Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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The trombomodulin lectin-like domain does not change host responses to tuberculosis

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ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium (M.) tuberculosis*, is a devastating infectious disease causing many deaths worldwide. Thrombomodulin (TM) is a multidomain glycoprotein expressed on all vascular endothelial cells. We here studied the role of the lectin-like domain of TM, responsible for a variety of anti-inflammatory properties of TM, during TB. We compared the extent of TM-expression in human lung tissue of TB and control patients. The role of the lectin-like domain of TM was investigated by comparing mice lacking this domain (TM<sup>Ld/Ld</sup> mice) with wild type (WT) mice during experimental lung TB induced by infection with *M. tuberculosis* via the airways. Lungs were harvested for analyses at 2, 6 and 29 weeks after infection. Lung TM-expression was downregulated in TB patients, which was not related to changes in the amount of endothelium in infected lungs. TM<sup>Ld/Ld</sup> mice showed unaltered mycobacterial loads in lungs, liver and spleen during experimental TB. Additionally, lung histopathology and cytokine concentrations were largely similar in TM<sup>Ld/Ld</sup> and WT mice, while total leukocyte counts were increased in lungs of TM<sup>Ld/Ld</sup> mice after 29 weeks of infection. Mortality did not occur in either group. The lectin-like domain of TM does not play an important role in the host response to *M. tuberculosis* infection in mice.
INTRODUCTION

Tuberculosis (TB) is a highly prevalent chronic infectious disease worldwide, with one-third of the population being infected with the causative pathogen Mycobacterium (M.) tuberculosis. In 2012, 8.7 million new cases of TB and 1.4 million deaths were registered. Most infected individuals do not develop clinical disease, although in these people, viable tubercle bacilli remain present inside granulomas, which are tissue nodules comprising infected macrophages surrounded by lymphocytes and a fibrous capsule. Clinically manifest infection can emerge upon a temporary decrease in resistance, and as such, failure to eradicate M. tuberculosis bacilli following primary exposure renders the host at risk of developing fulminant disease. Additionally, multi-drug resistant (MDR) TB has become a serious threat to effective treatment. Hence, there is a clear need to increase our understanding of host defense mechanisms to TB in hopes of developing more effective preventative and therapeutic strategies.

Thrombomodulin (TM, CD141) is a multidomain transmembrane glycoprotein primarily expressed on the surface of vascular endothelial cells, but also found on monocytes, neutrophils, osteoblasts, synovial cells and dendritic cells. Due to its strategic location and its interactions with thrombin, protein C (PC), thrombin activatable fibrinolysis inhibitor (TAFla), complement factors, Lewis Y antigen and high-mobility group box-1, TM has multiple properties in modulating inflammation, coagulation, fibrinolysis and cell proliferation. TM comprises five structural domains: the N-terminal C-type lectin-like domain, six epidermal growth factor (EGF)-like repeats, a serine/threonine-rich region, a single transmembrane domain and a cytoplasmic tail. The EGF-domain is the best characterized, in that it is a cofactor for thrombin mediated generation of activated protein C (APC) which in turn cleaves and inactivated coagulation factors Va and VIIIa. The EGF-domain also supports thrombin-mediated generation of the carboxypeptidase B, TAFila. We have previously shown that the EGF-domain of TM plays an important role in murine TB, i.e., mice with a mutation in the TM-gene resulting in reduced TM expression and minimal capacity to generate APC (TMpro/pro mice), respond to M. tuberculosis infection with increased lung inflammation, a diminished capacity to form well-shaped granulomas, elevated levels of pro-inflammatory cytokines and reduced survival. The lectin-like domain of TM lacks anti-coagulant and anti-fibrinolytic properties, but rather, regulates inflammation, cell adhesion and cell proliferation. Several murine models of human disease using TMLeD/LeD mice have revealed that the lectin-like domain of TM has anti-inflammatory properties.

Little is known about the role of the lectin-like domain of TM during chronic infections such as TB. Since TMpro/pro mice have reduced capacity to generate APC as well as reduced expression of TM, we could not definitively exclude a role for the lectin-like domain in explaining the pro-inflammatory phenotype. In view of the documented role of the lectin-like domain in regulating inflammation, we aimed to assess whether it participates in the host response to M. tuberculosis lung infection.
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MATERIALS AND METHODS

Patients
Lung tissues of pulmonary TB patients ($n=8$; mean age 62 years ($y$), range 32-85$y$; 38% male) were obtained after partial lobectomy ($n=2$) or from post-mortem samples ($n=6$) of patients who died from (disseminated) TB or TB-related causes. TB infection was confirmed in these tissues by Ziehl-Neelsen (ZN) positive staining for acid-fast bacilli. Control tissues ($n=10$; mean age 57$y$, range 18-77$y$; 50% male) were obtained from patients after (partial) lobectomy because of (suspicion of) lung cancer or pulmonary metastases. Control tissues were free of signs of pulmonary infection or cancer, as confirmed by reviewing all cases by a qualified histopathologist.

All patients were diagnosed and treated in the Academic Medical Center, University of Amsterdam, the Netherlands. According to Dutch law, tissue samples harvested for diagnostic purposes can be freely used after anonymizing the tissues, provided these are handled according to national ethical guidelines (“Code for Proper Secondary Use of Human Tissue”, Dutch Federation of Medical Scientific Societies).

Histology and immunohistochemistry on human lung tissues
Four μm-lung tissue sections were cut from paraffin-embedded tissues, dewaxed in xylene and rehydrated in graded alcohols. Tissues were stained for TM and a double staining for TM and CD31 (an epitope abundantly present on endothelium) was performed. First, on all tissue sections heat-induced epitope retrieval was performed with 10 mM TrisEDTA pH 9.0 in the PreTreatment Module (PTModule, Thermo Fisher Scientific/Labvision, Fremont, CA) for 20 min at 98˚C and a cool-down to 75˚C, followed by application of Ultra-V Block (Thermo Fisher Scientific/Labvision) for 10 min at room temperature. Then, TM-staining with anti-TM antibodies (monoclonal mouse IgG1, clone 1009; Dako, Glostrup, Denmark) was performed. To reveal co-localization of TM and CD31 (monoclonal mouse IgG1, clone JC70A, Dako), a sequential double alkaline phosphatase (AP) staining was performed starting with the TM antibody, detected with anti-mouse IgG AP-conjugated BrightVision polymer (ImmunoLogic, Duiven, The Netherlands) and AP-activity developed with Vector Blue (Vector Labs, Burlingame, CA). After a short 10 min heat step in the PTModule using TrisEDTA pH 9.0 HIER buffer to remove the first set of immunoreagents, but leaving the blue AP reaction product unchanged, the CD31-antibody was detected by a second AP staining using Vector Red (Vector Labs). Sections were mounted with VectaMount (Vector Labs), without a nuclear counterstain. The total tissue area of the TM-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, NIH, Bethesda, MD) and the immunopositive (TM+) area was expressed as the percentage of the total lung surface area. Analysis of double stained slides was performed with spectral imaging using the Nuance VIS-FL Multispectral Imaging System (Caliper Life Sciences, Hopkinton, MA, USA).

Data sets were acquired from 420-720 nm at 20 nm intervals. Spectral library of single-Vector Red and single-Vector Blue, brown pigment in erythrocytes and black pigment in lung alveolar space was applied to unmix the double staining into the individual components. Using the Nuance
software version 3.0, an exclusive image of TM/CD31 co-localization was created \(^7\) and the percentage CD31/TM co-localization was calculated. This pixel-based quantization represents the CD31/TM co-localization as a percentage of all CD31-positive staining.

**Mice**
Pathogen-free 8-10-week old female wild type (WT) C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands). TM\(^{LeD/LeD}\) mice were generated as described \(^18\) and backcrossed for at least eight generations onto a C57BL/6 genetic background. TM\(^{LeD/LeD}\) mice express normal antigenic levels of TM and unaltered thrombin-mediated activation of PC \(^18\). Activation of TAFI is also expected to be unaffected as this is mediated by the EGF-like repeats of TM. 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**
A virulent laboratory strain of *M. tuberculosis* (Erdman strain) was grown for 4 days in liquid Dubois medium containing 0.01% Tween-80. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed with sterile 0.9%NaCl.Experimental TB was induced by intranasal inoculation with 150 CFU of *M. tuberculosis* in 50 µL 0.9% NaCl, as previously described \(^17, 22-24\). Eight mice per time-point were sacrificed 2 and 6 weeks after infection under intraperitoneal anesthesia containing ketamin (Eurovet Animal Health, Bladel, The Netherlands) and medetomidin (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands). In addition, 16 WT and 16 TM\(^{LeD/LeD}\) mice were observed for 29 weeks after infection. Surviving mice were euthanized for that time point for immunohis-tochemical studies of harvested tissues. Organs were processed as previously described \(^17, 22-24\). Briefly, lungs, liver and spleen were removed aseptically and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU were determined from serial dilutions of organ homogenates that were plated in tenfold dilutions on Middlebrook 7H11 plates and incubated at 37°C 5%CO\(_2\) for 21 days before colonies were counted.

**Histology of mice lung tissues**
Four µm-sections were cut from paraffin-embedded tissues of mice lungs removed 2, 6 or 29 weeks after inoculation with *M. tuberculosis*. Parameters of lung histopathology were scored as previously described \(^22, 24, 27, 28\). Briefly, after deparaffinization, tissues were stained for H&E and scored from 0 (absent) to 4 (most severe) for the following parameters: interstitial inflammation, endothelialitis, peri-bronchitis, edema, granuloma formation and pleuritis by a pathologist blinded for the experimental groups. The total ‘lung inflammation score’ is expressed as the sum of the scores for each parameter, the maximum being 24. In addition, the percentage inflammation of the lung, representing the percentage of the total lung surface occupied by confluent (diffuse) inflammatory infiltrates, was quantified.
Flow cytometry
Lung cell suspensions were obtained by crushing lungs through a 40-μm pore-size cell strainer (BD, San Jose, CA) as described previously17, 22-24. Erythrocytes were lysed using ACK lysing buffer (BioWhittaker, Verviers, Belgium); the remaining cells were washed twice with FACS-buffer (phosphate buffered saline (PBS) supplemented with 0.5% BSA, 0.01% NaN₃ and 0.35 mM EDTA) and counted using a haemocytometer. Cells were brought to a concentration of 1 x 10⁷ cells/mL in FACS-buffer. Immunostaining for cell-surface molecules was performed for 30 minutes at 4°C using directly labeled antibodies against CD3 (CD3-phycoerythrin), CD4 (CD4-allophycocyanin), CD8 (CD8-peridinin chlorophyl protein) or Gr-1 (Gr-1-fluorescein isothiocyanate (FITC)). All antibodies were used in concentrations recommended by the manufacturer (BD Pharmingen, San Diego, CA). After staining, cells were fixed in 4% paraformaldehyde and the percentages of cells were determined by flow cytometric analysis using a fluorescence-activated cell sorter (FACS Calibur; BD Immunocytometry Systems, San Jose, CA). The percentages of polymorphonuclear cells (PMNs), macrophages and lymphocytes were determined using Gr-1 expression (Gr-1 high, intermediate and low, respectively) and T-cell surface proteins were analyzed on CD3+ cells within the lymphocyte gate.

Splenocyte stimulation
Single cell suspensions were obtained by crushing spleens through a 40 μm pore-size cell strainer (BD, San Jose, CA) as described22, 24. Erythrocytes were lysed with ACK lysis buffer (BioWhittaker, Walkersville, MD). The remaining cells were washed twice with RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic (GiboBRL, Rockville, MD). Cells were seeded in 96-well round bottom culture plates at a cell density of 1 x 10⁶ cells/well in quadruplicate and stimulated with 20 μg/mL tuberculin purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark). Supernatants were harvested after 48h incubation at 37°C in 5% CO₂ and cytokine levels were analyzed by ELISA.

Assays
For cytokine measurements, organ 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) and incubated on ice for 30 min. Homogenates were centrifuged at 1500 g at 4°C for 15 min and supernatants were sterilized using a 0.22-μm pore-size filter (Corning Inc., Corning, NY) and stored at -20°C until analysis. Levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, IL-10, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured by ELISA according to the manufacturer’s instructions (all from R&D Systems, Minneapolis, MO).
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Statistical analysis
Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Comparisons between groups were performed using a Mann-Whitney *U* test. Analyses were completed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). *P*-values < 0.05 were considered statistically significant.

RESULTS

TM expression in human pulmonary TB
The lungs are the primary site of infection during human TB. TB-infected lungs display typical ‘granulomas’ as part of the host immune response, tissue nodules consisting of a kernel of infected macrophages, surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components. In order to obtain insight into the extent of pulmonary TM expression during human pulmonary TB, we stained lung tissues of proven TB-infected and control patients with an anti-TM antibody. Overall, expression of TM in lung tissues of TB-infected patients was lower when compared to controls (*P* < 0.05; Figure 1A-C). TM is mainly present on vascular endothelium. In granuloma of TB-infected lungs the number of penetrating blood vessels has been shown to be markedly diminished. In order to find out whether the decrease in TM-expression was only due to a decreased amount of blood vessels in TB-infected tissues, we determined TB/CD31 co-expression in control and TB-infected tissues by double immunohistochemistry. CD31 is abundantly present on endothelium and is therefore used as an immunohistochemical marker. Clearly, TB-infected lungs displayed significantly less TM expression on CD31 positive cells when compared to control lung tissues (*P* < 0.05; Figure 1D), indicating that in TB-infected lung tissues TM-expression is not decreased due to a lower number of blood vessels. Representative photographs of TM/CD31 co-expression are displayed in Figure 1E-H.

Lack of the lectin-like domain of TM does not affect mycobacterial growth or dissemination
To study the possible role of the lectin-like domain of TM on mycobacterial growth, we infected WT and TM<sub>LeD/LeD</sub> mice with *M. tuberculosis* and determined mycobacterial loads in the lungs at 2, 6 and 29 weeks. During the 29-week observation period, none of the mice died and body weights were not different between WT and TM<sub>LeD/LeD</sub> mice (data not shown). The numbers of CFU detected in the lungs were similar in WT and TM<sub>LeD/LeD</sub> mice at all time points (Figure 2A). Although dissemination of the mycobacteria occurred over time, no genotype-dependent differences in mycobacterial loads were detected in the liver or spleen over the 29 week observation period (Figure 2B-C).
Figure 1. TM-expression on endothelium in human lung tissues. TM-staining of lung tissues of TB-positive patients (n = 8) compared with control lung tissues of uninfected patients (n = 10), as described in the Methods section. TM-stained areas are presented as the percentage of the total lung surface area (A). Representative photographs of uninfected controls (B) and TB-positive (C) patients (TM-staining, original magnification x200). Co-expression of TM and CD31, a universal marker for endothelium, was measured in lung tissues of uninfected controls and TB-positive patients. Positive TM-staining was expressed as percentage of CD31-positivity (D). After capturing spectral data set and spectral unmixing by Nuance 3.0, the following images were obtained: TM (blue) and CD31 (red)-staining on a RGB image of bright field microscopy of control (E) and TB-infected lung tissues (F); CD31/TM co-localization (yellow) and CD31 (grey) in control (G) and TB-infected lung tissues (H) (original magnification x200). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney U test). *P < 0.05 versus control.

Lack of the lectin-like domain of TM has limited impact on lung histopathology

We also examined the role of the lectin-like domain of TM in lung inflammation in response to murine TB. For this lung tissue sections obtained after 2, 6 or 29 weeks were scored according to the scoring system described in the Methods section (Figure 3). At 2 weeks of infection lung pathology scores were relatively low in both groups; at this early time point TM^{Ldr}/Ldr mice displayed modestly but statistically significant enhanced lung inflammation (Figure 3A). The extent of lung inflammation progressively increased at 6 and 29 weeks, revealing no differences between mouse strains. Of note, at 29 weeks, lung inflammation was so extensive in both groups that pathol-
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Figure 2. Lack of the lectin-like domain of TM does not affect mycobacterial growth and dissemination in lungs, liver and spleen. Mycobacterial loads in lungs (A), liver (B) and spleen (C) from wild type (WT, grey boxes) and TMLeD/LeD mice (white boxes) 2, 6 and 29 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. n = 8 (2 and 6 weeks) or 16 (29 weeks) mice per group. Data expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney *U* test). Differences between groups were not significant at any time point.

Figure 3D-I show representative photographs of lung histology of WT (3D-F) and TMLeD/LeD mice (3G-I) at 2, 6 and 29 weeks. Overall these data indicate that the lectin-like domain of TM has a limited if any role in the regulation of lung inflammation during experimental lung TB.

**Cellular composition of lung infiltrates in WT and TMLeD/LeD mice**

We characterized the cellular composition of the pulmonary infiltrates in WT and TMLeD/LeD mice following infection with *M. tuberculosis*. Whole lung cell suspensions were prepared at 2, 6 and 29 weeks and the cellular composition was analyzed by FACS (Table 1). At 2 and 6 weeks, total leukocyte counts were similar in both WT and TMLeD/LeD mice, although at 2 weeks, TMLeD/LeD mice had fewer macrophages in their lungs (*P* < 0.05). At 29 weeks, there were significantly more leukocytes in the lungs of TMLeD/LeD mice (*P* < 0.05). Determination of differential leukocyte counts showed that the increase in total leukocyte counts was caused by increased numbers of all leukocyte subsets (Table 1). As CD4+ and CD8+ lymphocytes are important in cellular immunity during TB, we analyzed whole-lung CD3+ lymphocytes with respect to expression of CD4 and CD8. Our data show that the percentages of both CD3+/CD4+ and CD3+/CD8+ lymphocytes were responsible for the increase in the total number of lymphocytes measured at 29 weeks (Table 1).

**Cytokine and chemokine response during experimental TB**

Cytokines and chemokines play pivotal roles in the immune response to TB. We measured the concentrations of TH1-cytokines (IFN-γ, TNF-α), TH2-cytokines (IL-4, IL-6, IL-10) and chemokines (MIP-2, KC) in lysated lung homogenates obtained 2, 6 and 29 weeks after infection (Table 2). The concentrations of all mediators were similar in lungs of WT and TMLeD/LeD mice at
Figure 3. Lack of the lectin-like domain of TM has limited impact on lung histopathology.
Mean histological scores (A), percentages of inflammation (B) and lung weights (C) for WT (grey boxes) and TM<sup>LeD/LeD</sup> mice (white boxes) 2, 6 and 29 weeks after inoculation with 150 CFU of <i>M. tuberculosis</i>. <i>n</i> = 8 (2 and 6 weeks) or 16 (29 weeks) mice per group. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney <i>U</i> test). Representative histological sections of lungs of WT (D-F) and TM<sup>LeD/LeD</sup> mice (G-I) infected with <i>M. tuberculosis</i> 2 (D, G), 6 (E, H) and 29 (F, I) weeks earlier (H&E, original magnification 100x). B.D. below detection limits. N.D. not determinable. *<i>P</i> < 0.05 for the difference between groups at the indicated time point. All other differences between groups were not statistically significant.

2 weeks after infection. Very modest differences between groups were detected at later time points, i.e. the lungs of TM<sup>LeD/LeD</sup> mice had higher IL-10 at 6 weeks and higher IL-6 at 29 weeks (both <i>P</i> < 0.05).

Similar IFN-γ production upon ex vivo stimulation of TM<sup>LeD/LeD</sup> splenocytes
We next determined the capacity of splenocytes obtained from mice infected with <i>M. tuberculosis</i> to respond to the recall antigen PPD. Splenocytes were harvested at 2, 6 and 29 weeks after infection and then stimulated with PPD for 48 hours after which IFN-γ (indicative of a T<sub>H1</sub>-1-response) and IL-4 (indicative of a T<sub>H2</sub>-2-response) were measured in the supernatant (Figure 4). IL-4 levels were undetectable in supernatants from both groups at all time-points (data not shown). Antigen-specific IFN-γ production by splenocytes harvested from the mice was below detection limits after 2 weeks. At 6 weeks IFN-γ was readily detectable but not different between genotypes. After 29 weeks of infection TM<sup>LeD/LeD</sup> splenocytes tended to release more IFN-γ upon stimulation with PPD (<i>P</i> = 0.08).
Table 1. Effect of lack of the lectin-like domain of TM on total leukocyte counts and differentials in the lungs.

<table>
<thead>
<tr>
<th></th>
<th>Total leukocyte counts x 10^7/mL</th>
<th>Mφs x 10^5/mL</th>
<th>PMNs x 10^5/mL</th>
<th>Lymphocytes x 10^5/mL</th>
<th>CD4^+ x 10^5/mL</th>
<th>CD8^+ x 10^5/mL</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>32 (22-36)</td>
<td>12.9</td>
<td>0.6</td>
<td>18</td>
<td>12</td>
<td>6.4</td>
</tr>
<tr>
<td>M^LeDLeD</td>
<td>24 (20-40)</td>
<td>8.8</td>
<td>0.8</td>
<td>14</td>
<td>9.3</td>
<td>5.1</td>
</tr>
<tr>
<td>6w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>61 (46-67)</td>
<td>11.2</td>
<td>1.6</td>
<td>48</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>M^LeDLeD</td>
<td>42 (36-56)</td>
<td>7.3</td>
<td>1.0</td>
<td>34</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>29w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>32 (22-44)</td>
<td>19.8</td>
<td>1.5</td>
<td>10</td>
<td>6.8</td>
<td>3.1</td>
</tr>
<tr>
<td>M^LeDLeD</td>
<td>50 (28-92)^*</td>
<td>34.7</td>
<td>1.9</td>
<td>13</td>
<td>9.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Total leukocyte counts and differential cell counts in lungs of WT and M^LeDLeD mice 2, 6 and 29 weeks after intranasal infection with 150 CFU of M. tuberculosis (Erdman strain). Absolute numbers of polymorphonuclear cells (PMNs) macrophages (Mφs) and lymphocytes were determined on Gr-1 expression (Gr-1 high, intermediate and low respectively) and T-cell subsets (CD4^+ and CD8^+) are presented as number of positive cells in the CD3+ gate. Data are expressed as the median ± interquartile range for 8 mice per group. ^P < 0.05 versus WT, **P < 0.01 versus WT (Mann-Whitney U test).

Table 2. Effect of lack of the lectin-like domain of TM on pulmonary cytokine and chemokine levels.

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ pg/mL</th>
<th>TNF-α pg/mL</th>
<th>IL-4 pg/mL</th>
<th>IL-6 pg/mL</th>
<th>IL-10 pg/mL</th>
<th>KC pg/mL</th>
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<tbody>
<tr>
<td>2w</td>
<td>104 (80-144)</td>
<td>221 (183-254)</td>
<td>53 (22-82)</td>
<td>207 (177-261)</td>
<td>455 (391-587)</td>
<td>148 (141-163)</td>
</tr>
<tr>
<td>WT</td>
<td>122 (92-137)</td>
<td>238 (177-278)</td>
<td>75 (44-80)</td>
<td>229 (201-278)</td>
<td>586 (406-619)</td>
<td>150 (135-211)</td>
</tr>
<tr>
<td>M^LeDLeD</td>
<td>58 (50-109)</td>
<td>734 (681-812)</td>
<td>39 (33-41)</td>
<td>32 (28-39)</td>
<td>214 (179-240)</td>
<td>1023 (888-1065)</td>
</tr>
<tr>
<td>6w</td>
<td>37 (24-63)</td>
<td>713 (685-761)</td>
<td>44 (36-55)</td>
<td>36 (33-39)</td>
<td>254 (220-319)^*</td>
<td>908 (762-1122)</td>
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<tr>
<td>WT</td>
<td>173 (108-271)</td>
<td>649 (550-843)</td>
<td>142 (97-176)</td>
<td>30 (14-37)</td>
<td>B.D.</td>
<td>472 (281-621)</td>
</tr>
<tr>
<td>M^LeDLeD</td>
<td>119 (108-182)</td>
<td>697 (641-759)</td>
<td>99 (95-120)</td>
<td>44 (39-52)^*</td>
<td>B.D.</td>
<td>B.D.</td>
</tr>
</tbody>
</table>

Cytokine and chemokine concentrations in lysated lung homogenates of WT and M^LeDLeD mice 2, 6 and 29 weeks after intranasal infection with 150 CFU of M. tuberculosis. Data are expressed as the median ± interquartile range for 8 mice per group. ^P < 0.05 versus WT (Mann-Whitney U test). B.D. below detection limits.
Chapter 4

Figure 4. IFN-γ response upon PPD re-stimulation of splenocytes of WT and TMLeD/LeD mice 2, 6 and 29 weeks after infection. Splenocytes were isolated from WT (grey boxes) and TMLeD/LeD mice (white boxes), 2, 6 and 29 weeks after infection with 150 CFU of *M. tuberculosis*, counted and stimulated with PPD for 48 hours. Stimulated splenocytes from TMLeD/LeD mice released equal amounts of IFN-γ compared to infected WT mice. \( n = 8 \) (2 and 6 weeks) or 16 (29 weeks) mice per group. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney U test). B.D. below detection limits. Differences between groups were not significant at any time point (\( P \) value at 29 weeks: 0.08).

DISCUSSION

TM is a primarily vascular endothelial glycoprotein receptor that is involved in multiple physiologically important biological systems, regulating the innate immune response to injury\(^7\),\(^8\). We previously demonstrated that mice with reduced total TM and minimal capacity to generate APC, respond to mycobacterial infection with more lung inflammation\(^17\). These findings supported an important role for the EGF-domain of TM but did not exclude participation of the lectin-like domain, a structure that in most mouse models, exhibits anti-inflammatory properties. In this study we characterized the role of the lectin-like domain of TM in the host response to lung infection with *M. tuberculosis*. Using a genetic approach in mice and several quantitative outcome measures, we determined that the lectin-like domain of TM does not have a significant impact on the ensuing inflammatory response or the resultant lung pathology. Our laboratory previously reported that TM expression becomes strongly downregulated in lung tissue of mice with experimentally induced lung TB\(^17\). We here report for the first time that TM expression is also diminished in patients with pulmonary TB. Using double stainings with a specific endothelial cell marker (CD31) we demonstrate that TM expression was reduced on the vascular endothelium. These data are in line with earlier findings of reduced TM expression in lungs of mice with acute lung inflammation elicited by endotoxin or bacteria\(^31\). Likewise, TM expression was found to be reduced in the dermal microvasculature of patients with severe bacterial sepsis\(^32\).

Several studies have confirmed that the lectin-like domain of TM plays a key role in regulating the response to a wide range of inflammatory stimuli. TMLeD/LeD mice exhibit a hyper-inflammatory response in models of endotoxemia\(^18\), arthritis\(^13\), ischemia-reperfusion of the heart and lung\(^19\), in shiga toxin-associated HUS\(^21\) and diabetic glomerulopathy\(^30\). In only one study, in which mice were
infected with *Streptococcus pneumoniae* to induce pneumonia, was the lectin-like domain of TM shown to have pro-inflammatory properties. The role of the lectin-like domain of TM has not been directly assessed in a model of chronic infection and thus ours, using *M. tuberculosis*, is unique.

We studied the chronic phase of infection at 29 weeks. Previous studies from our group that used the same infectious dose and *M. tuberculosis* strain showed limited mortality in C57Bl/6 WT mice after 25-35 weeks of infection and strong lung inflammation in surviving mice. Hence, while this time point should be sufficient to demonstrate strongly enhanced susceptibility of a particular mouse strain, it also enables to study the chronic inflammatory response. Neither this late time point nor the earlier time points (2 and 6 weeks) revealed differences in mycobacterial loads or inflammatory responses of importance. Together these data suggest that the lectin-like domain of TM does not significantly contribute to the host response during lung infection with *M. tuberculosis*.

The immune response to infection with *M. tuberculosis* features an influx of macrophages into the lung. At least in some *in vitro* and *in vivo* models, lack of the lectin-like domain of TM has been associated with an increase in leukocyte migration into the lungs, and these were identified as neutrophils and macrophages. Thus, it was somewhat surprising that at 2 weeks following infection with *M. tuberculosis*, the TMLeD/LeD mice did not exhibit a different response as compared to WT mice. Nonetheless, at 29 weeks, total leukocyte counts in the lungs of TMLeD/LeD mice were increased as compared to the WT mice, which was caused by increases in the numbers of macrophages, neutrophils and lymphocytes. Considering the unaltered mycobacterial loads and similar overall lung pathology, this finding suggests an inhibitory effect of the TM lectin-like domain on leukocyte recruitment during chronic TB, albeit without consequences for host defense. It is however possible that the impact of the differential cellular response due to lack of the lectin-like domain of TM might not be manifest within the 29 week observation period, but rather, is even more delayed. Of note, lung weights were not different between mouse strains at any time point in spite of increased leukocyte numbers at 29 weeks of infection in TMLeD/LeD mice, which may be explained by other factors impacting on lung weight, such as the extent of edema.

Cytokine release is a major component of the host response to *M. tuberculosis*. Mycobacteria reside in macrophages and a strong Th1-response, in which IFN-γ, IL-12 and CD4+ T-cells are involved, is crucial for control of TB, wherein macrophage activation by IFN-γ is supported by TNF-α. Our data revealed similar levels of Th1-type cytokines (IFN-γ and TNF-α) in WT and TMLeD/LeD mice throughout the study. Except for a slight increase in IL-10 at 6 weeks and IL-6 at 29 weeks, levels of Th2-cytokines and chemokines were also not notably different. In line with these findings, TMLeD/LeD splenocytes harvested from infected mice displayed an unaltered capacity to produce IFN-γ upon antigen specific stimulation. In line with these findings, TMLeD/LeD splenocytes harvested from infected mice displayed an unaltered capacity to produce IFN-γ upon antigen specific stimulation, although at 29 weeks of infection TMLeD/LeD splenocytes tended to produce more IFN-γ upon restimulation with PPD. Overall, these data indicate that the lectin-like domain
of TM has limited effect on cytokine release during experimental TB. In conclusion, this is the first study in which the lectin-like domain of TM has been examined in the setting of chronic infection. This structure, which otherwise has potential therapeutic value in suppressing inflammation in several acute models, has little or no effect in modulating the host response to experimental TB.

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REFERENCES

Chapter 4


