Interaction between inflammation, coagulation and fibrinolysis during infection

Weijer, S.

Citation for published version (APA):
Chapter 10

Thrombomodulin influences the pulmonary immune response in murine tuberculosis

Sebastiaan Weijer ¹, Sandrine Florquin², Cathrien Wieland¹, Tom van der Poll¹

From the Laboratory of Experimental Internal Medicine¹, Department of Pathology², Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
Abstract

Thrombomodulin (TM) plays an essential role in the generation of activated protein C (APC), a mediator with both anticoagulant and anti-inflammatory properties. TM is preferentially expressed in lungs. To investigate the role of TM in the coagulant and inflammatory response in the lung during tuberculosis, mice with a mutation in the TM gene that results in a minimal capacity for APC generation in both the alveolar space and in the circulation (TM*pro/pro mice) were intranasally infected with live virulent *Mycobacterium tuberculosis*. Pulmonary tuberculosis was not associated with activation of coagulation in either wild type or TM*pro/pro* mice, as reflected by unaltered levels of thrombin-antithrombin complexes in lung homogenates and plasma and the complete absence of fibrin deposits in lung tissue during the 5-week observation period. Lung inflammation and mycobacterial loads did not differ either between both mouse strains at 2 weeks postinfection. In sharp contrast, after 5 weeks TM*pro/pro* mice displayed an uncontrolled inflammatory response in their lungs, as reflected by higher lung weights, a diminished ability to form well-shaped granulomas, an enhanced recruitment of neutrophils accompanied by a reduced influx of lymphocytes and macrophages, elevated levels of proinflammatory cytokines and concurrently reduced concentrations of anti-inflammatory cytokines. Mycobacterial loads were lower in TM*pro/pro* mice than in wild type mice at 5 weeks after infection. These data suggest that a TM mutation that impairs APC generation results in uncontrolled lung inflammation during tuberculosis.

Introduction

Thrombomodulin (TM) is a widely expressed glycoprotein receptor that plays a pivotal role in the generation of the natural anticoagulant activated protein C (APC) by virtue of its capacity to bind thrombin with high affinity. Formation of the thrombin–TM complex limits the procoagulant and cellular-activating functions of thrombin and results in thrombin-mediated catalytic transformation of protein C (PC) into APC, an effect that is facilitated by the endothelial protein C receptor (EPCR). APC acts as an anticoagulant, together with its co-factor protein S (PS), by inactivating factors Va and VIIIa, and in addition exerts profibrinolytic effects through inhibition of plasminogen activator inhibitor type I (PAI-1), the main inhibitor of plasminogen activation. Moreover, several anti-inflammatory effects have been ascribed to APC, including inhibition of leukocyte activation, inhibition of E-selectin mediated cell adhesion to the vascular endothelium and reduction of tumor necrosis factor (TNF)-α production.

Evidence exists that exogenously administered APC can inhibit inflammatory responses in the pulmonary compartment. Intravenous infusion of APC protected against lung injury by inhibiting leukocyte activation in rats challenged with lipopolysaccharide (LPS) systemically and direct intrapulmonary delivery of APC reduced bleomycin-induced lung inflammation in mice. Considering that TM is abundantly expressed in the lungs, our laboratory recently investigated the...
influence of endogenous TM on acute lung inflammation induced by either live bacteria or LPS. Mice with a single amino acid substitution (Glu 404 → Pro) in the gene for TM (TM\textsuperscript{pro/pro} mice), which results in a profoundly reduced capacity to generate APC in both the circulation and in the alveolar space\textsuperscript{13,14}, were found to have an unaltered coagulant and inflammatory response in their pulmonary compartment after intranasal administration of \textit{Streptococcus pneumoniae} (a Gram-positive pathogen that is the most common cause of community-acquired pneumonia), \textit{Klebsiella pneumoniae} (a common Gram-negative respiratory pathogen) or LPS.\textsuperscript{13} Although this study indicated that the role of TM and endogenous APC in the regulation of acute lung inflammation is limited, we wondered whether the impaired capacity of TM\textsuperscript{pro/pro} mice to generate APC would influence lung inflammation in a more chronic setting. One of the most dramatic manifestations of chronic lung inflammation is tuberculosis. Indeed, experimentally induced pulmonary tuberculosis in mice results in a gradually developing local inflammatory reaction characterized by the recruitment of mainly mononuclear cells and the formation of well-shaped granulomas at the site of the infection\textsuperscript{15-17}. In the present study we compared the coagulant and inflammatory responses in the lungs of TM\textsuperscript{pro/pro} and normal wild type (WT) mice intranasally infected with live virulent \textit{Mycobacterium tuberculosis}.

Material and methods

\textit{Animals}

Mice with a single amino acid substitution (Glu 404 → Pro) in the gene for TM, kindly provided by dr. R.D. Rosenberg (Massachusetts Institute of Technology, Cambridge, Massachusetts) were generated on a C57BL/6 (and B6D2F1) background, as previously described\textsuperscript{14}. Homozygous mutant TM\textsuperscript{pro/pro} mice exhibit a decrease of approximately 1000-fold with respect to PC activation and approximately 100-fold with respect to binding of thrombin at physiological levels of the enzyme\textsuperscript{14}. In addition, TM\textsuperscript{pro/pro} mice produce < 4% of APC in their alveolar space generated by WT mice upon intratracheal administration of PC and thrombin.\textsuperscript{13} Yet in contrast to TM gene deficient mice, which die in the embryonic stage\textsuperscript{18}, TM\textsuperscript{pro/pro} mice develop to term, and possess normal reproductive performance\textsuperscript{14}. All mice were on a C57BL/6 background. The WT of the TM\textsuperscript{pro/pro} mice were derived from original littermates. All experiments were approved by the Committee on Use and Care of Animals of the Academic Medical Center, Amsterdam, the Netherlands.

\textit{Experimental infection}

Pulmonary tuberculosis was induced exactly as described previously\textsuperscript{15-17}. Briefly, a virulent laboratory strain of \textit{M. tuberculosis} H37Rv was grown in liquid Dubos medium containing 0.01% Tween 80 for 4 days. 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 twice with sterile 0.9% NaCl. Mice were anesthetized by inhalation with isoflurane (Abbott Laboratories, Kent, U.K.) and infected with $1 \times 10^5$ live bacilli in 50-μl saline, as determined by viable counts on
Middlebrook 7H11 agar plates. Groups of 8 mice per time point were sacrificed 2 or 5 weeks post infection, and lungs and one lobus of the liver were removed aseptically. Organs were homogenized with a tissue homogeniser (Biospec Products, Bartlesville, OK) in 5 volumes of sterile 0.9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21-day incubation at 37°C. Numbers of colony forming units (CFU) are provided as total in the lungs or as total per gram liver. For cytokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl\(\cdot\)H\(_2\)O, 1 mM CaCl\(_2\), 1% Triton X-100, 100 μg/ml pepstatin A, leupeptin, and aprotinin), and incubated on ice for 30 min. Supernatants were sterilized using a 0.22-μm filter (Corning, Corning, NY) and frozen at -20°C until assays were performed.

**Lung cell counts and differentiation**

Pulmonary-cell suspension was obtained using an automated disaggregation device (Medimachine System; DAKO A/S, Glostrup, Denmark) as described\(^ {15-17}\) and re-suspended in medium. Erythrocytes were lysed with ice-cold isotonic NH\(_4\)Cl solution, and the remaining cells were washed twice with RPMI. Total leukocytes in pulmonary-cell suspensions were counted using a hemacytometer and Turk’s solution (Merek KGaA, Darmstadt, Germany). The percentages of macrophages, neutrophils and lymphocytes were determined using cytoospin preparations stained with modified Giemsa stain (Diff-Quik; Baxter Healthcare Corp., McGraw Perk, Illinois).

**Histologic examination**

After 24h fixation of lungs in 10% formaline and embedding in paraffin, 4 μm thick sections were stained with haematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the genotype of mice and treatment. Staining for fibrin(ogen) and TM was done exactly as described\(^ {13,19,20}\). In brief, after quenching endogenous peroxidase activity and blocking non-specific binding, slides were incubated with a rat anti-mouse TM mAb (kindly provided by Dr. S.J. Kennel, Oak Ridge National Laboratory, Oak Ridge) followed by a biotinylated rabbit anti-rat polyclonal Ab (Dako). Slides were then incubated in a streptavidin-ABC solution (Dako) and developed using 1% H\(_2\)O\(_2\) and 3.3’-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin after a slight methyl green counterstaining.

**FACS analysis**

For FACS analysis pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and processed as described previously\(^ {15-17}\). Cells were brought to a concentration of 4 x 10\(^6\) cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN\(_3\), and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labeled Abs against CD3, CD4, CD25, CD69, Ly-6 (Gr-1) and CD11b. All Abs were used in concentrations recommended by the manufacturer.
To correct for aspecific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. Cells were fixed with 2% paraformaldehyde, and lymphocyte surface molecules were analyzed by gating of the CD3⁺ population. Expression of CD11b on neutrophils was analyzed by gating of the Gr-1 population. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining.

**Assays**

Thrombin-antithrombin complexes (TAT-c) were measured by ELISA as described. TNF-α, interleukin (IL)-4, IL-6, IL-10, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 were measured by cytometric bead array (CBA, PharMingen, San Diego, CA). Macrophage inflammatory protein (MIP)-2 and transforming growth factor (TGF)-β were measured by ELISA (R & D Systems, Abingdon, UK). All assays were done according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. P < 0.05 was considered to represent a statistically significant difference.

**Results**

**TM-expression**

We previously reported that TM expression becomes down regulated in lung tissue during the acute inflammatory response to intranasally administered bacteria or LPS, which is in line with earlier findings of reduced TM expression in the dermal microvasculature of patients with severe bacterial sepsis.

**Figure 1. Reduced pulmonary TM expression during tuberculosis** Immunostaining for TM in lungs of WT mice without infection (A) and 5 weeks after *M. tuberculosis* infection (B), and TM pr0/pr0 mice 5 weeks after infection (C). Slides are representative for 5 mice per group. Magnification x10.
Figure 2. The TM pro/pro mutation has no effect on coagulation during tuberculosis. TATc levels in plasma and lung homogenates. Mice were intranasally infected with \(10^5\) CFU *M. tuberculosis* and sacrificed 2 and 5 weeks post-infection. Filled bars represent WT mice; open bars represent TM pro/pro mice. Data are means ± SE of 8 mice per group. Dotted lines represent the mean values obtained form normal plasma and lung homogenate of mice without infection.

To obtain insight into the regulation of TM expression in the lung during chronic infection, we compared the expression of TM in lungs obtained from uninfected mice and from mice 5 weeks after intranasal infection with *M. tuberculosis* using anti-TM immunostaining. As shown in Figure 1, a dramatic reduction of TM immunoreactivity was observed 5 weeks after infection in WT (Figure 1B) and TM pro/pro mice (Figure 1C) compared to uninfected mice (Figure 1A).

**No evidence for coagulation activation in TM pro/pro mice**

Tuberculosis can be associated with a modest procoagulant state, which occasionally even can result in disseminated intravascular coagulation 22,23. Since the TM pro/pro mutation results in a mild prethrombotic state 14, we wondered whether endogenous TM is important for maintaining normal hemostasis in the lung during chronic infection by *M. tuberculosis*.

Figure 3. TM pro/pro mice show an increased lung weight. Total lung weights. Mice were intranasally infected with \(10^5\) CFU *M. tuberculosis* and sacrificed 2 and 5 weeks post-infection. Filled bars represent WT mice; open bars
For this, we measured TAT-c in lung homogenate and plasma 2 and 5 weeks post infection. Pulmonary tuberculosis was not associated with elevated TAT-c concentrations in lungs or plasma in either WT or TM<sup>pro/pro</sup> mice, when compared to uninfected mice (Figure 2). In addition, lung tissue did not show deposition of fibrin in either mouse strain (not shown). Thus, these data argue against a role for TM-APC in preventing coagulation during tuberculosis.

**TM<sup>pro/pro</sup> mice show uncontrolled pulmonary inflammation**

Next we evaluated whether the TM<sup>pro/pro</sup> mutation influenced the inflammatory response in the lung during tuberculosis. Remarkably, whereas at 2 weeks postinfection total lung weights were similar in both mouse strains, at 5 weeks after inoculation with *M. tuberculosi*s the lung weights of TM<sup>pro/pro</sup> mice were much higher than those of WT mice (Figure 3, P < 0.05). In line with these findings, histopathological examination of lung tissue did not reveal differences between TM<sup>pro/pro</sup> and WT mice at 2 weeks postinfection (not shown). However, profound differences were found between TM<sup>pro/pro</sup> and WT mice at 5 weeks after infection (Figure 4). Indeed, at that time point, lungs of WT mice displayed granulomatous inflammatory infiltrates primarily located around small bronchi and vessels (Figure 4A) composed of lymphocytes and macrophages (insert 4A), confirming earlier observations. In contrast, lungs of TM<sup>pro/pro</sup> mice displayed more confluent areas of inflammation (Figure 4B) predominantly composed of neutrophils and foamy macrophages (insert 4B). Moreover, edema and pleuritis were more pronounced in TM<sup>pro/pro</sup> than in WT mice.

![Figure 4](image-url)

**Figure 4. Lungs of TM<sup>pro/pro</sup> mice show more confluent inflammation and an enhanced neutrophil influx.** Representative histopathological sections of lungs of WT (A) and TM<sup>pro/pro</sup> (B) mice 5 weeks after inoculation with *M. tuberculosi*s showing granulomatous inflammatory infiltrates predominantly composed of lymphocytes and macrophages in WT mice (A) and confluent areas of inflammation in TM<sup>pro/pro</sup> mice (B) and an enhanced neutrophil influx (insert B). Figures are representative for n=5 for each group, H&E staining, magnification x10, insert x40.

**TM<sup>pro/pro</sup> mice show increased neutrophil influx into lungs**
To obtain more insight into the cellular composition of the pulmonary infiltrates in TM<sup>pro/pro</sup> and WT mice, we prepared whole lung suspensions at 2 and 5 weeks after infection (Table I). At 2 weeks, TM<sup>pro/pro</sup> and WT mice had similar leukocyte numbers in their lungs, albeit lungs of the former mouse strain already contained more neutrophils (P < 0.05 versus WT). At 5 weeks after infection, the lungs of TM<sup>pro/pro</sup> mice contained more leukocytes (P = 0.07 versus WT), which was caused by a profound rise in the number of neutrophils (P < 0.05 versus WT). Interestingly, this enhanced neutrophil influx was accompanied by the presence of less lymphocytes and macrophages in lungs of TM<sup>pro/pro</sup> mice (both P < 0.05 versus WT).

<table>
<thead>
<tr>
<th></th>
<th>T=2weeks</th>
<th>T=5weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TM&lt;sup&gt;pro/pro&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total cells (x10&lt;sup&gt;6&lt;/sup&gt;/ml)</strong></td>
<td>0.9 ± 0.7</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td>0.3 ± 0.7</td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td>0.2 ± 0.9</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.2</td>
</tr>
</tbody>
</table>

Table I. Leukocyte counts in lung homogenates Data are mean ± SE (n = 8 mice per group for each time point) at 2 and 5 weeks after intranasal administration of <i>M. tuberculosis</i> (10<sup>5</sup> CFU). * P < 0.05 versus WT mice.

To determine whether the diminished influx of lymphocytes in TM<sup>pro/pro</sup> mice was restricted to a certain subset, we analyzed whole lung cell suspensions obtained 5 weeks postinfection by FACS (Table II). This revealed that the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells within the CD3<sup>+</sup> population did not differ between TM<sup>pro/pro</sup> and WT mice. Moreover, in both strains the CD4<sup>+</sup> and CD8<sup>+</sup> T-cells equally expressed the activation markers CD25 and CD69. Finally, we also evaluated the activation status of Gr-1<sup>+</sup> neutrophils in the lungs by analyzing CD11b expression, and found no differences between TM<sup>pro/pro</sup> and WT mice.
Table II. Cellular composition in the lungs at 5 weeks after infection. Lymphocyte typing was performed on pulmonary cell suspensions 5 weeks post-infection as described in the Methods. Data are (mean ± SE of 8 mice per group). FACS results of the lymphocytes are expressed as the percentage of CD4+, CD8+, CD25+, and CD69+ within the CD3+ population; results of neutrophils as percentage CD11b+ cells within the Gr-1+ population. Differences between groups were not significant.

<table>
<thead>
<tr>
<th>% positive</th>
<th>WT</th>
<th>TM&lt;sup&gt;pro/pro&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>48.4 ± 4.2</td>
<td>47.8 ± 2.2</td>
</tr>
<tr>
<td>CD8+</td>
<td>37.3 ± 2.4</td>
<td>34.3 ± 2.1</td>
</tr>
<tr>
<td>CD4+/CD69+</td>
<td>15.1 ± 1.2</td>
<td>17.8 ± 2.0</td>
</tr>
<tr>
<td>CD4+/CD25+</td>
<td>2.3 ± 1.3</td>
<td>4.1 ± 2.1</td>
</tr>
<tr>
<td>CD8+/CD69+</td>
<td>12.9 ± 6.2</td>
<td>10.1 ± 3.2</td>
</tr>
<tr>
<td>CD8+/CD25+</td>
<td>4.9 ± 1.3</td>
<td>3.6 ± 1.4</td>
</tr>
<tr>
<td>Gr-1+/CD11b+</td>
<td>22.9 ± 2.1</td>
<td>19.5 ± 1.8</td>
</tr>
</tbody>
</table>

<sup>TM<sup>pro/pro</sup> mice have elevated levels of proinflammatory cytokines and reduced levels of anti-inflammatory cytokines in lungs</sup>

Cytokines and chemokines play a pivotal role in the regulation of the immune response to tuberculosis<sup>24,25</sup>. Therefore, we measured the concentrations of proinflammatory (IFN-γ, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10, TGF-β) in lung homogenates obtained 2 and 5 weeks after infection (Table III). At 2 weeks postinfection, the pulmonary concentrations of these mediators were similar in TM<sup>pro/pro</sup> and WT mice. However, at 5 weeks TM<sup>pro/pro</sup> mice displayed increased levels of IFN-γ and TNF-α in their lungs when compared to WT mice (P < 0.05), whereas the lung levels of IL-4, IL-10 and TGF-β were lower in TM<sup>pro/pro</sup> mice at that time point. In light of the altered recruitment of inflammatory cells to the pulmonary compartment in TM<sup>pro/pro</sup> versus WT mice, we also measured MCP-1 (a prototypic CC chemokine) and MIP-2 (a major CXC chemokine with neutrophil attracting properties)<sup>26</sup>. Both MCP-1 and MIP-2 concentrations were higher in TM<sup>pro/pro</sup> than in WT mice (P < 0.05).
Enhanced antigen-specific IFN-γ release by splenocytes from infected TM<sup>pro/pro</sup> mice.

To obtain insight into the capacity of infected animals to mount a protective antigen-specific type 1 response, the ability of spleen cells, harvested 2 and 5 weeks post-infection, to produce IFN-γ<em> ex vivo</em> upon stimulation with PPD was investigated (Figure 5). Splenocytes of TM<sup>pro/pro</sup> mice secreted more IFN-γ than splenocytes of WT mice upon recall stimulation (P < 0.05 at both time points).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TM&lt;sup&gt;pro/pro&lt;/sup&gt;</th>
<th>WT</th>
<th>TM&lt;sup&gt;pro/pro&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>191.2 ± 34.5</td>
<td>215.3 ± 52.8</td>
<td>219.3 ± 18.8</td>
<td>445.4 ± 70.8*</td>
</tr>
<tr>
<td>IL-4</td>
<td>23.5 ± 10.5</td>
<td>37.6 ± 8.5</td>
<td>488.3 ± 28.5</td>
<td>381.8 ± 28.5*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>461.2 ± 89.8</td>
<td>480.3 ± 135.3</td>
<td>376.6 ± 46.3</td>
<td>496.3 ± 55.2*</td>
</tr>
<tr>
<td>IL-10</td>
<td>44.4 ± 12.5</td>
<td>55.2 ± 0.9</td>
<td>235.3 ± 22.1</td>
<td>42.0 ± 21.3*</td>
</tr>
<tr>
<td>TGF-β</td>
<td>380.4 ± 85.1</td>
<td>337.3 ± 79.9</td>
<td>1306.4 ± 155.7</td>
<td>812.2 ± 63.5*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>458.2 ± 99.2</td>
<td>478.1 ± 78.4</td>
<td>154.2 ± 55.7</td>
<td>189.8 ± 17.1*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>1978.2 ± 68.2</td>
<td>1800.6 ± 89.0</td>
<td>1581.2 ± 469.1</td>
<td>2363.4 ± 125.9*</td>
</tr>
</tbody>
</table>

Table III. Cytokine and chemokine concentrations in lung homogenates Data are mean ± SE (<i>n = 8</i> mice per group for each time point) at 2 and 5 weeks after intranasal administration of <i>M. tuberculosis</i> (10<sup>5</sup> CFU). * P < 0.05 versus WT.
**Figure 5.** Enhanced antigen-specific IFN-γ release by splenocytes from infected TM^{pro/pro} mice. Splenocytes were harvested 2 and 5 weeks after infection and stimulated (1x10^6 cells/well) for 48 hours with PPD. Filled bars represent WT mice; open bars represent TM^{pro/pro} mice. Data are means ± SE of 8 mice per group. * P < 0.05 versus WT mice.

TM^{pro/pro} mice have a decreased bacterial outgrowth
We next determined the role of the TM^{pro/pro} mutation in containing the bacterial outgrowth (Figure 6). For this we compared the number of *M. tuberculosis* CFU's 2 weeks (upper panels) and 5 weeks (lower panels) after infection in lung homogenate (left panels), and in liver (right panels) of TM^{pro/pro} and WT mice. At 2 weeks mycobacterial loads were similar in lungs and livers of both mouse strains. However, at 5 weeks both lungs and liver of TM^{pro/pro} mice contained less mycobacteria in than lungs and liver of WT mice (both P < 0.05).

**Figure 6.** TM^{pro/pro} mice have a decreased bacterial outgrowth
Mycobacterial loads in CFU/ml in lungs (left panels) and livers (right panels) at 2 (upper panels) and 5 weeks (lower panels) after intranasal infection with 10^5 *M. tuberculosis* CFU. Data are means ± SE (n = 8 per group). Filled symbols represent WT mice; open symbols represent TM^{pro/pro} mice. Horizontal lines represent medians. * P < 0.05 between groups indicated.
Discussion

The central aim of this study was to examine the influence of TM, in particular the domain of TM that is crucial for the generation of APC, in the regulation of the pulmonary response to tuberculosis. In theory, a reduced capacity to produce APC could influence the local response to chronic infection with *M. tuberculosis* via two major mechanisms: in light of the anticoagulant properties of APC, the TM<sup>pro/pro</sup> mutation could result in an increased tendency to form pulmonary thrombosis, whereas in light of the anti-inflammatory properties of APC, this TM mutation could lead to an enhanced proinflammatory reaction. We here demonstrate that TM<sup>pro/pro</sup> mice do not display a diminished capacity to maintain a normal hemostatic balance during tuberculosis, but clearly have a reduced ability to control the inflammatory response to *M. tuberculosis* infection.

The role of TM in disease cannot be investigated using TM gene deficient mice since these mice die in the embryonic phase<sup>18</sup>. Therefore, we used TM<sup>pro/pro</sup> mice, which are viable yet have a strongly reduced capacity to produce APC. Indeed, TM<sup>pro/pro</sup> mice exhibit a 100-fold reduction with respect to binding of thrombin and a 1000-fold reduction with respect to PC activation in the circulation<sup>14</sup>. Similarly, TM<sup>pro/pro</sup> mice display a strongly reduced capacity to generate APC in their alveolar space (a 25-fold reduction when compared to WT mice)<sup>13</sup>. The incapacity of TM<sup>pro/pro</sup> mice to activate PC has been demonstrated using an indirect approach, i.e. mice received an intravenous<sup>14</sup> or intratracheal<sup>13</sup> dose of human PC with or without thrombin, after which human APC levels were determined in plasma or bronchoalveolar lavage fluid respectively using an immuno-capture assay. To the best of our knowledge, APC measurements in mice have not been reported previously. In addition, the “direct” demonstration of reduced APC levels in TM<sup>pro/pro</sup> mice during tuberculosis will be very difficult for several reasons. In particular, tuberculosis is accompanied by low PC levels in plasma<sup>22</sup>; together with our present finding of reduced TM expression in lungs during murine tuberculosis, these data suggest that this infection per se leads to a reduced capacity to generate APC. In line with this assumption, patients with interstitial lung disease demonstrated indirect evidence of diminished PC activation in bronchoalveolar lavage fluid<sup>27</sup>. Furthermore, patients with sepsis have low or undetectable APC concentrations in their circulation<sup>21</sup>. Hence, infection and inflammation likely results in a relative insufficiency to produce APC<sup>1,2</sup> and a further reduction of the APC production capacity, as in TM<sup>pro/pro</sup> mice, apparently has a profound influence on the regulation of inflammation during chronic pulmonary infection such as produced by *M. tuberculosis*.

TM<sup>pro/pro</sup> mice did not show any evidence for activation of coagulation during tuberculosis. In man, tuberculosis can be accompanied by modest alterations in the coagulant-anticoagulant balance<sup>22</sup>. Considering the preferential expression of TM in the lung<sup>11,12</sup>, we argued that TM mediated generation of APC may be important for inhibiting a sustained activation of coagulation at the site of chronic inflammation.
such as produced by *M. tuberculosis* infection in mice. This hypothesis was further supported by studies from other groups, reporting that TM<sup>pro/pro</sup> mice display a prethrombotic state and an increased susceptibility to thrombosis<sup>14,28</sup>. The current data clearly indicate that murine tuberculosis does not result in local activation of coagulation and that TM and APC do not contribute to prevention of thrombosis in this condition, i.e. TM<sup>pro/pro</sup> mice had normal TAT-c levels in plasma and lungs and a complete absence of fibrin deposits in their pulmonary compartment.

In contrast to the lack of an effect on coagulation, the TM<sup>pro/pro</sup> mutation exerted a profound effect on the inflammatory reaction to *M. tuberculosis* infection. Indeed, at 5 weeks after infection TM<sup>pro/pro</sup> mice displayed uncontrolled inflammation in their lungs, which was associated with a diminished capacity to form well-shaped granulomas, a reduced recruitment of lymphocytes and macrophages to the site of the infection and an increased influx of neutrophils. Moreover, TM<sup>pro/pro</sup> mice had higher levels of proinflammatory cytokines (IFN-γ and TNF-α) and lower levels of anti-inflammatory cytokines (IL-4, IL-10 and TGF-β) in their lungs, reflecting a net shift towards a proinflammatory cytokine environment. These findings are in line with several earlier studies examining the effect of TM and APC on inflammation. Exogenously administered TM and APC are both able to attenuate lung injury related to DIC by inhibiting leukocyte activation<sup>9,29-33</sup> and intratracheal administration of APC exerts anti-inflammatory effects in bleomycin-induced lung fibrosis<sup>10</sup>. Furthermore, leukocyte activation was also inhibited by APC after renal and spinal cord injury in rats<sup>6,7,34</sup>. In addition, APC can inhibit the production of TNF-α and other proinflammatory cytokines in vitro and in vivo<sup>6,9</sup>. The majority of the anti-inflammatory effects of APC in these models were unrelated to its anticoagulant effects. Our current data are also in accordance with recent findings in heterozygous protein C deficient mice, which demonstrated higher levels of proinflammatory cytokines and increased neutrophil invasion in their lungs after intraperitoneal injection of LPS<sup>19</sup>.

Notably, the present study contrasts with a previous investigation from our laboratory, in which the influence of the TM<sup>pro/pro</sup> mutation on the pulmonary coagulant and inflammatory response during acute lung inflammation models induced by intranasal administration with either bacterial respiratory pathogens or LPS was examined<sup>13</sup>. In that study we did not detect any difference between TM<sup>pro/pro</sup> and WT mice in either of these models, although in pneumococcal pneumonia fibrin deposition was modestly increased in TM<sup>pro/pro</sup> mice<sup>15</sup>. Thus, TM and APC apparently have a greater impact on the inflammatory reaction in the lung during chronic lung infection such as in murine tuberculosis.

Conway et al. recently reported several anti-inflammatory properties of endogenous TM that were unrelated to its anticoagulant properties<sup>35</sup>. These authors generated mice that lack the NH2-terminal (lectin) domain of TM; these TM<sup>LeD/LeD</sup> mice were shown to have normal TM antigen levels and to retain the capacity to generate APC. TM<sup>LeD/LeD</sup> mice were found to have elevated circulating cytokine levels upon
systemic challenge with LPS. Moreover, TM^{LeD/LeD} mice had higher neutrophil counts in bronchoalveolar lavage fluid after exposure to LPS via a nebulizer. This investigation indicates that TM, and in particular its lectin domain, has anti-inflammatory properties that can be dissected from the anticoagulant properties of TM. It should be noted that the TM^{pro/pro} mice used here have an intact TM lectin domain. It is therefore possible that intact TM may even have a more profound impact on the inflammatory response to pulmonary tuberculosis.

TM^{pro/pro} mice had less mycobacterial CFUs in lungs and liver than WT mice at 5 weeks postinfection, in spite of the fact that they were less able to form well-defined granulomas. It is conceivable that the enhanced local concentrations of the protective cytokines IFN-γ and TNF-α played a role in the modestly reduced mycobacterial loads in TM^{pro/pro} mice. In addition, TM^{pro/pro} mice displayed an enhanced protective antigen-specific type 1 response at 5 weeks postinfection.

Tuberculosis results in a chronic inflammatory reaction of the lungs that normally is not or to a very limited extent associated with activation of the coagulation system. We here tested the hypothesis that the capacity to generate APC in the lung is important for inhibiting the coagulant and the inflammatory response during pulmonary tuberculosis. By using TM^{pro/pro} mice we show that a strongly reduced ability to generate APC in the alveolar space does not result in enhanced activation of coagulation, but that the coordinated inflammatory response characterized by the recruitment of lymphocytes and macrophages to the site of the infection and the formation of well-shaped granulomas is disturbed. These data suggest that the presence of functional TM in the lung is of importance for the regulation of the immune response to M. tuberculosis.

References