreover, BALF MPO levels were not different (not shown).

We next quantified the levels of several chemokines and cytokines in lung homogenates at 6, 24 and 48 hours post-infection (Table 2). At 6 and 24 hours, no differences in lung chemokines and cytokines were detected between the groups. However, at 48 hours, lung homogenates from
TM<sup>LeD/LeD</sup> mice had significantly lower levels of the chemokines KC and MIP-2 (P < 0.05 and P < 0.01 respectively), and reduced levels of TNF-α (P < 0.001), IL-6, IL-10, IFN-γ and MCP-1 (P < 0.05, Table 2).

**Reduced plasma cytokine levels in TM<sup>LeD/LeD</sup> mice after *S. pneumoniae* infection**

To further investigate the role of the lectin-like domain of TM in response to pneumococcal pneumonia we measured inflammatory markers in plasma. The differential inflammatory response between TM<sup>LeD/LeD</sup> mice and WT mice was already evident in plasma at 24 hours post-infection, at which point levels of TNF-α, IL-6 and MCP-1 were significantly lower in TM<sup>LeD/LeD</sup> mice than in WT mice (Table 3), a difference which was sustained until 48 hours. Plasma levels of IL-10, IL-12 and IFN-γ were below the detection limit of the assays (not shown).

**TM<sup>LeD/LeD</sup> mice exhibit a dampened coagulation response to *S. pneumoniae***

We also evaluated whether the lectin-like domain of TM modified pneumococcal pneumonia-induced changes in coagulation activation by measuring TATc levels in lung homogenates and plasma. No differences in TATc levels were noted at 6 and 24 hours post-infection in plasma or in lung homogenates. However, at 48 hours, when plasma TATc levels remained similar in both groups of mice (data not shown), TATc levels were significantly lower in lung tissue from TM<sup>LeD/LeD</sup> mice as compared to WT mice (P < 0.05, Figure 5).

**Discussion**

Coagulation and inflammation are highly integrated and interactive participants in the immediate host response to infection. TM occupies a central role in the regulation of coagulation and
inflammation, with responsibility for its specific functions relying on the integrity of its distinct structural domains. Thus, the EGF-like repeats provide cofactor activity for thrombin-mediated activation of PC and TAFI, resulting in dampening of coagulation, fibrinolysis and complement, while the N-terminal lectin-like domain exhibits a number of anti-inflammatory properties [5-7].

Using mouse models to study the host response to bacterial pneumonia, we have previously investigated the role of the EGF-like repeat of TM, that is responsible for PC activation. TMPro/Pro mice, which have moderately diminished levels of TM antigen and a dramatic reduction in its capacity to support thrombin-mediated activation of PC, did not exhibit TM-dependent changes in the procoagulant or inflammatory response to bacterial pneumonia as compared to WT mice [18].

Here, we similarly studied the role of the lectin-like domain of TM in a mouse model of community-acquired pneumonia caused by *S. pneumoniae*. This domain of TM does not participate in the generation of APC. In contrast to sterile inflammation models in which loss of the lectin-like domain of TM renders mice more sensitive to lung and myocardial ischemia-reperfusion injury, inflammatory arthritis and endotoxemia [13-15], loss of the lectin-like domain of TM appears to protect the host in response to pneumococcal pneumonia, as reflected by improved survival, reduced bacterial growth and dissemination, and attenuated inflammatory and procoagulant response in TMLeD/LeD mice as compared to WT mice.

This is the first report in which viable gram-positive bacteria were used to evaluate the *in vivo* role of the lectin-like domain of TM. In our study, pneumococcal loads were transiently lower in the lungs of TMLeD/LeD mice 6 hours post-infection. This difference could not be attributed to altered inflammatory cell numbers, since BALF total cell counts and neutrophil numbers were not different between WT and TMLeD/LeD mice at this early time point after infection. Notably, uninfected TMLeD/LeD mice display a clearly enhanced neutrophil accumulation in the lung interstitium in peribronchial locations [13], which is expected to facilitate the immediate antibacterial response.
upon infection of the lower airways [21]. However, the difference became more prominent at 48 hours post-infection, when bacterial loads in the blood and liver were found to be markedly lower in TM\(^{LeD/LeD}\) than in WT mice. These data suggest that killing of \(S.\ pneumoniae\) at the lung-blood interface, i.e. at the endothelial cell layer or in the systemic compartment itself, is enhanced in the absence of the lectin-like domain of TM. It is possible that this may be due to excess complement activation at that site due to loss of the lectin-like domain of TM that otherwise downregulates complement activation. Indeed, TM\(^{LeD/LeD}\) mice have lower basal plasma C3 levels and a reduced CH50 (the amount of serum that causes 50% hemolysis of antibody-sensitized sheep erythrocytes), both of which are indicative of excess complement activation [15]. Considering the important role that complement activation plays in host defense against pneumococci \textit{in vivo} [23], it is conceivable that the protective phenotype of TM\(^{LeD/LeD}\) mice is at least in part explained by increased clearance of pneumococci from the bloodstream via augmented activation of the complement system.

In our study, TM\(^{LeD/LeD}\) mice displayed less evidence of local and systemic inflammation in response to \(S.\ pneumoniae\) infection. This was reflected by lower plasma levels of TNF-\(\alpha\), IL-6 and MCP-1, less histologic evidence of lung damage, lower levels of cytokines and chemokines in lung homogenates, fewer neutrophils in the infected lungs, reduced secondary activation of coagulation as measured by TATc, and finally by relative resistance to lethal doses of the bacteria. Although appearing to be contradictory to previous reports in which TM\(^{LeD/LeD}\) mice exhibited a heightened inflammatory response, those studies were performed with endotoxin or non-bacterial stresses, rather than with live gram-positive bacteria [13]. Indeed, the current findings suggest that TM via its lectin-like domain may mediate a differential host response to gram-negative \textit{versus} gram-positive bacteria. The lectin-like domain of TM binds to the carbohydrate Lewis Y antigen of gram-negative bacteria and induces agglutination and opsonization of gram-negative organisms, including \textit{E. coli} and \textit{Klebsiella pneumoniae}, enhancing their phagocytosis by macrophages [24]. By interacting with
the Lewis Y antigen, the lectin-like domain of TM may interfere with lipopolysaccharide binding to CD14, its cognate receptor, thereby dampening downstream pro-inflammatory signal pathways and the release of pro-inflammatory cytokines, and iNOS expression. Indeed, a recombinant soluble form of the lectin-like domain of TM reduced *Klebsiella* induced inflammatory responses and lethality [24]. Without invoking other mechanisms, this beneficial response would not necessarily be expected in our gram-positive pneumonia model, as endotoxin is not involved. The clinical relevance of this finding is important to recognize, as soluble thrombomodulin is being studied for use in the clinic [25] and identifying the appropriate application is critical to ensure optimal benefit. Also, whereas in non-bacterial stress models anti-inflammatory responses tend to ameliorate damage, in live bacteria models a substantial amount of inflammatory response is necessary to kill the bacteria; interfering with bacterial killing by reducing the inflammatory response can enhance bacterial outgrowth, in turn resulting in an adverse outcome.

Our study has limitations. As elaborated above, we studied a single pathogen in a single infection model. It is unclear if results from our study would be comparable to those in other models and/or using other bacteria. Also, it is possible that genetic knock-down of TMLeD/LeD has induced unknown compensatory changes in mice during growth and development. In this regard it would be interesting to study the effect of interfering with the function of the TMLeD/LeD domain as opposed to knocking down TMLeD/LeD, for example by using blocking antibodies.

In conclusion, we show that deletion of the lectin-like domain of TM in mice results in improved host defense against pneumococcal pneumonia, a common cause of serious community-acquired infection. Additional studies are necessary to obtain further insight into the possible differential role of the lectin-like TM domain in gram-positive versus gram-negative infections, as this will determine how to best design targeted therapies to either enhance the function or interfere with the
function of this interesting endothelial C-type lectin-like structure, thereby gaining maximal benefit in different clinical situations.

Acknowledgements

The authors thank the next persons, all working at the Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam; Marieke ten Brink and Joost Daalhuisen for their technical assistance during the animal experiments, Regina de Beer for performing (immuno)histopathological stainings and Achmed Achouiti and Florry E. van den Boogaard for assisting in cytokine measurements.
References


Table 1. Cell counts and differentials in bronchoalveolar lavage fluid in wild-type (WT) and TM^{LeD/LeD} mice 6, 24 and 48 hours post-infection with pneumococcal pneumonia.

<table>
<thead>
<tr>
<th>WT</th>
<th>TM^{LeD/LeD}</th>
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<td>T = 6</td>
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<td>n = 8</td>
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<td></td>
<td>T = 6</td>
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<td></td>
<td>n = 8</td>
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<tr>
<td>Total cell counts (10^6/ml)</td>
<td>3.69 (2.24-6.53)</td>
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<tr>
<td>Neutrophil count (10^7/ml)</td>
<td>0.71 (0.32-1.48)</td>
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Pneumonia was induced by intranasal inoculation with 5 x 10^4 S. pneumoniae CFU. Data are medians (interquartile ranges).
Table 2. Pulmonary cytokine and chemokine levels in wild-type (WT) and TM<sup>LeD/LeD</sup> mice 6, 24 and 48 hours post-infection with pneumococcal pneumonia.

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<tr>
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<td>WT n = 8</td>
<td>TM&lt;sup&gt;LeD/LeD&lt;/sup&gt; n = 8</td>
<td>WT n = 8</td>
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<tr>
<td>KC (ng/ml)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.19 (0.72-3.10)</td>
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<tr>
<td>MIP-2 (ng/ml)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3.66 (3.16-4.52)</td>
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<tr>
<td>TNF-α (pg/ml)</td>
<td>20.2 (12.7-26.0)</td>
<td>23.1 (15.5-30.1)</td>
<td>5.17 (2.50-6.31)</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>36.4 (25.4-41.5)</td>
<td>44.8 (30.5-67.2)</td>
<td>19.1 (5.40-123)</td>
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<tr>
<td>IL-10 (pg/ml)</td>
<td>6.03 (5.13-6.69)</td>
<td>6.74 (6.26-7.72)</td>
<td>2.50 (2.50-5.36)</td>
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<tr>
<td>IL-12 (pg/ml)</td>
<td>B.D.</td>
<td>B.D.</td>
<td>B.D.</td>
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<tr>
<td>IFN-γ (pg/ml)</td>
<td>1.46 (1.40-1.57)</td>
<td>1.68 (1.36-2.22)</td>
<td>2.19 (2.03-3.59)</td>
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<tr>
<td>MCP-1 (ng/ml)</td>
<td>299 (222-341)</td>
<td>315 (278-620)</td>
<td>0.19 (0.15-0.24)</td>
</tr>
</tbody>
</table>

Pneumonia was induced by intranasal inoculation with 5 x 10<sup>4</sup> S. pneumoniae CFU. Data are medians (interquartile ranges). KC = keratinocyte-derived chemokine, MIP-2 = macrophage inflammatory protein-2, TNF-α = tumor necrosis factor-α, IL = interleukin, IFN-γ = interferon-γ, MCP-1 = monocyte chemotactic protein-1. N.D. = not determined. B.D. = below detection. *, ** and *** indicate statistical significance compared to wild-type (WT) (P < 0.05, P < 0.01 and P < 0.001 respectively, Mann Whitney U test).
Table 3. Plasma cytokine and chemokine levels in wild-type (WT) and TM<sup>LeD/LeD</sup> mice 6, 24 and 48 hours post-infection with pneumococcal pneumonia.

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<td>WT n = 8</td>
<td>TM&lt;sup&gt;LeD/LeD&lt;/sup&gt; n = 8</td>
<td>WT n = 8</td>
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<tr>
<td>TNF-α  (pg/ml)</td>
<td>5.99 (3.28-8.05)</td>
<td>6.47 (5.53-7.99)</td>
<td>7.90 (6.38-9.75)</td>
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<td></td>
<td>1.30 (1.30-4.10) **</td>
<td>1.30 (1.30-4.10) **</td>
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<tr>
<td>IL-6   (pg/ml)</td>
<td>4.38 (1.00-19.5)</td>
<td>5.02 (1.00-7.02)</td>
<td>37.4 (21.3-85.2)</td>
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<td></td>
<td>6.25 (2.50-15.6) **</td>
<td>6.25 (2.50-15.6) **</td>
<td>6.25 (2.50-15.6) **</td>
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<tr>
<td>MCP-1  (pg/ml)</td>
<td>B.D.</td>
<td>B.D.</td>
<td>37.8 (30.6-80.9)</td>
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<td>10.0 (10.0-30.3) **</td>
<td>10.0 (10.0-30.3) **</td>
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|         | 15.8 (10.0-34.7) **         | 15.8 (10.0-34.7) **         |                             | ** indicates statistical significance compared to wild-type (WT) (P < 0.01, Mann Whitney U test).
Figure legends

Figure 1. Loss of the lectin-like domain of thrombomodulin reduces mortality in murine pneumococcal pneumonia. Survival of wild-type (WT) mice (black dashed line) and mice lacking the lectin-like domain of thrombomodulin (TM\textsuperscript{LeD/LeD}) (grey line) after intranasal infection with $5 \times 10^4$ S. pneumonias colony forming units (CFU) (14 mice per group). * indicates statistical significance as compared to wild-type (P < 0.05, log rank test).

![Figure 1](image.png)

Figure 2. Bacterial outgrowth in blood and liver is lower in mice lacking the lectin-like domain of thrombomodulin (TM\textsuperscript{LeD/LeD}) as compared to wild type (WT) mice after pneumococcal infection. Bacterial outgrowth in A. lung, B. blood, C. spleen and D. liver 6, 24, and 48 hours after induction of pneumonia by intranasal inoculation with $5 \times 10^4$ S. pneumonias colony forming units (CFU) in WT mice (white) and TM\textsuperscript{LeD/LeD} mice (grey). Data are expressed as
box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group). * indicates statistical significance as compared to WT (P < 0.05, Mann-Whitney U test).

Figure 2.

Figure 3. Lung neutrophil influx and myeloperoxidase (MPO) levels are lower in mice lacking the lectin-like domain of thrombomodulin (TM<sup>LeD/LeD</sup>) as compared to wild type (WT) mice after pneumococcal infection. Representative slides of lung Ly-6G staining (brown) 48 hours after induction of pneumonia by intranasal inoculation with 5 x 10<sup>4</sup> S. pneumoniae colony forming units (CFU) in A. WT mice and B. TM<sup>LeD/LeD</sup> mice (200 times original magnification). C. Quantitation of pulmonary Ly-6G 48 hours after induction of pneumococcal pneumonia in WT mice and TM<sup>LeD/LeD</sup> mice. D. Lung levels of MPO 48 hours post-infection with pneumococcal pneumonia in WT mice (white) and TM<sup>LeD/LeD</sup> mice (grey). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest
observation (8 mice per group). ** indicates statistical significance as compared to wild-type (P < 0.01, Mann-Whitney U test).

**Figure 3.**

A. WT mice and B. TMLeD/LeD mice (100 times original magnification). C. Total pathology scores 48 hours post-infection with pneumococcal pneumonia in WT mice and TMLeD/LeD mice. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group). * indicates statistical significance as compared to wild-type (P < 0.05, Mann-Whitney U test).

**Figure 4.** Total histopathology scores are lower in mice lacking the lectin-like domain of thrombomodulin (TMLeD/LeD) as compared to wild type (WT) mice after pneumococcal infection. Representative slides of lung haematoxylin and eosin stainings 48 hours after induction of pneumonia by intranasal inoculation with 5 x 10⁴ S. pneumoniae colony forming units (CFU) in A. WT mice and B. TMLeD/LeD mice (100 times original magnification). C. Total pathology scores 48 hours post-infection with pneumococcal pneumonia in WT mice and TMLeD/LeD mice. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group). * indicates statistical significance as compared to wild-type (P < 0.05, Mann-Whitney U test).
Figure 5. Activation of coagulation is reduced in the lung tissue of mice lacking the lectin-like domain of thrombomodulin (TM^LeD/LeD) as compared to wild type (WT) mice after pneumococcal pneumonia. Lung homogenate levels of thrombin-antithrombin complexes (TATc) 6, 24 and 48 hours post-infection with 5 x 10^4 S. pneumoniae colony forming units (CFU) in WT mice (white) and TM^LeD/LeD mice (grey). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group at each time point). * indicates statistical significance as compared to wild-type (P < 0.05, Mann-Whitney U test).
Figure 5.