Contribution of alveolar macrophages to the response of the TIMP-3 null lung during a septic insult

Erica L. Martin, Tanya A. Sheikh, Kevin J. Leco, James F. Lewis, and Ruud A. W. Veldhuizen

Departments of Physiology and Pharmacology and Medicine, Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada

Submitted 8 November 2006; accepted in final form 16 June 2007

Previous characterization of the TIMP-3 knockout (KO) lung phenotype found chronic air space enlargement, decreased lung function, no compensation of the other TIMPs, and an MMP-TIMP imbalance, thereby modeling the disease of pulmonary emphysema (39). To better understand the role of TIMP-3 in the development and progression of injury and disease, TIMP-3 null mice were exposed to various lung insults (44, 45). After indirect septic insults, TIMP-3 KO lungs developed a rapid increase in lung compliance and altered tissue structure (44, 45). However, when the TIMP-3 KO mice were exposed to direct lung insults (mechanical ventilation or hypoxia), lung compliance was not altered, suggesting that this response was specific to some component of the septic insult (44). Although it was unclear which cell types and molecules were involved in the TIMP-3 null lung’s susceptibility to sepsis, MMPs were believed to be involved in this response to sepsis, since they were increased after septic insults but remained unchanged after direct lung insults (44, 45).

Interestingly, this TIMP-3 null lung response to sepsis displays clinical similarities to exacerbations of COPD, which involve destructive structural alterations and permanently worsened airflow in response to a lung insult (57). Although respiratory infection is the leading cause of COPD exacerbations (57), sepsis is known to activate similar signaling pathways (10, 49, 56) and, therefore, may also be a risk factor for this condition. Since COPD patients experiencing frequent exacerbations deteriorate more rapidly (12), an understanding of the mechanisms underlying this condition has therapeutic value.

Given that TIMP-3 null lungs develop increased compliance selectively after septic insults, this response is thought to involve activation of the inflammatory cascade (40). Several inflammatory cells secrete MMPs during a septic insult (58, 67); however, the inflammatory cell population in the TIMP-3 null lung 6 h after a septic insult consists of >99% macrophages. Furthermore, alveolar macrophages are known to be pivotal to the pathophysiology of COPD, since they are correlated with disease severity and associated with increased compliance (11, 33, 52, 61). This pathophysiology may occur via secretion of various MMPs, cytokines, chemokines, and other proteases from the macrophage (61, 63), many of which can alter lung structure and increase compliance (9, 36, 37, 43, 73).

On the basis of this information, it was hypothesized that the alveolar macrophage is responsible for the increased compliance in the TIMP-3 null lung after a septic insult. In the present study we will test this hypothesis by depleting TIMP-3 null alveolar macrophages.
mice of alveolar macrophages and, subsequently, subjecting them to a septic insult.

**MATERIALS AND METHODS**

**Animal breeding and preparation.** A full description of the generation and breeding of the TIMP-3 null mice was previously reported (39, 45). Young adult (9–12 wk of age) male C57BL6 mice were group housed and had free access to standard rodent chow and water in an automatically controlled facility providing a 12:12-h light-dark cycle. All procedures were approved by the Animal Use Subcommittee of the University of Western Ontario.

**Depletion of alveolar macrophages via liposome administration.** Phosphatidylcholine and cholesterol, in a ratio of 43 mg:4 mg and dissolved in chloroform, were used to form liposomes containing dichloromethylene-diphosphonic acid (DMDP) or PBS, as previously described by van Rooijen and Sanders (68, 69). TIMP-3 wild-type (WT) and KO animals were anesthetized with a 0.45-ml intraperitoneal injection of 7.5 mg of ketamine (Sandoz, Quebec, PQ, Canada) and 0.75 mg of xylazine (Bayer, Toronto, ON, Canada). Mice were randomized to receive 100 μl of 5 mg/ml of DMDP- or PBS-encapsulated liposomes, which were intratracheally instilled using a 30-gauge needle. Researchers were blinded with respect to the experimental groups throughout the experimentation. Animals were then sutured and allowed to recover while being monitored every 12 h. During this time, animals were assigned health scores on the basis of respiration and stress as indicated by appearance, activity, and behavior.

**Induction of the cecal ligation-perforation insult.** At 42 h after instillation of liposomes, which previous studies determined to be a sufficient time for significant depletion of macrophages (2, 20), TIMP-3 WT and KO mice were reanesthetized with ketamine and xylazine (see above). Animals were randomized to cecal ligation and perforation-induced sepsis (CLP) or a laparotomy (sham) surgery. The CLP procedure, as previously described (45), involved a laparotomy for all experimental groups with regard to the specific settings for the perturbations as well as the order of the measurements. After assessment of lung mechanics, mice were split into two cohorts: 1) lavage, inflammatory cell assessment, cytokine/chemokine measurement, and zymography and 2) histology.

**Inflammatory cell assessment.** After the assessment of lung mechanics, two separate 1-ml volumes of 0.15 M NaCl were used to lavage the lungs through the endotracheal tube (1). The saline was instilled and withdrawn from the lung three times, the recovered lavage was combined, and the total volume was recorded. The remaining lung tissue was snap frozen in liquid nitrogen for zymographic analysis. The lavage was centrifuged for 10 min at 400 g for isolation of inflammatory cells within the lavage as previously described (55). The supernatant from this spin of the lavage was snap frozen in liquid nitrogen for cytokine analysis. The pellet, which contained the inflammatory cells, was resuspended in 200 μl of Plasmalyte, and from this resuspended solution, 50 μl were spun onto a cytoslide and stained by HemaColor (EM Science, Gibbstown, NJ) for differential cell analysis. In addition, the resuspended solution was combined with an equal volume of trypan blue stain, and total live cells were counted using a hemocytometer and light microscope.

**Histology and morphometric analysis.** Lungs were removed from the thoracic cavity, inflated with air to a pressure of 25 cmH2O and immediately fixed at this pressure in 10% phosphate-buffered formalin for 24 h. Lungs were rinsed in PBS for 1 h and then stored in 70% ethanol. Lungs were embedded in paraffin and sectioned at 7 μm. For structural analysis, the sections were deparaffinized to water and stained with hematoxylin and eosin. Ten random digital images of the peripheral alveolar structure of each animal were captured using a transmitted light microscope (Carl Zeiss, New York, NY) at a magnification of ×20. Automatic image analysis of lung sections for calculation of surface area, alveolar diameter, and alveolar wall thickness was performed with Northern Eclipse (version 7.0) morphometric analysis software (Empix Imaging, Mississauga, ON, Canada). Briefly, for acquisition of the surface area and alveolar diameter measurements, this software system first identified all tissue through threshold intensity, and then the exterior lung surface, which was present along one side of each photograph, and significant blood vessels were blocked out, resulting in identification of the remaining alveolar air spaces. The software then calculated the average diameter of each air space and
the total perimeter (or surface area) of these alveoli. For alveolar wall thickness, a grid of three horizontal lines was overlaid on the same photographs, and each time the grid crossed an alveolar wall, the thickness was measured. All measurements were performed by investigators blinded to the experimental group, and since each of the 10 photographs normally contain >100 alveoli, >1,000 alveoli were assessed for each animal.

Zymography. Zymography was used to analyze MMPs, as previously described (45). Briefly, frozen, lavaged lungs were homogenized in extraction buffer [50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1% SDS, and 1 EDTA-free Protease Inhibitor Cocktail Pill (Roche Diagnostics, Laval, QC, Canada) for every 10 ml of buffer], and protein content of the homogenate was determined using a Micro BCA protein assay reagent kit (Pierce Chemical, Biolyx, Brockville, ON, Canada). Subsequently, 50 μg of lung homogenate protein were separated by electrophoresis in 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (for analysis of MMP-2 and MMP-9) or 1 mg/ml casein (for analysis of MMP-8 and MMP-7). Gels were washed in 2.5% Triton X-100 solution, rinsed in double-distilled H2O, and then incubated at 37°C in incubation buffer [50 mM Tris–Cl (pH 7.5), 5 mM CaCl2, and 5 μM ZnCl2] for 18–45 h. Gels were stained in Coomassie brilliant blue dye for 60 min (Bio-Rad Laboratories, Hercules, CA) and destained in 30% methanol and 10% acetic acid for 15 min. Gels were analyzed by spot densitometry using an Alpha Innotech Imager 2200 and the AlphaEase image analysis software (Alpha Innotech, San Leandro, CA). Clear resolution of the gelatin zymography allowed for individual quantification of latent and active forms of MMP-2 and MMP-9. However, the lower resolution of casein zymography (a limitation of this analysis) prevented accurate quantitative separation of the latent and active bands, resulting in combined analysis for MMP-8 and MMP-7. Replicate gels containing the same samples were incubated in incubation buffer containing 5 mM EDTA to confirm that the proteolytic activity was due to metalloproteinase activity.

Cytokine/chemokine analysis. The lavage concentrations of 16 cytokines or chemokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, TNF-α, IFN-γ, keratinocyte-derived chemokine (KC), monocyte chemotactic protein-1, granulocyte-macrophage colony stimulating factor, macrophage inflammatory protein-1α, and regulated on activation, normal T cell expressed and secreted] were measured using the Luminex mouse inflammatory cytokine kit (Cedarlane Laboratories, Hornby, ON, Canada) according to the manufacturer’s instructions. Briefly, lavage samples (25 μl) were diluted in assay buffer and plated onto a 96-well filter plate prequenched with assay buffer. The samples were incubated with 25 μl of the antibody-coupled beads selected for the assay on a plate shaker overnight at 4°C. Detection antibody cocktail was added to the wells, and the samples were incubated on a plate shaker for 2 h at room temperature. Streptavidin-phycoerythrin was added to the wells, and the samples were incubated at room temperature for 30 min. The vacuum manifold was used to filter the unbound fraction out of the wells, and the bound beads were washed twice with wash buffer (200 μl/well). Finally, 100 μl of sheath fluid were added to each well, and the plate was placed on a plate shaker for 5 min. The samples were analyzed on the Luminex100 according to the manufacturer’s instructions (RCYTO, Linco Research) (13, 65, 70).

Statistical analysis. Values are means ± SE. Differences were considered statistically significant at P < 0.05. Significance was determined by a three-way ANOVA using genotype, liposome administration, and insult as grouping variables. This analysis determines main effects of each individual variable, as well as interactions between variables. Statistical analysis was performed using the SPSS statistical software package for Windows version 11.0 (SPSS, Chicago, IL).

RESULTS

Eighty-eight male mice were used in this study; there were no significant differences in age (9–12 wk) or body weight (22–24 g) among the groups. Results from health monitoring showed no significant differences after intratracheal instillation of DMDP or PBS liposomes, whereas mice further randomized to CLP had significantly poorer health scores than sham animals; however, this response was not significantly different between WT and KO mice (data not shown).

Inflammatory cells in the lavage. To determine the inflammatory cell populations within the lung and confirm our method of macrophage depletion, we performed total and differential cell counts. Analysis of the total number of macrophages (Fig. 1A) in the lavage revealed a significant decrease in DMDP groups compared with PBS controls. Figure 1B shows the number of polymorphonuclear neutrophils, which was significantly decreased in TIMP-3 KO vs. WT mice.

Respiratory mechanics. Results from the analysis of dynamic lung compliance and resistance are shown in Fig. 2. TIMP-3 KO lungs developed increased dynamic compliance (Fig. 2A) after sepsis; however, after DMDP liposome administration, TIMP-3 KO lungs were protected from this sepsis-
induced compliance alteration. This effect on whole lung compliance was confirmed by three-way ANOVA, which found significant main effects of all three variables (genotype, liposome administration, and insult). There were also significant interactions between genotype and liposomes, genotype and insult, and liposomes and insult. Additionally, there was a significant three-way interaction between genotype, liposome administration, and surgery revealed decreased tissue elastance after CLP in TIMP-3 KO lungs; however, DMDP liposome administration may be protective in regard to this septic alteration.

Histology and morphometric analysis. Typical alveolar structure is shown in Fig. 4, with corresponding morphometric analysis in Table 1. All measurements had a main effect of genotype, where alveolar diameter was significantly greater in TIMP-3 KO mice than in WT controls, and alveolar surface area and alveolar wall thickness were significantly lower in TIMP-3 KO mice than in WT controls. Additionally, alveolar wall thickness also had a main effect of liposome administration.

Fig. 2. Whole lung dynamic compliance (A) and resistance (B), indicating distensibility and level of constriction, respectively. Values are means ± SE of flexiVent measurements of WT groups [PBS sham (n = 10), PBS CLP (n = 13), DMDP sham (n = 12), and DMDP CLP (n = 10)] and KO groups [PBS sham (n = 12), PBS CLP (n = 10), DMDP sham (n = 11), and DMDP CLP (n = 10)]. Statistical significance (P < 0.05) is shown as main effect of genotype (a), main effect of liposome administration (b), main effect of insult [CLP vs. sham (c)], interaction of genotype and liposome administration (d), interaction of genotype and surgery (e), interaction of liposome administration and surgery (f), and interaction of genotype, liposome administration, and surgery (g).

Fig. 3. Central and peripheral lung mechanics: central airway resistance, tissue damping (or tissue resistance), and tissue elastance. Values are means ± SE of flexiVent measurements of WT groups [PBS sham (n = 10), PBS CLP (n = 13), DMDP sham (n = 12), and DMDP CLP (n = 10)] and KO groups [PBS sham (n = 12), PBS CLP (n = 10), DMDP sham (n = 11), and DMDP CLP (n = 10)]. Statistical significance (P < 0.05) is shown as main effect of genotype (a), main effect of liposome administration (b), main effect of insult [CLP vs. sham (c)], interaction of genotype and liposome administration (d), interaction of genotype and surgery (e), interaction of liposome administration and surgery (f), and interaction of genotype, liposome administration, and surgery (g).
MMP analysis. MMPs were assessed by gelatin and casein zymography, and band intensities were quantified by densitometry (Table 2). On the basis of a limitation of the number of samples that can be analyzed on one gel, TIMP-3 WT and KO samples were run on separate gels; however, all samples were standardized to the PBS sham control, and previous studies showed no significant difference in MMPs between TIMP-3 WT and KO control lungs (44, 45). Typical zymograms of WT mice are displayed in Fig. 5; results from zymograms of TIMP-3 KO mice were identical.

After gelatin zymography analysis, we observed that the abundance of both forms of MMP-2 (72 kDa) and the latent form of MMP-9 (105 kDa) were not significantly different between groups. The active form of MMP-9, however, was significantly increased in CLP groups compared with sham groups. Results from casein zymography analysis showed that MMP-7 (28 kDa) and MMP-8 (75 kDa) abundance was significantly increased in CLP groups compared with sham groups. Furthermore, there was a significant interaction for MMP-7 between liposome administration and surgery: MMP-7 was increased to a lesser extent after DMDP than after PBS liposome administration.

Fig. 4. Histological changes in overall gross lung alveolar structure in the 8 experimental groups described in Fig. 1 legend (n = 4). Images were obtained from fixed paraffin-embedded 7-μm lung sections stained with hematoxylin and eosin. Representative images show increased air spaces in KO mice compared with WT animals. This air space enlargement is enhanced by CLP in PBS-liposome-treated, but not in DMDP-treated, KO animals. All images were obtained from the lung periphery without knowledge of experimental group at ×20 magnification. Scale bar, 250 μm.
Table 1. Morphometric analysis of alveolar structure

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CLP</th>
<th>PBS</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT KO</td>
<td>WT KO</td>
<td>WT KO</td>
<td>WT KO</td>
</tr>
<tr>
<td>Diameter, μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>179.9±3.5</td>
<td>191.4±4.8a</td>
<td>172.0±8.4</td>
<td>226.1±23.4</td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>167.6±15.0</td>
<td>191.6±15.7b</td>
<td>170.7±4.8</td>
<td>195.4±9.9b</td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wall thickness, μm</td>
<td>18.3±1.4</td>
<td>16.7±0.3a</td>
<td>18.9±0.7</td>
<td>16.1±0.1e</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>24.5±2.3</td>
<td>22.3±1.2a</td>
<td>26.2±3.1</td>
<td>18.7±1.7b</td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>22.9±2.7b</td>
<td>16.9±0.8ab</td>
<td>20.0±0.6b</td>
<td>18.1±0.6ab</td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 4 in each group). DMDP, dichloromethylene-diphosphonic acid; CLP, cecal ligation and perforation; WT, wild type; KO, knockout. Statistical significance (P < 0.05) is as follows: main effect of genotype [KO vs. WT (*)] and main effect of liposome administration [DMDP vs. PBS (†)].

Cytokine/chemokine analysis. The lavage concentration of 16 inflammatory cytokines and chemokines was analyzed. To determine whether any of these molecules are mechanistically involved in the TIMP-3 KO lung’s altered response to sepsis, the concentrations of these molecules were examined in relation to the whole lung compliance data. This was accomplished by a linear regression correlation analysis of the cytokine/chemokine concentrations compared with compliance in each of the 24 TIMP-3 KO lungs that were lavaged, producing r² values to quantify the degree of correlation (Fig. 6). From this analysis, it was determined that none of the 16 cytokines/chemokines showed any correlation to changes in compliance of the TIMP-3 KO lung, since all r² values were <0.4. Figure 7 displays the lavage concentration values for three of these molecules, IL-6, KC, and TNF-α, which had a similar expression pattern. Statistically, IL-6 and KC had significant main effects of liposome administration and insult, both resulting in increased cytokine concentration, as well as a significant interaction between liposome administration and insult, showing a synergistic increase in these cytokines. Although TNF-α concentrations showed a similar synergistic pattern (Fig. 6C), it was not statistically significant.

DISCUSSION

The TIMP-3 KO mouse is an established in vivo model of MMP-TIMP imbalance that leads to progressive air space enlargement similar to the pathology of emphysema (39, 45). TIMP-3 null mice developed a rapid increase in compliance after a septic insult (44, 45), which may reflect aspects of the irreversible alterations to lung structure and function that patients with emphysema can develop in response to various lung insults (57). The increase in compliance was accompanied by significantly decreased collagen and fibronectin levels but no changes to the pulmonary surfactant system, indicating that a rapid degradation of extracellular matrix components due to MMP activity was, in part, responsible for the effect (44, 45). To further examine this phenomenon and since 1) the altered response of the TIMP-3 KO lung was specific to a septic insult, 2) alveolar macrophages are activated by the inflammatory cascade during sepsis (30, 34, 62), and 3) the main inflammatory cell found in the lungs of this model are alveolar macrophages, the main focus of this study was to determine whether depletion of alveolar macrophages in the TIMP-3 KO lung before the septic insult could prevent the alterations in pulmonary compliance.

The technique used to deplete macrophages was established by van Rooijen and Sanders (68, 69) and is highly specific to macrophages due to 1) the high phagocytic action of macrophages toward liposomes, 2) the short (~15-min) half-life of DMDP, and 3) the inability of free DMDP to cross cell membranes, limiting its effect on other cell types and quarantining it within the lung.

Examination of respiratory mechanics showed that depletion of alveolar macrophages completely eliminated the sepsis-induced compliance increase in the TIMP-3 KO lungs, thereby supporting our original hypothesis that the alveolar macrophage is required for this response. In addition to compliance, this study explored other respiratory mechanics to elucidate the mechanisms responsible for the TIMP-3 KO response to sepsis. Although central airway resistance and peripheral tissue damping may influence the initial null phenotype, they were not affected by sepsis. The peripheral tissue elastance mea-

Table 2. MMP levels as analyzed by gelatin and casein zymography

<table>
<thead>
<tr>
<th>MMP</th>
<th>PBS</th>
<th>CLP</th>
<th>DMDP</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Latent</td>
<td>1±0.04</td>
<td>1±0.06</td>
<td>1.13±0.15</td>
<td>0.98±0.15</td>
</tr>
<tr>
<td>Active</td>
<td>1±0.14</td>
<td>1±0.04</td>
<td>0.96±0.08</td>
<td>1.28±0.18</td>
</tr>
<tr>
<td>MMP-7</td>
<td>1±0.15</td>
<td>1±0.08</td>
<td>2.48±0.60ab</td>
<td>2.17±0.28b</td>
</tr>
<tr>
<td>MMP-8</td>
<td>1±0.09</td>
<td>1±0.05</td>
<td>1.36±0.17c</td>
<td>1.41±0.21c</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1±0.04</td>
<td>1±0.04</td>
<td>1.32±0.21</td>
<td>1.11±0.07</td>
</tr>
<tr>
<td>Active</td>
<td>1±0.10</td>
<td>1±0.09</td>
<td>1.85±0.38b</td>
<td>1.80±0.62b</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6 in each group). Data are presented as relative values compared with PBS sham control group within each genotype. MMP, matrix metalloproteinase. Statistical significance (P < 0.05) is as follows: main effect of insult [CLP vs. sham (*)] and interaction of liposome administration and surgery (†).
sured using the constant-phase model was consistent with compliance measured using the single-compartment model, since this was decreased in the lungs of TIMP-3 KO mice after sepsis, yet it was maintained if lungs were depleted of alveolar macrophages. It is acknowledged that there are some limitations when the constant-phase model is used to assess central and peripheral lung mechanics, including a tendency to under-estimate central airway resistance and/or overestimate peripheral tissue damping (64). However, since our results show an increase in central airway resistance (which may be underestimated) and a decrease in peripheral tissue damping (which may be overestimated), this limitation of the constant-phase model would reduce the magnitude of the changes observed in our experiments. Furthermore, Thamrin et al. (64) reported that an alteration in tissue damping can be clouded by an alteration in central airway resistance; however, they also showed that a corresponding change in tissue elastance, as seen in our data, supports the validity of the change in tissue damping. The alterations in alveolar morphometry, including increased alveolar diameter and decreased alveolar surface area and wall thickness, showed trends similar to the lung mechanics, which supports the more sensitive findings of lung compliance and elastance. These histologically based measurements reflect the static properties of the lung and suggest that the dynamic alteration of compliance is the result of structural modifications. However, although fixation of lung tissue at a set pressure was utilized to histologically visualize alterations in lung compliance and that all tissues appeared adequately fixed during sectioning, a limitation of this external fixation tech-

![Fig. 5](image-url) Typical zymogram of samples from WT mice quantified in Table 2. MMP, matrix metalloproteinase. Molecular sizes are as follows: 72 kDa (MMP-2), 28 kDa (MMP-7), 75 kDa (MMP-8), and 105 kDa (MMP-9).

![Fig. 6](image-url) Correlation analysis of tissue inhibitor of metalloproteinase-3 (TIMP-3) KO lungs. Dynamic lung compliance and cytokine/chemokine concentrations in lavage in 24 TIMP-3 KO mice described in Fig. 1 legend were compared using linear regression analysis. KC, keratinocyte-derived chemokine; GMCSF, granulocyte-macrophage colony stimulating factor; MIP-1α, macrophage inflammatory protein-1α; MCP-1, macrophage chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed and secreted.
The technique is the variation of wall thickness, which could result in uneven fixative penetration and, thereby, uneven fixation.

On the basis of the lung mechanics measurements, it was concluded that the alveolar macrophage is essential for the sepsis-induced increased compliance in the TIMP-3 KO lung and that this phenomenon occurs through a mechanism that specifically alters peripheral tissue elastance. This implied role of the macrophage is based on the significant decrease in macrophage cell number in the lung when the macrophage depletion model is used. Depletion was confirmed in our study, which showed an 88% decrease in alveolar macrophages in lung lavage of TIMP-3 KO and WT mice 48 h after DMDP administration, which is comparable to other studies in the literature (2, 14). A limitation of this model, however, is that alterations in lung homeostasis due to side effects of macrophage depletion may contribute to these findings. For example, this technique has recently been observed to increase levels of pulmonary surfactant in the lung (18), which could potentially alter lung function and inflammation (15, 24, 41). However, because 1) DMDP treatment alone does not alter any of our measured lung mechanics, histology, or inflammatory markers and 2) increased surfactant levels by surfactant administration does not increase compliance above normal values (55, 74), it is unlikely that this side effect of DMDP administration plays a primary role in the TIMP-3 KO response to sepsis.

The secondary focus of this study was to assess MMPs, cytokines, and chemokines to potentially further our understanding of how the macrophage induces physiological changes in compliance. Various MMPs were investigated on the basis of the knowledge that 1) macrophages secrete various MMPs during an inflammatory insult (17, 22, 50), 2) the increased compliance in the TIMP-3 KO lung after sepsis is believed to result from altered lung structure (45), 3) excessive MMP activity can alter lung structure, resulting in an emphysema-like phenotype (8, 9, 17, 19), and 4) MMPs are less inhibited in the TIMP-3 KO lung, resulting in an MMP-TIMP imbalance (39, 45). MMP-2, MMP-7, MMP-8, and MMP-9 were analyzed using zymography, and consistent with previous findings, MMP-7, MMP-8, and MMP-9 were increased in CLP groups compared with sham groups (44, 45). Because of the absence of regulation by TIMP-3, it was previously thought that the increased abundance of these MMPs was the major cause of the sepsis-induced compliance alteration in the TIMP-3 KO lung (45). Nevertheless, the results of this study indicate that MMP-2, MMP-8, and MMP-9 are not involved in the sepsis-induced compliance alterations of the TIMP-3 KO lung. Among the MMPs measured, only the increase in MMP-7 during sepsis was attenuated after macrophage depletion, which suggests that MMP-7 may be involved in the development of altered compliance of the TIMP-3 KO lung after sepsis. This potential role of MMP-7 is supported by other studies. It has been shown that macrophage cells directly produce MMP-7 (16, 21, 25) and that this expression can be stimulated after LPS exposure (4). Macrophages may also activate lung epithelial cells, leading to increased MMP-7 production in response to bacterial exposure (42). Furthermore, MMP-7 produced from macrophages has previously been implicated in the development of acquired pulmonary emphysema, which is characterized by altered lung structure and increased compliance (7). Despite this evidence, our results in the TIMP-3 KO animals showed that macrophage depletion caused only an attenuation of MMP-7 levels after CLP, not a complete reduction to baseline values. In contrast, the increase in compliance observed in the KO animals after CLP was completely eliminated by alveolar macrophage depletion, suggesting that it is unlikely that MMP-7 is solely responsible for the increased compliance after sepsis. In this regard, it is important to realize that only 4 of 26 members of the MMP family were assessed in our study, and it remains likely that other MMPs contribute to an MMP-TIMP imbalance that is involved in this process. For example, MMP-12, which is produced primarily by mouse macrophages (32) and is a known contributor to emphysema phenotypes (28, 29, 36, 46, 47), may also contribute to the septic response and, therefore, should be investigated in future studies.

During an inflammatory injury such as sepsis, macrophages secrete many cytokines and chemokines that are involved in...
MECHANISM OF THE TIMP-3 NULL MOUSE RESPONSE TO SEPSIS

L787

the propagation of the inflammatory cascade (23, 34). Overexpression of several of these molecules, including IL-1, IL-13, TNF-α, and IFN-γ, has been shown to induce an increase in lung compliance (36, 37, 43, 73). Therefore, a large panel of inflammatory cytokines/chemokines was analyzed in lung lavage in an attempt to identify any molecules that may contribute to the mechanism involved in the CLP-induced compliance alterations of the TIMP-3 KO lung. As expected, many of these cytokines were increased in response to the CLP-induced septic insult, which confirmed our inflammatory model (only data for IL-6 and KC are shown). However, none of the lavage concentrations of these molecules correlated with the alteration of TIMP-3 KO lung compliance; therefore, they do not appear to have a causative role in this response. Interestingly, a few cytokines, namely, IL-6, KC, and TNF-α, appeared to be reduced in lavage concentrations after CLP-induced sepsis in TIMP-3 KO vs. WT mice. Although these changes did not reach statistical significance, this difference between genotypes may be related to the fact that TIMP-3 KO mice had significantly fewer neutrophils than WT controls. Furthermore, previous studies have shown that macrophages are involved in the downregulation of neutrophil infiltration and inflammation (54, 66). Specifically, Elder et al. (14) found that alveolar macrophages play an important suppressive role in the pulmonary inflammatory response to an LPS injury. Similarly, in this study, WT mice have a significantly greater lung inflammatory response to a CLP-induced septic insult in the absence of alveolar macrophages; however, it is believed that since the TIMP-3 KO lung has significantly fewer neutrophils, they are protected from this response.

The clinical implication of this study is that since the TIMP-3 KO mice have a lung phenotype similar to that of emphysema, emphysemic patients may be susceptible to a sepsis-induced injury possibly mimicking the symptoms of an exacerbation. Furthermore, since our results show that the macrophage is essential for the susceptibility of TIMP-3 KO mice to sepsis and in COPD patients macrophage numbers correlate with severity of disease (11), the macrophage may also play a significant role in the response of COPD patients to insults and their resulting exacerbations. This concept is supported by a recent review by Vlahos et al. (71) that implicated a role for granulocyte-macrophage colony stimulating factor, a major regulator of macrophages, in the development and treatment of COPD exacerbations.

In conclusion, the present study demonstrates that the alveolar macrophage is required for the increased compliance observed in the TIMP-3 KO lung after a septic insult. Furthermore, we have shown that although the initial TIMP-3 KO phenotype involves alterations to central and peripheral lung mechanics, the altered response of these mice to sepsis only involves alterations to peripheral tissue. Although the specific enzymes and mediators through which the alveolar macrophage influences pulmonary compliance in TIMP-3 KO mice during sepsis remains unclear, the results of this study indicate that MMP-2, MMP-8, and MMP-9 and the major inflammatory cytokines/chemokines are not involved, whereas MMP-7 may play a partial role in this process.

ACKNOWLEDGMENTS

The authors thank Dr. Li-Juan Yao, Lynda McCaig, Cynthia Pape, and Pamela Joseph for technical assistance, Dr. Martin Post’s laboratory for assistance with the Luminex cytokine assay, Kalamo Farley for teaching the DMDP liposome technique, and Dr. Yves Bureau for statistical consultation. Present address of E. L. Martin: Università di Torino, Dipartimento di Discipline Medico Chirurgiche, Sezione di Anestesiologia e Rianimazione, Ospedale S. Giovanni Battista, Turin 10126, Italy.

GRANTS

This work was funded by Ontario Thoracic Society Grant RO361A10 (to R. A. W. Veldhuizen) and Canadian Institutes of Health Research Grant MOP-42556 (to R. A. W. Veldhuizen). Salary support for E. L. Martin was provided in part by an Ontario Graduate Scholarship.

REFERENCES

L788  MECHANISM OF THE TIMP-3 NULL MOUSE RESPONSE TO SEPSIS


