Targeting CD162 protects against streptococcal M1 protein-evoked neutrophil recruitment and lung injury

Songen Zhang,1 Lei Song,1 Yongzhi Wang,1 Heiko Herwald,2 and Henrik Thorlacius4
1Department of Clinical Sciences, Malmö, Section of Surgery, Skåne University Hospital and Lund University, Sweden; and 2Department of Clinical Sciences, Lund, Section for Clinical and Experimental Infection Medicine, Skåne University Hospital and Lund University, Sweden

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Zhang S, Song L, Wang Y, Herwald H, Thorlacius H. Targeting CD162 protects against streptococcal M1 protein-evoked neutrophil recruitment and lung injury. Am J Physiol Lung Cell Mol Physiol 305: L756–L763, 2013. First published September 13, 2013; doi:10.1152/ajplung.00220.2013.—Streptococcus pyogenes of the M1 serotype can cause streptococcal toxic shock syndrome and acute lung damage. CD162 is an adhesion molecule that has been reported to mediate neutrophil recruitment in acute inflammatory reactions. In this study, the purpose was to investigate the role of CD162 in M1 protein-provoked lung injury. Male C57BL/6 mice were treated with monoclonal antibody directed against CD162 or a control antibody before M1 protein challenge. Edema, neutrophil infiltration, and CXC chemokines were determined in the lung, 4 h after M1 protein administration. Fluorescence intravitral microscopy was used to analyze leukocyte-endothelium interactions in the pulmonary microcirculation. Inhibition of CD162 reduced M1 protein-provoked accumulation of neutrophils, edema, and CXC chemokine formation in the lung by >54%. Moreover, immunoneutralization of CD162 abolished leukocyte rolling and firm adhesion in pulmonary venules of M1 protein-treated animals. In addition, inhibition of CD162 decreased M1 protein-induced capillary trapping of leukocytes in the lung microvasculature and improved microvascular perfusion in the lungs of M1 protein-treated animals. Our findings suggest that CD162 plays an important role in M1 protein-induced lung damage by regulating leukocyte rolling in pulmonary venules. Consequently, inhibition of CD162 ameliorates M1 protein-evoked leukocyte adhesion and extravasation in the lung. Thus, our results suggest that targeting the CD162 might pave the way for novel opportunities to protect against pulmonary damage in streptococcal infections.

Address for reprint requests and other correspondence: H. Thorlacius, Dept. of Clinical Sciences, Malmö, Section of Surgery, Skåne Univ. Hospital, Lund Univ., 205 02 Malmö, Sweden (e-mail: henrik.thorlacius@med.lu.se).

Clinical manifestations of Streptococcus pyogenes infections vary from uncomplicated cases to severe and fatal conditions, such as streptococcal toxic shock syndrome (STSS), which is associated with a mortality exceeding 50% (7, 15). Despite significant investigative efforts, management of patients with STSS is largely restricted to antibiotics and supportive care, which is partly due to an incomplete understanding of the basic pathophysiology in STSS. S. pyogenes express several different virulence factors, including M proteins, of which there are more than 80 different serotypes described in the literature (15, 26). The M1 serotype of Streptococcus pyogenes is most commonly linked to STSS (7). Numerous studies have documented that M1 protein is a powerful stimulator of innate immune cells, such as monocytes (26) and neutrophils (15). In addition, the M1 protein has the capacity to induce formation of cytokines (26), chemokines (9), and tissue factor (25), which all contribute to M1 protein-induced edema formation and tissue damage in the lung. Several studies have shown that lung failure is an insidious feature in STSS patients (37, 38). Excessive accumulation infiltration of neutrophils is a rate-limiting step in septic lung damage (11, 17). For example, it has been shown that depletion of neutrophils abolishes M1 protein-induced neutrophil lung damage (35, 41).

Extravascular localization of neutrophils is coordinated by secreted CXC chemokines, including KC (CXCL1) and MIP-2 (CXCL2) (45). A recent study demonstrated that M1 protein-provoked pulmonary accumulation of neutrophils is critically dependent on the synthesis and action of CXC chemokines (44). Thus, the chemokine-mediated mechanisms behind accumulation of neutrophils in the lung are relatively well known, whereas the adhesive mechanisms regulating M1 protein-induced neutrophil recruitment and tissue damage in the lung remain elusive. It is widely held that leukocyte recruitment is a multistep process in venules initiated by a rolling adhesive interaction followed by firm adhesion and transendothelial migration in tissues, such as the colon (31), liver (19), pancreas (13), brain (3), and striated muscle (21). However, the recruitment process of leukocytes in the lung appears to be more complex, involving both venules and capillaries. Under homeostatic conditions, most neutrophils, which have a diameter larger than that of pulmonary capillaries, must deform to pass through the pulmonary microcirculation (23, 40). Upon activation, neutrophil stiffness increases, promoting mechanical sequestration of neutrophils in lung capillaries (20, 34). It is widely held that leukocyte rolling is mediated by the selectin family of adhesion molecules, including P-, E- and L-selectins although their relative roles appear to be both tissue- and stimulus-dependent (2, 28). Selectin ligands are complex glycoproteins and, in terms of selectin specificity and function, are poorly understood. Nonetheless, CD162 is a well-accepted high-affinity ligand of P-selectin in spite of the fact that CD162 also binds to E-selectin (24, 47). Convincing studies have shown that inhibition of CD162 effectively decreases leukocyte recruitment in different models of inflammation (1, 8, 22). However, the role of CD162 in mediating M1 protein-induced leukocyte-endothelial cell interactions in the pulmonary microcirculation is not known.

Based on the considerations above, we hypothesized that CD162 might be involved in the pathophysiology of streptococcal lung injury. Thus, the purpose of this study was to define the detailed role of CD162 in M1 protein-induced neutrophil recruitment and acute lung injury.
MATERIALS AND METHODS

Animals. Male C57BL/6 mice, weighing 20–25 g, were used for experiments and kept under standard laboratory conditions, maintained on a 12:12-h light-dark cycle, and fed a laboratory diet and water ad libitum. Animals were anesthetized with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body wt. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University (Sweden).

Experimental model. M1 protein was purified from the MC25 strain (derived from the AP1 S. pyogenes strain 40/58 from the World Health Organization Collaborating Center for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic) as described previously (6). M1 protein was purified from a mutated S. pyogenes strain, making the likelihood of endotoxin contamination close to zero. Nevertheless, we also measured the endotoxin content in the M1 protein samples and confirmed that endotoxin levels were below the detection limit (1 ng/ml). Mice were injected intravenously with 15 μg of M1 protein in PBS. To examine the role of CD162 in leukocyte recruitment, mice were pretreated intravenously 10 min before M1 protein challenge with an anti-CD162 antibody (1.5 mg/kg, clone 2PH1, rat IgG1; BD Biosciences Pharmingen, San Jose, CA) or an isotype-matched control antibody (1.5 mg/kg, clone R3–34, rat IgG1; BD Biosciences Pharmingen). Sham mice received PBS only, and M1 protein-treated animals that received PBS only served as positive controls. Animals were anesthetized 4 h after M1 protein challenge. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) to quantify neutrophils. Next, the lung was excised, one lobe was fixed in formaldehyde for histology, and the remaining lung tissue was snap-frozen in liquid nitrogen and stored at −80°C for later MPO assays and ELISA, as described subsequently.

Systemic leukocyte count. Blood was collected from the tail vein and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid; 6.25% vol/vol) in a 1:20 dilution. Leukocytes were identified as polymorphonuclear leukocytes (PMNL) and mononuclear leukocytes (MNL) in a Burker chamber.

Lung edema. The left lung was excised, washed in PBS, gently dried using a blotting paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet weight to dry weight was used as an indicator of lung edema formation.

MPO activity. Lung tissue was thawed and homogenized in 1 ml 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed, after which, the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (450 nm; with a reference filter, 580 nm emission) at 25°C). Values were expressed as the MPO activity of the supernatant per gram tissue.

Fluorescence intravital microscopy. In separate animals, leukocyte-endothelium interactions and perfusion were examined in the pulmonary microcirculation by use of fluorescence intravital microscopy as described previously (29). Briefly, the right diaphragm was incised to create a right-sided pneumothorax under transient lowering of the stroke volume to 100 μl. A parasternal thoracotomy was performed up to the level of the fourth intercostal space to avert the right thorax wall to the side. During the preparation, great care was taken not to manipulate the lung tissue directly, and the lung surface was rinsed intermittently by saline (37°C). A micromanipulator was used to fix a cover slip horizontally on the surface of right lung. Horizontal movements of the lung tissue could be minimized by modulating a positive end-expiratory pressure between 5 and 7 cmH2O and adjusting stroke volume (minimum: 150 μl) and stroke frequency (minimum: 100 strokes/min) by use of a ventilator (Mini-vent type 845; Hugo Sachs Elektronik-Harvard Apparatus, March-Hustetten, Germany). Immediately after surgical preparation, mice were put on the microscopic stage. Fluorescence intravital microscopy was performed after intravenous injection of 0.1 ml 0.1% rhodamine 6G (Sigma-Aldrich, Taufkirchen, Germany) for direct staining of leukocytes and 0.1 ml 5% FITC-dextran (mol wt 150,000; contrast enhancement; Sigma Chemical) for representing the background. The subpleural pulmonary microvasculature was visualized by means of a modified Olympus microscope (BX50WI; Olympus Optical, Hamburg, Germany) equipped with a 100-W mercury lamp and filter sets for blue (450–490 nm excitation and >520 nm emission wavelength) and green (530–560 nm excitation; >580 nm emission) light epillumination. Microscopic images were televised by using a charge-coupled device video camera and recorded digitally. With this setup, all parts of the subpleural pulmonary microvasculature, i.e., arterioles, venules, and capillaries, could be identified. For measurements, five venules and capillaries were randomly selected in each animal. Leukocyte rolling was determined by counting the number of such cells passing a reference point in venule per 20 s and expressed as cells per minute. Firm adhesion was measured by counting the number of cells adhering to 100-μm-long venular segment for at least 20 s. Leukocyte trapping in capillaries was determined in five regions of interest. Functional capillary density served as a measurement of microvascular perfusion and was defined as the length of all red blood cell-perfused nutritive capillaries per observation area and is given in centimeters per square centimeter.

ELISA. Levels of CXCL1 and CXCL2 in lung homogenates were analyzed by using double-antibody Quantikine ELISA kits (R&D Systems, Europe, Abingdon, Oxon, UK) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm. Each cDNA was synthesized by reverse transcription from 10 μg of total RNA by use of the StrataScript First-Strand Synthesis System and random hexamer primers (Stratagene, AH diagnostics, Stockholm, Sweden). Real-time PCR was

Fig. 1. Edema formation in the lung. Mice were treated with an anti-CD162 antibody (Anti-CD162 Ab, 2PH1) or an isotype-matched control antibody (Control Ab, R3–34) 10 min before M1 protein injection. Sham mice received PBS iv only, and M1 protein-treated animals that received PBS only served as positive controls. Data represent means ± SE. *P < 0.05 vs. Sham and #P < 0.05 vs. Control Ab + M1 protein, n = 5.
performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of CXCL1, CXCL2, and β-actin were as follows: CXCL1 (forward) 5'-GCT TCC TCG GGC ACT CCA GAC-3', CXCL1 (reverse) 5'-TTA GCC TTG CCT TGG TTT AGT AT -3'; CXCL2 (forward) 5'-GCC AAT GAG CTC CGC TGT CAA TGC -3', CXCL2 (reverse) 5'-CTT GGG GAC ACC TTT TAG CAT CTT -3'; and β-actin (forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3', β-actin (reverse) 5'-TCT CCA GGG AGG AAG AGG AT-3'. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 μl, containing 25 μl of SYBRgreen PCR master mix, 2 μl of 0.15 μM each primer, 0.75 μl of reference dye, and one 1 μl cDNA as a template adjusted up to 50 μl with water. PCR reactions were started with 10 min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min) and 1 min of elongation at 72°C. The relative differences in expression between groups were expressed by using cycling time values. Cycling time values for the specific target genes were first normalized with that of β-actin in the same sample, and then relative differences between groups were expressed as percentage of control.

Histology. Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six-micrometer sections were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a preexisting scoring system as described (14), including size of alveolar collapse, thickness of alveolar septum, alveolar fibrin deposition, and neutrophil infiltration graded on a 0 (absent) to 4 (extensive) scale. In each tissue sample, five random areas were scored, and mean value was calculated. The histology score is the sum of all four parameters.

Statistics. Data are presented as mean values ± SE. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on ranks, followed by multiple comparisons vs. the control group (Dunnett’s method). P < 0.05 was considered significant, and n represents the number of animals.

RESULTS

Lung edema and injury. M1 protein challenge triggered lung injury, indicated by a clear-cut increase in pulmonary edema formation (Fig. 1). Thus, the lung wet-to-dry ratio increased...
from 4.5 ± 0.05 to 5.2 ± 0.02 in response to M1 protein (Fig. 1). Pretreatment with the anti-CD162 antibody reduced the lung wet-to-dry ratio to 4.8 ± 0.05 in mice exposed to the M1 protein (Fig. 1). Thus, inhibition of CD162 attenuated M1 protein-evoked lung edema by >57%. Moreover, morphological examination revealed normal tissue structure in lungs from sham-operated mice (Fig. 2A), whereas M1 protein caused massive destruction of the lung microarchitecture, characterized by edema of the interstitial tissue, capillary congestion, and infiltration of neutrophils (Fig. 2B). Immunoneutralization of CD162 decreased M1 protein-triggered changes of the tissue architecture and neutrophil accumulation in the lung (Fig. 2D). Quantification of the morphological injury revealed that M1 protein significantly increased the lung damage score and that inhibition of CD162 function decreased the lung damage score by 63% in animals exposed to the M1 protein (Fig. 2E).

**Neutrophil recruitment.** Challenge with M1 protein increased levels of MPO by 8.5-fold in the lung (Fig. 3A). Administration of the anti-CD162 antibody reduced M1 protein-evoked pulmonary MPO activity by 58% (Fig. 3A). Analysis of BALF revealed a dramatic enhancement in the number of neutrophils, 4 h after challenge with M1 protein (Fig. 3B). Immunoneutralization of CD162 reduced M1 protein-induced pulmonary infiltration of neutrophils (Fig. 3B). Thus, pretreatment with the anti-CD162 antibody decreased the number of pulmonary neutrophils from 95.2 ± 4.6 × 10^7 to 48.0 ± 2.8 × 10^7, corresponding to a 63% reduction, 4 h after M1 protein administration (Fig. 3B).

**Leukocyte-endothelium interactions.** To study the detailed role of CD162 in M1 protein-provoked leukocyte-endothelial cell interactions in the pulmonary microvasculature, we used a recently developed method to study the lung microcirculation by use of fluorescence intravital microscopy (29). It was found that M1 protein challenge triggered a clear-cut increase in leukocyte-endothelial cell interactions in venules and capillaries of the pulmonary microvasculature (Fig. 4). Administration of the anti-CD162 antibody abolished M1 protein-induced venular leukocyte rolling (Fig. 4A) and reduced firm adhesion by 98% (Fig. 4B). Notably, we also found that inhibition of CD162 significantly decreased capillary trapping of leukocytes by 98% in the lungs of animals challenged with M1 protein (Fig. 4C). In addition, we observed that M1 protein significantly decreased functional capillary density in the lung microcirculation and that immunoneutralization of CD162 significantly improved functional capillary density in the lungs of septic animals (Fig. 4D). We observed that challenge with M1 protein decreased levels of PMNLs and MNLs in the circulation (Table 1). This M1 protein-induced leukopenia was attenuated by inhibition of CD162 function (Table 1).

**CXC chemokines.** CXC chemokines are known to regulate neutrophil trafficking in the lung. Pulmonary levels of CXC chemokines in sham animals were low but detectable (Fig. 5). We observed that M1 protein administration enhanced pulmonary levels of CXCL1 and CXCL2 by 42- and 124-fold, respectively (Fig. 5, A and B). Pretreatment with the anti-CD162 antibody reduced M1 protein-induced formation of CXCL1 from 92.8 to 43.5 ng/g tissue in the lung (Fig. 5A). Moreover, immunoneutralization of CD162 decreased M1 protein-evoked pulmonary levels of CXCL2 from 48.2 to 15.5 ng/g tissue (Fig. 5B). Thus, inhibition of CD162 attenuated CXCL1 and CXCL2 formation in the lung of M1 protein-treated animals by >54% (Fig. 5, A and B). We next isolated alveolar macrophages from the BALF in animals challenged with M1 protein. M1 protein challenge markedly increased gene expression of CXCL1 and CXCL2 in alveolar macrophages (Fig. 5, A and B). We observed that inhibition of CD162 significantly attenuated M1 protein-induced mRNA levels of CXCL1 and CXCL2 in alveolar macrophages (Fig. 5, C and D).

**DISCUSSION**

Treatment of patients with STSS is a major challenge for clinicians, which is related to an incomplete understanding of the mechanisms behind streptococcal-induced acute lung injury. In the present study, we demonstrate that CD162 plays an important role in mediating M1 protein-induced lung damage. It was found that CD162 supports neutrophil recruitment in pulmonary microvasculature and subsequent accumulation in the bronchoalveolar space. Thus, targeting CD162 function might be a useful way to protect against lung injury caused by streptococcal infections.
The M1 serotype of *S. pyogenes* is frequently associated with STSS and high mortality (7, 15). During infection, *S. pyogenes* shed M1 protein from their surface, which subsequently triggers activation of neutrophils and monocytes (15, 25, 26). Although M1 protein challenge is not identical to an infection with whole bacteria, M1 protein is a dominant virulence factor on *S. pyogenes*, and the M1 serotype of these bacteria is most frequently associated with STSS (7). Acute lung damage is a key feature in STSS, and accumulating data suggest that activation of neutrophils is a critical component in the onset of the M1 protein-provoked lung injury (7, 35, 37, 41). Herein, we show for the first time that targeting CD162 decreases edema formation and tissue injury in the lungs of mice exposed to M1 protein, suggesting that CD162 plays a key role in regulating acute lung damage in streptococcal infections. Although this study provides the first direct data indicating a role of CD162 in streptococcal lung injury, Asaduzzaman et al. (4) reported that CD162 attenuates lung damage in a model of abdominal peritonitis, supporting the concept that CD162 is an important molecule exerting proinflammatory effects in the lung. Knowing that lung accumulation of neutrophils is a central feature in M1 protein-induced lung damage (26, 35), it was of interest to examine the impact of CD162 inhibition on pulmonary neutrophilia. We found that inhibition of CD162 decreased the M1 protein-evoked increase in MPO levels in the lung by 58%, suggesting that CD162...
function plays a significant role in mediating pulmonary accumulation of neutrophils in streptococcal infections. This inhibitory effect on MPO activity correlated well with the decrease in the number of neutrophils in the alveolar space (63%) of M1 protein-treated mice observed after immunoneutralization of CD162. Considering the intimate relationship between neutrophil infiltration and lung damage (12), it is suggested that the CD162. According to a newly developed model of intravital fluorescence microscopy of the lung microcirculation, which has the advantage that the different steps in the leukocyte extravasation process can be visualized and quantified in a temporal and spatial manner in vivo (29).

In general, recruitment of leukocytes is a multistep process mediated by sequential interactions between leukocytes and endothelial cells in the microvasculature (27).

Endothelial cell activation and surface upregulation of selectins are necessary parts in the localization of leukocytes at sites of tissue injury (2). Indeed, numerous studies have shown that P-, E-, and L-selectin support early interactions between leukocytes and endothelial cells at sites of inflammation (13, 19, 21, 28). Selectins bind to complex carbohydrates expressed on specific glycoproteins. The importance of the selectin ligands is illustrated by the leukocyte adhesion deficiency syndrome II, in which patients lack proper formation of selectin ligands and suffer from life-threatening episodes of recurrent infections (32, 36). The best-characterized selectin ligand is CD162, which binds to both P- and E-selectin (24). However, the detailed roles of these specific adhesion molecules in mediating leukocyte-endothelium interactions in the pulmonary microvasculature remain elusive. We used a newly developed model of intravital fluorescence microscopy of the lung microcirculation, which has the advantage that the different steps in the leukocyte extravasation process can be visualized and quantified in a temporal and spatial manner in vivo (29).

Fig. 5. CXC chemokine production in the lung. Mice were treated with an anti-CD162 antibody (2PH1, 1.5 mg/kg) or an isotype-matched control antibody (R3-34, 1.5 mg/kg) 10 min before M1 protein injection. Sham mice received PBS only, and M1 protein-treated animals that received PBS only served as positive controls. ELISA was used to quantify CXCL1 (A) and CXCL2 (B) levels in the lung tissue. Gene expression of CXCL1 (C) and CXCL2 (D) in alveolar macrophages was determined 30 min after M1 protein injection. Levels of CXCL1 and CXCL2 mRNA were normalized to mRNA levels of β-actin. Data represent means ± SE. *P < 0.05 vs. Sham and #P < 0.05 vs. Control Ab + M1 protein, n = 5.

Table 1. Systemic leukocyte differential counts

<table>
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<tr>
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<th>MNL</th>
<th>PMNL</th>
<th>Total</th>
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<tbody>
<tr>
<td>Sham</td>
<td>5.8 ± 0.9</td>
<td>1.9 ± 0.2</td>
<td>7.7 ± 1.1</td>
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<tr>
<td>Vehicle + M1 protein</td>
<td>1.5 ± 0.2*</td>
<td>0.4 ± 0.0*</td>
<td>2.0 ± 0.3*</td>
</tr>
<tr>
<td>Control Ab + M1 protein</td>
<td>1.6 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
<td>2.4 ± 0.2*</td>
</tr>
<tr>
<td>Anti-CD162 Ab+</td>
<td>5.2 ± 0.3#</td>
<td>2.0 ± 0.2#</td>
<td>7.2 ± 0.3#</td>
</tr>
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</table>

Data represent means ± SE (10⁶ cells/ml); n = 5 experiments. Blood was collected from sham animals receiving PBS iv only as well as mice treated iv with vehicle (PBS) or an isotype-matched control antibody (Control Ab, 1.5 mg/kg) or an anti-CD162 antibody (Anti-CD162 Ab, 1.5 mg/kg) 10 min before M1 protein challenge for 4 h. Cells were identified as monomorphonuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL). *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + M1 protein.
capillaries (10), it is not very likely that CD162 supports direct adhesion to endothelial cells in the capillaries. One explanation might be that M1 protein triggers aggregate formation between circulating leukocytes and platelets. Leukocyte-platelet complexes are larger in size and have been suggested to be susceptible for size-restricted microvascular trapping (18). Nonetheless, this issue on leukocyte-platelet aggregate formation and capillary leukocyte trapping requires further studies. One hallmark of septic lung injury is a decrease in microvascular perfusion (39). Herein, we found that M1 protein challenge caused a significant reduction in microvascular perfusion in the lung, which was markedly improved in mice pretreated with the antibody directed against CD162. The relationship between CD162-mediated leukocyte recruitment on one hand and microvascular perfusion on the other hand is not clearly established at present but might be related to the lung protective effect exerted by reducing inflammatory tissue injury. For example, tissue edema and endothelial cell swelling associated with organ injury appear to be significant components behind microvascular compression and decreased organ perfusion (16).

Orchestration of neutrophil trafficking in the extravascular space is coordinated by secreted CXC chemokines, including CXCL1 and CXCL2, which are the murine homologs of human interleukin-8 (33). A functional role of CXC chemokines has been proposed in streptococcal infections (44), and we have demonstrated that M1 protein is a potent stimulator of CXCL1 and CXCL2 production in the lung (41). Herein, we found that inhibition of CD162 reduced M1 protein-induced formation of CXCL1 and CXCL2 in the lung. This observation is somewhat surprising considering that lung CXC chemokines are secreted by tissue resident cells, such as epithelial cells and alveolar macrophages (30, 42, 43). We therefore next analyzed CXC chemokine formation in alveolar macrophages. As expected, it was found that M1 protein markedly increased CXCL1 and CXCL2 mRNA levels in alveolar macrophages. Notably, immunoneutralization of CD162 markedly decreased M1 protein-provoked gene expression of CXCL1 and CXCL2 in alveolar macrophages. Taken together, these findings suggest that CD162-dependent neutrophil infiltration regulates subsequent production of CXC chemokines in alveolar macrophages in streptococcal infections. The association between neutrophil infiltration and CXC chemokine formation in alveolar macrophages is speculative but might be related to proinflammatory compounds released from infiltrating neutrophils, which, in turn, activate tissue-resident cells in the lung. Nonetheless, this reduction of CXC chemokine formation in the lung might help to further reduce neutrophil accumulation in the lung when targeting CD162 function in M1 protein-provoked lung injury.

We conclude that CD162 is a dominant adhesion molecule in supporting leukocyte rolling and adhesion in the lung microvasculature and that inhibition of CD162-mediated adhesive interactions abolishes bronchoalveolar accumulation of leukocytes in M1 protein-induced lung injury. Thus, our novel data suggest that CD162 may be a useful target to protect respiratory function in streptococcal infections.

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DISCLOSURES
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
Author contributions: S.Z., L.S., and Y.W. performed experiments; S.Z., L.S., and Y.W. analyzed data; S.Z and L.S. interpreted results of experiments; S.Z. prepared figures; S.Z., L.S., Y.W., and H.T. drafted manuscript; S.Z., Y.W., H.H., and H.T. edited and revised manuscript; S.Z. and H.T. approved final version of manuscript; H.H. and H.T. conception and design of research.

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