Plasminogen activator inhibitor type I may contribute to transient, non-specific changes in immunity in the subacute phase of murine tuberculosis.

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ABSTRACT

Tuberculosis, caused by *Mycobacterium (M.) tuberculosis*, is a devastating infectious disease causing many deaths world-wide. Non-specific host defense mechanisms such as the coagulation and fibrinolytic system may give insight in possible new therapeutic targets. Plasminogen activator inhibitor type-1 (PAI-1), an important regulator of inflammation and fibrinolysis, might be of interest as tuberculosis patients have elevated plasma levels of PAI-1. In this study we set out to investigate the role of PAI-1 during tuberculosis *in vivo*. Wildtype (WT) and PAI-1 deficient (PAI-1-/-) mice were intranasally infected with *M. tuberculosis* H37rv and sacrificed after 2, 5 and 29 weeks. Five weeks post-infection, bacterial loads in lungs of PAI-1-/- mice were significantly higher compared to WT mice, while no differences were seen 2 and 29 weeks post-infection. At two weeks post-infection increased influx of macrophages and lymphocytes was observed. PAI-1 deficiency was associated with a reduced cytokine response in the lungs; however, upon stimulation with tuberculin purified protein derivative (PPD), PAI-1-/- splenocytes released increased levels of IFN-γ compared to WT. No clear differences were found between PAI-1-/- and WT mice at 29 weeks after infection. In conclusion, these data suggest that PAI-1 contributes to transient, non-specific changes in immunity during the early phase of murine tuberculosis.
INTRODUCTION

Tuberculosis, caused by the acid-fast bacterium *Mycobacterium (M.) tuberculosis*, is one of the most devastating infectious diseases worldwide, with one-third of the world population being infected1, 2. In 2009 there were 9.4 million new cases and 1.3 million people died from this disease3. Most infected individuals do not progress to a full-blown disease because, due to a strong protective T helper -1 (Th1) response, the tubercle bacilli are 'walled-off' inside granuloma, tissue nodules consisting of infected macrophages surrounded by lymphocytes and a fibrotic capsule4. *M. tuberculosis* bacilli that are not fully eliminated from the lungs remain a potential danger to the infected individual1, 4. Additionally, multi-drug resistant (MDR) tuberculosis has become a serious threat3, 5. This emphasizes the importance of understanding host defense mechanisms during tuberculosis.

During severe acute pulmonary infections a range of specific and non-specific host defense mechanisms becomes activated, resulting in a strong inflammatory response together with increased procoagulant activity and suppression of the fibrinolytic system6-9. Plasminogen activator inhibitor type 1 (PAI-1) is considered to be the main inhibitor of the fibrinolytic system10. By inactivating both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), PAI-1 inhibits generation of the key fibrinolytic enzyme plasmin and subsequent fibrin degradation. Besides its role in maintaining normal hemostasis by regulating the fibrinolytic system, PAI-1 has also been implicated in other processes and diseases that are not or only partially related to its capacity to inhibit plasminogen generation, including leukocyte trafficking, neutrophil effrocytosis (phagocytosis of apoptotic cells), extracellular matrix (ECM) degradation, tumor angiogenesis, wound healing, atherosclerosis and metabolic diseases10-13. These processes may depend on the antiproteolytic activity of PAI-1 and/or be associated with its interference in cellular migration and ECM binding10. Indeed, previous studies showed that the role of PAI-1 as a chemotactic factor promoting the migration of leukocytes into inflammatory sites contributed to its inflammatory effects12-14. Effective host defense against *M. tuberculosis* is primarily dependent on the interplay between macrophages, T-cells and dendritic cells2. As this interaction requires migration and activation of leukocytes, it is conceivable that PAI-1 plays a role in this process.

Patients with lung tuberculosis have shown increased plasma PAI-1 levels, accompanied by elevations in the plasma levels of tPA, fibrinogen and fibrin(ogen) degradation products, reflecting concurrent activation and inhibition of both procoagulant and fibrinolytic pathways15, 16. In pleural fluid, increased PAI-1 levels and a positive PAI-1/tPA ratio were found, indicating that an imbalance of tPA and PAI-1 in pleural spaces may lead to pleural thickening and loculation of pleural effusions17, 18. To the best of our knowledge only one study examined the functional role of fibrinolytic mediators in mycobacterial infection19. Using mice deficient for either plasminogen, tPA, uPA or uPA receptor, this report demonstrated that none of these proteins played a significant role in initiating or maintaining the immune response against a *M. avium* infection19. Knowledge on the role of PAI-1 in host defense during mycobacterial infection in general and during pulmonary tuberculosis in vivo in particular is lacking. Therefore, in the present study we aimed to characterize the role of PAI-1 in the host response after lung infection by *M. tuberculosis* using our established mouse model20-22.
MATERIALS AND METHODS

Mice and experimental infection
Pathogen-free 8 to 10 week old wild-type (WT) C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). PAI-1−/− mice on a C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbour, ME) and bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments. A virulent laboratory strain of M. tuberculosis H37Rv (American Type Culture Collection, Rockville, MA) was grown for 4 days in liquid Dubos 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. Tuberculosis was induced as described previously\(^2\)\(^3\). Briefly, a replicate culture of M. tuberculosis H37Rv was incubated at 37°C and stirred gently, harvested at midlog phase, and stored in aliquots at −70°C. Before each experiment, a vial was thawed and washed twice with sterile saline, to clear the mycobacteria of medium. We anesthetized the mice with isoflurane (Abott Laboratories, Kent, UK) delivered by inhalation. During this brief anesthesia, intranasal inoculation was conducted by placing 10⁵ viable M. tuberculosis organisms in 50 µL NaCl on the nares. The inoculum was placed on Middlebrook 7H11 plates immediately after inoculation to determine viable counts. This model of experimental tuberculosis is designed to mimic pulmonary tuberculosis, with the lungs as primary site of infection with subsequent dissemination of bacteria distant organs such as liver, as demonstrated by earlier reports from our laboratory\(^2\)\(^0\)-\(^2\)\(^2\),\(^2\)\(^4\). Groups of eight mice per time-point were sacrificed 2 and 5 weeks after infection. In addition, 14 WT and 11 PAI-1−/− mice were followed for 29 weeks after infection; surviving mice were killed at that time point to obtain insight in the role of PAI-1 in chronic infection. Organs were processed as described previously\(^2\)\(^1\),\(^2\)\(^2\). Briefly, lungs and liver were removed aseptically and homogenized in 5 volumes of sterile 0.9% NaCl. Ten-fold dilutions were plated on Middlebrook 7H11 plates to determine bacterial loads. Colonies were counted after 21 days incubation at 37°C. Numbers of CFUs were provided per g of lungs and liver.

Histology
Lungs were removed 2, 5 or 29 weeks after inoculation with M. tuberculosis, fixed in formalin for 24h and embedded in paraffin. Hematoxilin and eosin stained slides were scored from 0 (absent) to 4 (most severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, granuloma formation and pleuritis by a pathologist blinded for the groups, as described earlier\(^2\)\(^2\),\(^2\)\(^5\). The total ‘lung inflammation score’ was expressed as the sum of the scores for each parameter, the maximum being 24. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung area.
Flow cytometry
Lung cell suspensions were obtained by crushing lungs through a 40-μm pore-size cell strainer (BD, San Jose, CA) as described previously. Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4). 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 per mL. The percentages of macrophages, polymorphonuclear cells (PMNs) and lymphocytes were determined using flow cytometric analysis using FACSCalibur (BD, San Jose, CA). 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), CD69 (CD69-fluorescein isothiocyanate) or GR-1 (GR-1-fluorescein isothiocyanate). All antibodies were used in concentrations recommended by the manufacturer (all from BD Pharmingen, San Diego, CA). After staining, cells were fixed in 2% paraformaldehyde and T-cell surface molecules were analyzed on CD3+ cells within the lymphocyte gate.

Assays
For PAI-1 protein and cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 10 ng/mL; pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at 1500 x 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 PAI-1, interferon (IFN)-γ, tumour 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 (PAI-1: Kordia, Leiden, The Netherlands, all others: R&D, Minneapolis, MO).

Splenocyte stimulation
Single cell suspensions were obtained by crushing spleens through a 40 μm pore-size cell strainer (BD, San Jose, CA) as described. Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution. The remaining cells were washed twice with RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic (GibobRL, Life Technologies, Rockville, MD). Cells were seeded in 96-well round bottom culture plates at a cell density of 1 x 10⁶ cells per 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.

Statistical analysis
Data are expressed as dot plots (CFU data) and box and whisker plots (all other data) showing the median, lower and upper quartiles and range of values. Normality was tested using a D’Agostino
& Pearson omnibus normality test. As not all data were normally distributed, comparisons between groups were conducted using the Mann-Whitney U test. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered statistically significant.

RESULTS

Endogenous pulmonary PAI-1 is upregulated during M. tuberculosis infection
PAI-1 is considered to be the main inhibitor of the fibrinolytic system\textsuperscript{10}. Patients with lung tuberculosis show increased plasma PAI-1 levels, accompanied by elevations in the plasma levels of tPA, fibrinogen and fibrin(ogen) degradation products\textsuperscript{15,16}, which is a reflection of concurrent activation and inhibition of both procoagulant and fibrinolytic pathways during this disease. To obtain insight into local PAI-1 concentrations in the lung during experimental M. tuberculosis infection, we measured PAI-1 protein levels in lung homogenates of uninfected WT mice and in WT mice infected with M. tuberculosis. Pulmonary PAI-1 was detected at levels 0.9 - 1.5 ng/mL in lung homogenates of uninfected mice. Infection with M. tuberculosis was associated with a significant increase in pulmonary PAI-1 levels after 2 and 5 weeks of infection, as compared to uninfected mice (P < 0.05 and P < 0.01 respectively; Fig. 1A).

PAI-1 limits early pulmonary mycobacterial growth
To study the potential impact of PAI-1 on mycobacterial growth during tuberculosis, we first determined M. tuberculosis loads in the lungs during the early phase of infection. At 2 weeks after infection the numbers of CFU of M. tuberculosis recovered from the lungs of WT and PAI-1\textsuperscript{-/-} mice did not differ. However, at 5 weeks after infection lungs from PAI-1\textsuperscript{-/-} mice contained increased numbers of mycobacteria as compared to WT mice (P < 0.01; Fig. 1B). No differences in bacterial loads after 2 and 5 weeks in liver homogenates were observed (Fig. 1C). Considering that tuberculosis is a chronic infection, we next studied the influence of PAI-1 deficiency on mycobacterial growth in mice infected for 29 weeks. During that period 1 of 14 WT and 2 of 11 PAI-1\textsuperscript{-/-} mice died (not significantly different). The lungs and liver of the surviving WT and PAI-1\textsuperscript{-/-} mice contained similar amounts of mycobacteria (Fig. 1B-C). Hence, these data suggest a role for PAI-1 in limiting the growth of M. tuberculosis in the pulmonary compartment during the early but not the late phase of the infection.

PAI-1 deficiency does not impact lung histopathology
To investigate the role of PAI-1 in the regulation of lung inflammation during tuberculosis, we performed histopathologic analyses of lung tissue slides prepared from WT and PAI-1\textsuperscript{-/-} mice 2, 5 and 29 weeks after infection. Figure 2 shows representative histological sections of lungs of mice
PAI-1 in tuberculosis

infected with *M. tuberculosis* after 2, 5 and 29 weeks of WT (Fig. 2A, C, E) and PAI-1−/− mice (Fig. 2B, D, F). Together, these data suggest that PAI-1 did not impact the extent of lung pathology during tuberculosis. After 2, 5 and 29 weeks, both groups displayed granulomatous inflammation which increased in extent and severity in time after infection in both mouse strains. At early time points histopathological scores did not differ between WT and PAI-1−/− mice (Table 1); after 29 weeks lung histopathology scores could not be adequately determined due to the presence of extensive inflammation in both mouse strains. Similarly, lung weights, a rough index for lung inflammation, were similar in both mouse strains at all time points (Table 1).

**PAI-1 deficiency enhances early macrophage and lymphocyte recruitment to the lungs**

To obtain more insight into the cellular composition of the pulmonary infiltrates in WT and PAI-1−/− mice, we prepared whole lung cell suspensions at 2 and 5 weeks after infection and determined subsets of inflammatory cells by FACS-analysis (Table 2). Two weeks after infection total leukocyte counts were enhanced in PAI-1−/− mice as compared to WT mice (*P* < 0.01), which was caused by an increase in the numbers of macrophages (*P* < 0.01) and lymphocytes (*P* < 0.01). Five weeks after inoculation total pulmonary leukocyte counts and differential cell counts were similar in both mouse strains. To determine whether the relative increase in lymphocytes in PAI-1−/− mice was restricted to a certain subset, we analyzed whole lung CD3+ lymphocytes with respect to expression of CD4, CD8 and the activation marker CD69 (Table 2). This analysis revealed that at 2 weeks post infection PAI-1−/− mice had an increased percentage of CD8+ T cells in their lungs (*P* < 0.01 versus WT mice); this difference between the two mice strains had disappeared at 5 weeks after infection. The percentage of CD4+ and CD8+ T cells expressing CD69 was increased in PAI-1−/− mice at 2 weeks (*P* < 0.05 and *P* < 0.01 respectively, versus WT mice), while after 5 weeks the differences between the two mice strains had disappeared. The percentages of CD4+ T cells did not differ

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**Figure 1.** Pulmonary PAI-1 protein levels in WT mice increase during *M. tuberculosis* infection and mycobacterial loads are significantly higher in lungs of PAI-1−/− mice 5 weeks after infection. Wildtype (WT) mice (grey boxes) were sacrificed before (0h) and 2 and 5 weeks after intranasal inoculation with *M. tuberculosis* to induce experimental tuberculosis. PAI-1 protein concentrations were measured in lung homogenates by ELISA (A). Mycobacterial loads per gram lung (B) and liver (C) of WT (black dots) and PAI-1−/− (white dots) mice 2, 5 and 29 weeks after infection with 10^5 CFU of *M. tuberculosis*. N = 7-8 mice per group. Data are expressed as box and whisker plots showing the median, lower and upper quartiles and range of values (1A) and dot plots showing all individual observations and the median (Figure 1B-C). *P* < 0.05 and **P* < 0.01 versus 0 hours (A) or WT (B-C) (Mann-Whitney U test).
significantly between groups at either time point, although at 2 weeks a clear trend was seen towards reduced percentages of CD4+ T-cells in PAI-1-/- mice ($P = 0.05$; Table 2).

**Cytokine and chemokine response during experimental tuberculosis**

Cytokines and chemokines play a pivotal role in the regulation of the immune response to tuberculosis. Therefore 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 lung homogenates obtained 2 and 5 weeks after infection (Table 3). The concentrations of all mediators were similar in lungs of WT and PAI-1-/- mice at 2 weeks after infection, except for KC which was decreased in lungs of PAI-1-/- mice ($P < 0.05$). At 5 weeks after infection lung levels of the pro-inflammatory TNF-α ($P < 0.01$), anti-inflammatory IL-10 ($P < 0.01$), IL-4 ($P < 0.05$) and the chemokines KC ($P < 0.01$) and MIP-2 ($P < 0.05$) were lower in PAI-1-/- mice as compared to WT mice, while the concentrations of the prototypic type 1 cytokine IFN-γ and the pro-inflammatory IL-6 were similar in both mouse strains.

![Figure 2. Histopathology of the lung during murine tuberculosis.](image)
Table 1. Effect of PAI-1 deficiency on lung inflammation during tuberculosis.

<table>
<thead>
<tr>
<th></th>
<th>Lung weighta (g)</th>
<th>Histopathological score (0-24)</th>
<th>Confluent inflammation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2w</td>
<td>WT</td>
<td>0.13 (0.12-0.16)</td>
<td>10.5 (9.0-12.5)</td>
</tr>
<tr>
<td></td>
<td>PAI-1+/−</td>
<td>0.12 (0.12-0.16)</td>
<td>8.0 (7.0-11.0)</td>
</tr>
<tr>
<td>5w</td>
<td>WT</td>
<td>0.20 (0.19-0.23)</td>
<td>14.0 (13.3-15.8)</td>
</tr>
<tr>
<td></td>
<td>PAI-1+/−</td>
<td>0.20 (0.18-0.23)</td>
<td>14.4 (14.0-16.8)</td>
</tr>
<tr>
<td>29w</td>
<td>WT</td>
<td>0.46 (0.43-0.52)</td>
<td>N.D. c</td>
</tr>
<tr>
<td></td>
<td>PAI-1+/−</td>
<td>0.46 (0.40-0.54)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

aData are expressed as medians ± interquartile ranges of 7-8 mice per group (for the 2 and 5 weeks time point) and 9-13 mice per group (for the 29 weeks time point). bConfluent inflamed areas were not present (N.P.) yet 2 weeks after infection. cNo histopathological scores could be determined adequately 29 weeks after infection (N.D.) due to the presence of extensive inflammation in both mouse strains. *P < 0.05 versus WT (Mann-Whitney U test).

Table 2. Effect of PAI-1 deficiency on total and differential lung cell counts and T-cell subsets in lungs during tuberculosis.

<table>
<thead>
<tr>
<th></th>
<th>Leukocytesa (absolute cell counts x 10^6)</th>
<th>Mφs b (x 10^6)</th>
<th>PMNs c (x 10^6)</th>
<th>Lymphocytes (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2w</td>
<td>WT</td>
<td>23 (22-31)</td>
<td>9 (8-10)</td>
<td>4 (3-5)</td>
</tr>
<tr>
<td></td>
<td>PAI-1+/−</td>
<td>41 (32-45)**</td>
<td>15 (12-17)**</td>
<td>5 (4-6)</td>
</tr>
<tr>
<td>5w</td>
<td>WT</td>
<td>57 (40-90)</td>
<td>13 (10-21)</td>
<td>2 (2-3)</td>
</tr>
<tr>
<td></td>
<td>PAI-1+/−</td>
<td>70 (54-100)</td>
<td>14 (10-20)</td>
<td>2 (1-3)</td>
</tr>
</tbody>
</table>

aData are expressed as medians ± interquartile ranges of 7-8 mice per group. bMφs macrophages, PMN polymorphonuclear cells. *P < 0.05 versus WT, **P < 0.01 versus WT (Mann-Whitney U test).
Enhanced IFN-γ production upon ex vivo stimulation of PAI-1 deficient splenocytes

Next, we determined the capacity of splenocytes obtained from infected mice to respond to the recall antigen PPD. Splenocytes were harvested at 2 and 5 week after infection and then stimulated with PPD for 48 hours after which IFN-γ (indicative for a Th1 response) and IL-4 (indicative for a Th2 response) were measured in the supernatant. After 2 weeks splenocytes from infected WT and PAI-1-/- mice produced similar IFN-γ levels. However, at 5 weeks after infection IFN-γ production by PAI-1-/- splenocytes was significantly increased compared to WT splenocytes ($P < 0.01$, Fig. 3). IL-4 levels were undetectable in supernatants from both groups at both time-points (data not shown).

DISCUSSION

Tuberculosis is a devastating disease, causing almost 2 million deaths every year. As a prerequisite to understanding the progression of disease post-infection, it is important to understand specific and non-specific host defense mechanisms during this infection. Besides the strong protective Th1-cell response elicited to control the growth of *M. tuberculosis*, other protective host defense mechanisms are likely to play a role as well. Pulmonary tuberculosis is associated with increased plasma levels of PAI-1\(^1\),\(^2\). In models of (sub)acute bacterial infection of the lung, local production of PAI-1 has been found to improve host defense.\(^2\),\(^7\),\(^8\). In bleomycin-induced chronic lung inflammation, pulmonary PAI-1 plays an important protective role against the development of fibrosis.\(^2\),\(^9\),\(^3\). Considering these roles of PAI-1 as a regulator of lung inflammation, we here investigated the contribution of endogenous PAI-1 to the host response against *M. tuberculosis* using our established mouse model. We found that during murine tuberculosis PAI-1 is upregulated in the lungs. PAI-1 deficiency was associated with enhanced pulmonary bacterial loads in the subacute phase after infection: five weeks post-infection, bacterial burdens in lungs of PAI-1-/- mice were significantly higher compared to WT mice. However, no differences were seen in the acute phase, 2 weeks post-inoculation and differences between PAI-1-/- and WT mice were modest and subsided during the more chronic phase of the infection. These data suggest a transient, non-specific role for PAI-1 in limiting mycobacterial growth during the early phase of experimental tuberculosis. To our knowledge, this is the first study that examined the functional role of PAI-1 during tuberculosis.

Recruitment of inflammatory cells to the site of infection is crucial for an adequate immune response. During tuberculosis acid-fast bacteria invade macrophages, after which T-lymphocytes are recruited to produce a protective Th1 response.\(^2\),\(^4\). The results obtained in this study indicate that PAI-1 contributes to the control of mycobacterial growth, as we observed increased bacterial loads in PAI-1-/- mice compared to WT mice 5 weeks after induction of infection. Increased bacterial growth in PAI-1-/- mice was preceded by an increased influx of T lymphocytes, in particular
Table 3. Effect of PAI-1 deficiency on pulmonary cytokine and chemokine levels during tuberculosis.

<table>
<thead>
<tr>
<th></th>
<th>2w</th>
<th>5w</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pg/mL</td>
<td>WT</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100 (80-112)</td>
<td>101 (55-133)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>487 (460-555)</td>
<td>443 (352-493)</td>
</tr>
<tr>
<td>IL-4</td>
<td>120 (96-132)</td>
<td>96 (84-110)</td>
</tr>
<tr>
<td>IL-6</td>
<td>206 (144-250)</td>
<td>298 (174-524)</td>
</tr>
<tr>
<td>IL-10</td>
<td>173 (118-220)</td>
<td>99 (67-139)</td>
</tr>
<tr>
<td>KC</td>
<td>1792 (1478-1953)</td>
<td>1334 (862-1646)</td>
</tr>
<tr>
<td>MIP-2</td>
<td>311 (292-332)</td>
<td>298 (248-423)</td>
</tr>
</tbody>
</table>

*aData are expressed as medians ± interquartile ranges of 7-8 mice per group. *<i>P</i> < 0.05 versus WT, **<i>P</i> < 0.01 versus WT (Mann-Whitney <i>U</i> test).

Figure 3. Increased IFN-γ response upon PPD re-stimulation of splenocytes of PAI-1<sup>+</sup> mice 5 weeks after infection. Splenocytes were isolated from WT (grey boxes) and PAI-1<sup>+</sup> (white boxes) mice 2 and 5 weeks after infection with <i>M. tuberculosis</i>, counted and stimulated with PPD for 48 hours. Stimulated splenocytes from PAI-1<sup>+</sup> mice released more IFN-γ in response to PPD than splenocytes from infected WT mice. Data are expressed as box and whisker plots showing the median, lower and upper quartiles and range of values of eight mice per group. **<i>P</i> < 0.01 versus WT (Mann-Whitney <i>U</i> test).
CD8+ T cells, which relative to WT mice, showed enhanced expression of the activation marker CD69. Although CD8+ T cells have been implicated to play a supportive role in host defense against tuberculosis[2, 4], their modestly enhanced recruitment apparently did not result in an improved antibacterial defense. Notably, the percentage of CD4+ T cells was reduced in PAI-1−/− mice 2 weeks after infection, possibly contributing to the subsequently enhanced mycobacterial growth. A recent study showed increased CD25+ T-lymphocyte infiltration in the lungs of PAI-1−/− mice during LPS endotoxemia, while the opposite phenotype was observed in PAI-1 overexpressing mice, suggesting a role for PAI-1 in controlling lymphocyte recruitment[31].

Cytokine release is an important component of the host response to \textit{M. tuberculosis}[2]. Mycobacteria mainly reside in phagosomes in macrophages and phagosomal maturation is promoted by activation with IFN-γ, which stimulates anti-mycobacterial mechanisms. At 5 weeks after infection the pulmonary levels of several cytokines were reduced in PAI-1−/− mice, especially so IL-4, IL-10 and TNF-α. Whereas the Th2 cytokines IL-4 and IL-10 play less important roles during tuberculosis, the reduced TNF-α levels may have facilitated mycobacterial growth in PAI-1−/− mice[2]. Although in lungs IFN-γ levels were not different between mouse strains, \textit{ex vivo} restimulation of splenocytes showed increased IFN-γ release in PAI-1−/− mice compared to WT mice, 5 weeks after infection. These results are in line with previous data showing an enhanced capacity for IFN-γ production by splenocytes from PAI-1−/− mice after incubation with LPS or staphylococcal enterotoxin B, relative to WT splenocytes[32]. If anything, this apparently increased ability to release IFN-γ is expected to result in a more effective anti-mycobacterial host defense response in PAI-1−/− mice, however, this was not observed here. Together, some responses detected in PAI-1−/− mice were altered in an opposite direction as could have been expected based on the increased mycobacterial loads 5 weeks post infection, in particular the enhanced early recruitment of CD8+ T cells and the enhanced IFN-γ production capacity of splenocytes upon stimulation with PPD ex vivo. A clear explanation is lacking, although these increased and presumably protective responses my present a failed compensatory reaction in light of the higher bacterial loads in PAI-1−/− mice.

Previous experiments using mice deficient for plasminogen, tPA, uPA or the uPA receptor failed to show any differences in pulmonary bacterial growth, compared with WT mice, 5 or 10 weeks after infection with \textit{Mycobacterium (M.) avium}; PAI-1−/− mice were not studied[19]. A limited role for plasminogen was shown in spreading of \textit{M. avium} infection from the lung, as reflected by increased mycobacterial dissemination to the spleen and liver of plasminogen-deficient mice 1 week after induction of infection[19]. We did not find such an effect of PAI-1 on dissemination of \textit{M. tuberculosis} from the lung. Differences in the pathogen used, as well as PAI-1 effects unrelated to plasminogen activation[10] may at least in part explain the different outcomes in this earlier study[19] and the current investigation. In conclusion, the current study shows that PAI-1 contributes to transient, non-specific changes in immunity during early phase of murine tuberculosis. Further studies are warranted to clarify the specific mechanisms that contribute to this effect.
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