Anchoring Fusion Thrombomodulin to the Endothelial Lumen Protects against Injury-induced Lung Thrombosis and Inflammation

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Rationale: Endothelial thrombomodulin (TM) regulates thrombosis and inflammation. Diverse forms of pulmonary and vascular injury are accompanied by down-regulation of TM, which aggravates tissue injury. We postulated that anchoring TM to the endothelial surface would restore its protective functions.

Objectives: To design an effective and safe strategy to treat pulmonary thrombotic and inflammatory injury.

Methods: We synthesized a fusion protein, designated scFv/TM, by linking the extracellular domain of mouse TM to a single-chain variable fragment of an antibody to platelet endothelial cell adhesion molecule-1 (PECAM-1). The targeting and protective functions of scFv/TM were tested in mouse models of lung ischemia-reperfusion and acute lung injury (ALI) caused by intratracheal endotoxin and hyperoxia, both of which caused approximately 50% reduction in the endogenous expression of TM.

Measurements and Main Results: Biochemical assays showed that scFv/TM accelerated protein C activation by thrombin and bound mouse PECAM-1 and cytokine high mobility group-B1. After intravenous injection, scFv/TM preferentially accumulated in the mouse pulmonary vasculature. In a lung model of ischemia-reperfusion injury, scFv/TM attenuated elevation of early growth response-1, inhibited pulmonary deposition of fibrin and leukocyte infiltration, and preserved blood oxygenation more effectively than soluble TM. In an ALI model, scFv/TM, but not soluble TM, suppressed activation of nuclear factor- κ B, inflammation and edema in the lung and reduced mortality without causing hemorrhage.

Conclusions: Targeting TM to the endothelium using an scFv anchor enhances its antithrombotic and antiinflammatory effectiveness in models of ALI.

Keywords: vascular targeting; acute lung injury; PECAM-1; protein C; pulmonary endothelium

The thrombomodulin (TM)–protein C system helps control coagulation and inflammation (*see* Video E1 in the online supplement) (1, 2). TM, a transmembrane protein located on

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Thrombomodulin (TM) is a pivotal endothelial molecule that helps maintain tissue homeostasis and prevents tissue injury. However, TM expression and activity are suppressed in disorders such as acute lung injury (ALI), aggravating thrombosis, inflammation, and tissue injury.

What This Study Adds to the Field

Endothelial anchoring of a recombinant TM fusion construct targeted to platelet-endothelial cell adhesion molecule-1 attenuated tissue damage in mouse models of lung ischemia-reperfusion and ALI to a greater extent than nontargeted soluble TM, without causing bleeding. Vascular targeting of TM may provide a novel approach to prevent or treat various forms of ALI.

the endothelial lumen, binds thrombin through its epidermal growth factor (EGF)–like domains and modulates its substrate specificity to generate activated protein C (APC) (2), which has tissue-protective antithrombotic and antiinflammatory activities (3–6). TM also binds cytokines through the lectin-like domain, thereby inhibiting the proinflammatory effect of activating nuclear factor (NF)- κ B in vascular cells (7, 8).

Pulmonary and vascular pathologies, including oxidative stress, inflammation, and ischemia-reperfusion (I/R) (e.g., vascular bypass and organ transplantation) suppress TM expression or activity (*see* Video E1) (9–15), which correlates with increased thrombotic and inflammatory injury to underlying tissue (11, 16). In animal studies, a deficit in TM expression or function results in a greater propensity to develop thrombosis and inflammation (2, 7).

Intravascular administration of recombinant APC has shown encouraging results in these injury settings (2–5), but its rapid elimination from the circulation and side effects (including bleeding) limit dosing and efficacy (Video E1) (17). Moreover, APC exerts only some of the protective features of the TM/APC pathway (1, 2). Soluble TM (sTM) may offer safer multifaceted effects (18), but it is also rapidly eliminated from blood (16). Furthermore, spatial separation of APC and sTM from key endothelial auxiliary proteins (e.g., endothelial protein C receptor [EPCR]) limits their activity (19, 20). We thus postulated that replenishing TM by anchoring it to endothelial luminal surfaces might circumvent these limitations (Video E1). The antithrombotic effect of TM transfected into rabbit artery supports this concept (21), but this approach cannot be applied in acute settings. In theory, it may be possible to achieve the

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same goal by anchoring TM to platelet endothelial cell adhesion molecule-1 (PECAM-1), a noninternalizable determinant stably expressed by endothelium (22). After intravenous injection, therapeutic proteins conjugated to PECAM-1 antibodies bind to endothelium, where they accumulate, remain, and express their activity on the pulmonary vasculature (23, 24).

To avoid any Fc fragment-mediated side effects and unwanted internalization of PECAM-1 that might be induced by multivalent antibody conjugates (25), we have previously generated fusion proteins consisting of a monovalent single-chain variable fragment of a PECAM-1 antibody fused with urokinase-plasminogen activator (scFv/uPA) that accumulates along the pulmonary vasculature and provides local fibrinolysis (26, 27). This prototype supports the concept of vascular targeting, but does not provide antiinflammatory activity. We hypothesized that endothelial anchoring of TM in a similar manner would mitigate both inflammation and thrombosis after lung injury (Video E1).

To test this hypothesis and develop a biotherapeutic prodrug with clinical potential, we generated a novel fusion protein composed of anti-PECAM-1 scFv fused with the extracellular domain of mouse TM (Leu¹⁷-Ser⁵¹⁷) (designated scFv/TM) intended to retain the activities of both constituents and preferentially accumulate on the mouse pulmonary vasculature. We then tested the effects of scFv/TM in mouse models of lung injury caused by ischemia-reperfusion (I/R) and intratracheal

endotoxin injection combined with hyperoxia (endotoxin/hyperoxia).

METHODS

An expanded methods description can be found in the online supplement. Chemicals were from Sigma (St. Louis, MO) unless specified otherwise.

Production and Characterization of scFv/TM

The extracellular domain of TM (Leu¹⁷-Ser⁵¹⁷) was amplified from mouse lung cDNA. sTM and scFv/TM were constructed with a triple-FLAG tag at the 3' end and purified by anti-FLAG affinity (26). APC activation was assayed by incubating scFv/TM or sTM with thrombin and protein C and measuring the optical density (OD_{405nm}) with Spectrozyme PCa (American Diagnostica, Inc., Stamford, CT). Binding of scFv/TM to PECAM-1 was measured by ELISA using an anti-TM antibody (27).

Organ Distribution of scFv/TM and sTM

C57BL/6 mice were used following National Institutes of Health guidelines. Anesthetized mice were injected intravenously with scFv/TM or sTM. Anti-FLAG immunoblotting was used to detect their levels in tissue homogenates. Cryostat sections were made from the lungs and stained with anti-FLAG, anti-VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-E-cadherin (BD Biosciences,

Figure 1. Molecular design and biochemical properties of single-chain variable fragment of thrombomodulin (scFv/TM). (A) Molecular design of scFv/TM. The extracellular domain of mouse TM was fused with the anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) scFv using the modular technique previously described (26). (B) Homogeneity and size of scFv/TM. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showing the migration of purified scFv/TM and soluble thrombomodulin (sTM). Reduction with dithiothreitol caused an upward shift in migration, confirming the disruption of the compact secondary structure dependent on disulfide bonding. (C) Activation of protein C by scFv/TM. scFv/TM showed comparable protein C-activating cofactor ability to sTM. All experiments were performed in triplicate unless otherwise specified. (D) scFv/TM binds mouse PECAM-1, revealed by anti-TM ELISA. (E) Activation of protein C by PECAM-associated scFv/TM. PECAM-coated wells preincubated with scFv/TM, but not sTM, generated activated protein C (APC) activity on addition of thrombin. Dashed line represents APC activity generated from the wells incubated with phosphate-buffered saline (PBS)-bovine serum albumin (BSA). **P* < 0.05 versus sTM.

San Jose, CA) antibody. Plasma APC activity in mice injected with scFv/TM or sTM was measured as described (20).

Effects of scFv/TM in Lung I/R

Left lung I/R, protein extraction, and detection of TM and fibrin were performed as described (27). Thirty minutes before I/R, mice were injected intravenously with 50 μ g scFv/TM, the same amount of sTM, or phosphate-buffered saline (PBS). Fibrin deposition and nuclear expression of early growth response-1 (Egr-1), lung myeloperoxidase activity, and arterial oxygen tension were measured (27).

Binding of scFv/TM to High Mobility Group-B1

Binding of scFv/TM to high mobility group-B1 (HMGB1) was detected using an anti-FLAG ELISA. A fusion uPA containing the same scFv (scFv/uPA-T) (27) served as a negative control. Binding of HMGB1 by scFv/TM and sTM were compared by immunoprecipitation–Western Blot.

Effects of scFv/TM in Acute Lung Injury

Acute lung injury (ALI) was induced by intratracheal injection of lipopolysaccharide (LPS, 8 mg/kg), followed by a 5- or 18-hour exposure to hyperoxia (98% O₂) and the levels of TM, HMGB1, and fibrin in lung tissue were measured. To assess protection, mice were injected intravenously with 50 μ g scFv/TM, the same amount of sTM,

or PBS at indicated time points. Bronchoalveolar lavage fluid (BALF) was obtained, and the levels of macrophage-inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), and interleukin-6 (IL-6) were measured by ELISA (R&D systems, Minneapolis, MN). Pulmonary expression of I κ B α , intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 were detected by immunoblotting. Frozen lung sections were stained to measure expression of granulocyte antigen Gr-1 and VCAM-1. To detect activation of NF- κ B, lung nuclear extracts were analyzed by electrophoretic mobility shift assay (Panomics, Fremont, CA). Lung wet/dry ratios were measured (24). In other experiments, Evans blue dye was injected intravenously before killing, extracted from lung homogenates, and quantified. To examine survival, mice were injected with 20 mg/kg LPS and exposed to hyperoxia for 84 hours. scFv/TM (50 μ g), the same amount of sTM, or PBS was injected 5 hours after injury and every 24 hours thereafter.

Tail Bleeding Time

Bleeding times were measured 60 minutes after mice were injected with 50 μ g scFv/TM or 20 μ g APC (28).

Data Analysis

All data are presented as mean $\pm$ SEM. Statistical differences between groups were tested using analysis of variance.

Figure 2. Biodistribution of single-chain variable fragment of thrombomodulin (scFv/TM). (A) Organ distribution of intravenous scFv/TM and soluble thrombomodulin (sTM) detected by anti-FLAG immunoblot. scFv/TM accumulated preferentially in the mouse lungs, whereas sTM uptake in the lungs was undetectable. Immunoblot for actin shown below confirms comparable protein loading. Each lane represents a sample from one mouse here and throughout. (B) Endothelial targeting of scFv/TM augments activated protein C (APC) generation. Activated protein C (APC) level in plasma collected 1 hour after thrombin injection was elevated in mice preinjected with scFv/TM. (C) Endothelial localization of scFv/TM in the pulmonary vasculature. Immunostaining for FLAG tag confirmed enhanced lung uptake of scFv/TM compared with sTM (left column). Dual staining for the endothelial marker VE-cadherin or epithelial marker E-cadherin (middle column) revealed the localization of scFv/TM on endothelium (right column). Magnification, $\times 40$; insets, $\times 100$.

RESULTS

Molecular Design and Biochemical Properties of scFv/TM

We fused the extracellular portion of cDNAs encoding TM with the anti-PECAM-1 scFv, to produce the scFv/TM cDNA construct (Figure 1A). The C-terminus of TM was tagged with triple-FLAG peptide (DYKDHDGDYKDHDIDYKDDDDK) for purification and detection. Purified scFv/TM and sTM migrated as predicted at 84 kD and 56 kD under nonreduced conditions, respectively, and exhibited the expected slight upward shift in mobility on reduction (Figure 1B). scFv/TM facilitated protein C activation in a thrombin-dependent manner to the same extent as sTM (Figure 1C). scFv/TM, but not sTM, bound to mouse PECAM-1 (Figure 1D), and PECAM-associated scFv/TM generated APC in the presence of thrombin (Figure 1E).

In Vivo Biodistribution of scFv/TM in Mice

After intravenous injection, scFv/TM, but not sTM, accumulated preferentially in the pulmonary vasculature relative to

other organs (Figure 2A). Approximately 35% of the injected dose of scFv/TM accumulated per gram of lung, which is similar to the accumulation of other anti-PECAM-1 conjugates (23, 24, 26, 27) in this highly vascularized organ (29). After injection of thrombin, APC generation in plasma of mice pretreated with scFv/TM was increased by almost an order of magnitude more than those given sTM (Figure 2B). This outcome may reflect the more favorable pharmacokinetics of scFv/TM caused by prolonged retention in the vascular lumen and/or its localization in proximity to EPCR (20). Dual-label immunostaining with VE-cadherin and E-cadherin showed that scFv/TM, but not sTM, bound to the pulmonary endothelium, but not the epithelium, after IV injection (Figure 2C).

Anticoagulant and Antiinflammatory Effects of scFv/TM in Lung I/R

We then studied the anticoagulant and antiinflammatory effects of scFv/TM in a mouse model of lung I/R injury, which simulates the pathophysiology after transplantation or cardiopulmonary bypass surgery (30–32). Thrombin generated during

Figure 3. Single-chain variable fragment of thrombomodulin (scFv/TM) prevents lung fibrin deposition and inflammation caused by ischemia-reperfusion (I/R). (A) Lung I/R reduces endogenous TM and increases fibrin deposition. Anti-TM and anti-fibrin immunoblots (upper panel) showed suppression of endogenous TM and increased fibrin deposition in I/R-damaged lungs. The lower bar graph presents the percentage of TM in the I/R lung compared with sham-operated mice (* $P < 0.05$). (B) Treatment schedule to assess the effects of scFv/TM in lung I/R. (C) Effects of scFv/TM and soluble thrombomodulin (sTM) on fibrin deposition in I/R-damaged lung. Lung fibrin deposition was quantified, as previously described (27). Representative immunoblots are shown. Attenuation in fibrin deposition is indicated. scFv/TM reduced fibrin deposition by 80% (* $P < 0.05$; $n = 6$). (D) scFv/TM reduces up-regulation of proinflammatory early growth response (Egr)-1 protein. Lung nuclear Egr-1 expression was detected by immunoblot. Stable factor SP1 served to normalize for loading. Representative immunoblots are shown. scFv/TM significantly ameliorated up-regulation of Egr-1 induced by I/R compared with sTM and phosphate-buffered saline (PBS) (* $P < 0.05$ vs. PBS; * $P < 0.05$ vs. sTM; $n = 6$). (E) scFv/TM inhibits leukocyte infiltration. scFv/TM decreased lung myeloperoxidase activity stimulated by I/R more effectively than sTM (* $P < 0.05$; $n = 5-6$). (F) scFv/TM improves lung gas exchange. Arterial blood oxygen tension in mice was compared ($n = 3-6$). The dashed line represents the PaO₂ in sham-operated animals. scFv/TM preserved oxygenation more efficiently than sTM (* $P < 0.05$ vs. PBS; * $P < 0.05$ vs. sTM).

I/R causes thrombosis and inflammation (32–38). We detected reduced expression of TM and a concomitant increase in the deposition of fibrin in lungs after I/R (Figure 3A). Injection of scFv/TM, but not sTM, 30 minutes before I/R (Figure 3B) reduced fibrin deposition in the lung by approximately 70% (Figure 3C).

In agreement with previous studies, I/R elevated the expression of Egr-1, a key transcription factor in the inflammatory response (Figure 3D) (31). scFv/TM interfered with inflammation-induced accumulation of nuclear Egr-1 (Figure 3D) and the leukocyte marker myeloperoxidase after I/R more effectively than sTM (Figure 3E) and preserved arterial oxygen pressure to a greater extent (Figure 3F).

LPS/Hyperoxia Suppresses TM Expression and Induces Acute Inflammatory Lung Injury

To further test the antiinflammatory profile of scFv/TM, we used a model of ALI initiated by intratracheal injection of LPS followed by continuous inhalation of 98% O₂ (hyperoxia, imitating the clinical setting of ventilation support) (39). LPS/hyperoxia suppressed endogenous TM in the lungs (Figure 4A) to the extent seen in the I/R model (~60% and 50%, respectively), although only modest fibrin deposition in the lungs was observed (*see* Figure E1A in the online supplement). In contrast, pulmonary expression of HMGB1, an inflammatory cytokine that is released by necrotic and immune cells and contributes to lethality in sepsis and ALI (4, 40, 41), was elevated in mice exposed to LPS/hyperoxia (Figure 4B), but not in those

exposed to I/R (Figure E1B). It has been reported that TM binds and neutralizes HMGB1 (8). Consistent with this finding, scFv/TM binds HMGB1 in a dose-dependent manner (Figure 4C). Immunoprecipitation–Western blot confirmed comparable binding of HMGB1 to scFv/TM and sTM (Figure 4C, *inset*), suggesting a mechanism by which scFv/TM may exert antiinflammatory effects.

Antiinflammatory Effect of scFv/TM in LPS/Hyperoxia

A kinetic analysis was performed to study the phosphorylation and degradation of I κ B α , a key step in the activation of NF- κ B (Figure E1C). Significant degradation of I κ B α and elevation of HMGB1 was seen in BALF 5 hours after LPS injection. Therefore, we chose this time point to evaluate protection of scFv/TM against I κ B α degradation. scFv/TM or sTM was given intravenously 1 hour after initiation of LPS/hyperoxia (Figure 5A). scFv/TM, but not sTM, significantly inhibited I κ B α degradation (Figure 5B) and reduced the levels of chemokines MIP-2 and KC, and cytokine IL-6 in the BALF (Figures 5C, 5D, and 5E).

In other experiments, scFv/TM or sTM was given intravenously at various times before or after initiation of the LPS/hyperoxia, and various parameters of inflammation were analyzed 18 hours after the onset of injury (Figure 6A). scFv/TM, but not sTM, injected either before or after the insult, blunted the increase in lung myeloperoxidase (Figure 6B). Immunostaining for the granulocyte antigen Gr-1 with either peroxidase or fluorescein isothiocyanate (FITC)-conjugated antibodies

Figure 4. (A and B) Mouse acute inflammatory lung injury. (A) Mice given an intratracheal injection of lipopolysaccharide (LPS) followed by exposure to 98% O₂ (LPS/hyperoxia) showed marked reduction in lung thrombomodulin (TM) and (B) an increase in cytokine high mobility group-B1 (HMGB1) (immunoblots are shown in Figure E1; **P* < 0.05 vs. sham group). (C) Interaction between single-chain variable fragment of thrombomodulin (scFv/TM) and HMGB1. scFv/TM bound HMGB1, whereas a thrombin-activated urokinase-plasminogen activator (uPA) fused with the same scFv moiety (scFv/uPA-T) (26) did not. Immunoprecipitation–Western blot confirmed the similar binding of scFv/TM and sTM to HMGB1 (*inset*).

Figure 5. Single-chain variable fragment of thrombomodulin (scFv/TM) attenuates activation of proinflammatory genes. (A) Overview of the experimental design. scFv/TM, soluble thrombomodulin (sTM), or phosphate-buffered saline (PBS) was administered intravenously (drug injection) 1 hour post insult (lipopolysaccharide [LPS] intratracheal injection). (B) Effects of scFv/TM on IκBα expression in the inflamed lungs. scFv/TM preserved approximately 80% of total IκB protein compared with sham group, which was significantly higher than in the cohort given sTM (* $P < 0.05$, $n = 4$). Representative immunoblot is shown (upper panel), and each lane represents results from one animal. (C–E) scFv/TM inhibits elevation of inflammatory cytokines and chemokines in bronchoalveolar lavage fluid (BAL). scFv/TM significantly attenuated the elevation of macrophage-inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), and IL-6 in BAL compared with PBS-treated mice challenged with LPS/hyperoxia. Dashed line represents expression in sham-group mice (* $P < 0.05$, $n = 5–6$).

showed that scFv/TM injected after initiation of the insult was more effective than sTM in reducing neutrophil accumulation in the pulmonary tissue (Figure 6C).

The proinflammatory cell adhesion molecules ICAM-1 and VCAM-1 were expressed in the lungs of LPS/hyperoxia-challenged mice (Figures 7A and 7B; Figure E2) (42). Immunostaining confirmed that scFv/TM injected after the injury inhibited VCAM-1 expression (Figure 7C). Furthermore, scFv/TM, but not sTM, injected before or after insult inhibited NF-κB activation by approximately 70% compared with unprotected animals (Figure 7D), thereby blocking one of the primary inflammatory pathways involved in ALI and sepsis (43). Pre-injection of even higher amounts of the control construct scFv/uPA-T induced little if any additional suppression of pulmonary NF-κB and myeloperoxidase in this model (Figure E3), indicating that it is unlikely that these antiinflammatory effects of scFv/TM are due to inhibition of thrombosis or blockage of PECAM-1.

scFv/TM Attenuates Edema in the LPS/Hyperoxia Lungs and Reduces Mortality Without Bleeding Complications

To explore the therapeutic effects of scFv/TM, we focused on the postinjury group (Figure 6A). By assessing both the lung

wet/dry ratio and extravasation of Evans blue (Figures 8A and 8B), we found that scFv/TM decreased pulmonary edema and vascular permeability, pathological hallmarks of ALI (43). scFv/TM also attenuated development of apoptosis in the LPS/hyperoxia-injured lung (Figure E4) and improved the survival of LPS/hyperoxia-challenged mice, in contrast to sTM (Figure 8C).

Bleeding times were not prolonged in mice injected with scFv/TM at a dose effective in the I/R and LPS/hyperoxia models (Figure 8D). In contrast, injection of APC at a dose that blunted pulmonary edema comparably to scFv/TM (Figure E5) caused a significant prolongation in bleeding times (Figure 8D).

DISCUSSION

Acute lung injury remains a major cause of morbidity and mortality, but as yet there is no intervention that improves the prognosis in the majority of patients. Loss of intravascular TM activity is characteristic of ALI and some success has been seen using APC in specific circumstances. However, the ability to modulate the TM/APC pathway in a sustained way by infusing APC (or sTM) is inherently limited by unfavorable pharmacokinetics, which require that high systemic levels of drug be given

Figure 6. Single-chain variable fragment of thrombomodulin (scFv/TM) reduces neutrophil lung infiltration. (A) Experimental design. scFv/TM, soluble thrombomodulin (sTM) or phosphate-buffered saline was administered intravenously (drug injection) at the two indicated time points: 30 minutes before or 1 hour after insult (intratracheal injection of lipopolysaccharide [LPS]) followed by injection 5 hours after the insult. (B) Effects of scFv/TM on myeloperoxidase (MPO) levels in the inflamed lungs. Injection of scFv/TM either before or after lung injury attenuated the increase in lung MPO activity to a significantly greater extent than sTM ($*P < 0.05$, $n = 5$). (C) Effect of scFv/TM on neutrophil infiltration revealed by expression of granulocyte antigen Gr-1 detected by immunostaining. Secondary antibody was labeled with peroxidase (upper panel) or fluorescein isothiocyanate (lower panel), respectively. Compared with the sham group, neutrophil infiltration induced by LPS/hyperoxia was attenuated more effectively by scFv/TM than by sTM. Magnification, $\times 20$.

to affect lung function with the attendant risk of complications, such as bleeding. TM fused with a tissue factor antibody has potent antithrombotic activity in a rat model (44). However, tissue factor, which is not expressed on undamaged vasculature, is not suitable for prophylaxis, and its rapid disappearance once the provocation for its expression has passed limits its utility as a target to prolong the therapeutic window of antithrombotic drugs.

Because expression of surface TM is commonly decreased in lung injury, we hypothesized that anchoring exogenous TM to the luminal surface of the endothelium might increase and prolong expression at sites of injury. To achieve this goal, we developed a fusion protein scFv/TM that targets the luminal surface of endothelium via PECAM-1, mediates thrombin-dependent activation of protein C, and binds the cytokine HMGB1. scFv/TM accumulates preferentially in the lungs, which contain approximately 30% of the total endothelial surface in the body and receive 100% of venous cardiac output, consistent with preferential pulmonary targeting of other PECAM-1 targeted fusions (26, 27). In contrast, sTM at concentrations equipotent in terms of thrombin-mediated protein C activation (Figure 1C) and binding of HMGB1 (Figure 4C), but

lacking affinity for PECAM-1, did not accumulate preferentially in the lungs and was less effective in animal models of pulmonary injury caused by thrombosis or inflammation. This indicates that anchoring and retention of scFv/TM on the endothelial lumen contributes to the enhanced efficacy of scFv/TM compared with sTM in our animal models. Both prolonged intravascular retention of scFv/TM and its localization in close proximity to key endothelial cofactors (e.g., EPCR) likely contribute to enhance its efficacy. Although scFv/TM and sTM generate equivalent amounts of APC in response to thrombin *in vitro*, mice pre-treated with the targeted protein generate an order of magnitude more APC in response to thrombin *in vivo*, suggesting that endothelial targeting avoids both rapid elimination and promotes proximity of TM to EPCR and other potential vascular cofactors (Figure 2B).

In a lung model of I/R, characterized by downregulation of TM along with activation of coagulation and inflammation (30–33, 35), scFv/TM was superior to sTM in reducing fibrin deposition and cytokine production and provided greater protection of lung function. scFv/TM also inhibited elevation of Egr-1, a pivotal participant in I/R-mediated lung injury (31, 33, 45).

Figure 7. (A and B) Single-chain variable fragment of thrombomodulin (scFv/TM) attenuates expression of inflammatory cell adhesion molecules. Administration of scFv/TM reduced lung (A) ICAM-1 and (B) VCAM-1 expression (representative immunoblots are shown in Figure E2) more effectively than soluble thrombomodulin (sTM) (** $P < 0.01$; * $P < 0.05$, $n = 5-6$). (C) Reduction of VCAM-1 activation by scFv/TM shown by immunostaining. Secondary antibody was labeled with peroxidase (upper panel) or fluorescein isothiocyanate (lower panel), respectively. Magnification, $\times 20$. (D) Inhibition of nuclear factor- κ B (NF- κ B) activation by scFv/TM. NF- κ B activation in the lipopolysaccharide /hyperoxia lungs was assayed by electrophoretic mobility shift assay and representative results are shown in the panels on the left. Ten μ g of input nuclear extract was used, and each lane represents results from one animal. Activation was diminished by scFv/TM to a significantly greater extent than by sTM (** $P < 0.01$ vs. sTM, $n = 6$).

ALI, the leading cause of morbidity and mortality in sepsis, is caused by uncontrolled inflammatory responses that may lead to severe edema and hypoxemia (4, 43). Mechanical ventilation and supplemental oxygenation used in the treatments frequently exacerbate lung inflammation and dysfunction (39). Intratracheal injection of LPS causes an ALI-like pattern in animals (39, 42). We used LPS/hyperoxia model to explore the antiinflammatory properties of scFv/TM in a setting of inflammatory lung injury during oxygen therapy. In this model, scFv/TM markedly attenuated lung leukocyte infiltration, reduced NF- κ B activation (Figure 7), and suppressed expression of the cell adhesion molecules, ICAM-1 and VCAM-1, implicated in leukocyte infiltration in ALI (42).

Although there is extensive evidence indicating cross-talk between coagulation and inflammation (34, 37, 46), the involvement of thrombin and signaling through protease-activated receptor (PAR) in LPS-mediated inflammation has not been fully delineated (47-49). Neither pharmacological inhibition of thrombin nor the genetic deletion of thrombin receptors (PAR-1 and -4) fully protect against LPS-induced injury (48). Mice with impaired generation of APC do not exhibit enhanced pulmonary inflammation after intratracheal injection of LPS (49). Furthermore, upregulation of Egr-1 contributes minimally to LPS-induced

injury (31). These facts, along with the absence of fibrin deposition, suggest that the antiinflammatory functions of scFv/TM in the LPS/hyperoxia model are not mediated primarily by neutralizing thrombin. Rather, the increased expression of HMGB1 in the injured lungs suggest that the antiinflammatory effect of scFv/TM may be due to other effects, for example neutralization of HMGB1-mediated inflammatory responses (7, 8, 41, 50). Thus, vascular-anchored scFv/TM may provide a novel approach to inhibiting cytokine-mediated tissue injury.

Together these data demonstrate that anchoring scFv/TM to endothelial PECAM-1 protects against lung injuries mediated by both thrombotic and inflammatory insults that characterize lung transplantation, cardiopulmonary bypass, and ALI. Importantly, from a clinical perspective, administration of scFv/TM was effective, even after lung injury had been initiated. In these animal models, the prodrug and endothelial targeting properties of scFv/TM avoided the need to achieve high systemic levels of APC and may thereby reduce the risk of hemorrhagic complications associated with its use (Figure 8D, Figure E5) (17). Restoration of endothelial protective pathways by vascular targeting of TM provides a new approach to prevent vascular injury in these and, perhaps, other common and serious clinical settings.

Figure 8. (A and B) Prevention of lung edema by single-chain variable fragment of thrombomodulin (scFv/TM). scFv/TM injected after injury attenuates (A) the increased lung wet-to-dry ratio ($*P < 0.05$, $n = 6$) and (B) the pathological elevation of lung vascular permeability revealed by diminished extravasation of Evans blue ($*P < 0.05$, $**P < 0.01$, $n = 5-6$). Dashed lines show levels in sham-operated animals. (C) scFv improves survival in acute lung injury. The 84-hour survival rate was monitored. Fifty percent of mice survived 84 hours after scFv/TM compared with 48 hours for 50% survival in the phosphate buffered saline (PBS)-treated group ($n = 10$). The statistical analysis using Kaplan-Meier test showed that scFv/TM significantly delayed the mortality ($P < 0.05$ vs. PBS-treated mice), whereas soluble thrombomodulin treatment failed to provide significant protection. (D) scFv/TM does not prolong the tail bleeding time. Injection of 50 μ g scFv/TM caused little increase in bleeding times, in contrast to 20 μ g of activated protein C (APC) ($*P < 0.05$). Determination of the APC dose that inhibited edema to the same extent as scFv/TM is presented in Figure E5.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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