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Phase-directed therapy: TSG-6 targeted to early inflammation improves bleomycin-injured lungs

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Bleomycins are a family of antibiotics that are currently used in the treatment of cancer and high rate of mortality (16, 40). Up to 46% of bleomycin-treated patients develop some form of lung toxicity leading to death in 3% of treated patients (43). Bleomycin exerts its toxic effects by intercalating into double-stranded DNA to degrade the DNA and trigger the intrinsic apoptotic pathway (41). Within the initial 48 h after administration, bleomycin produces damage/necrosis of the alveolar epithelium, capillary congestion, perivascular permeability leading to edema, and the formation of hyaline membranes (10, 11, 46). Concomitantly, there is an increase in inflammatory cell infiltrates in the bronchoalveolar lavage fluid (BALF) (17, 46). There is also a marked influx of immune cells such as lymphocytes during this acute phase (11, 46). As the injury progresses, pulmonary fibrosis develops with an excessive deposition of extracellular matrix deposition in the lung interstitium (15, 17, 44). In the long term, there is compromised lung function with impaired transfer of oxygen and carbon dioxide gases.

Several reports demonstrated that bleomycin-induced injury of lungs in mice can be improved by administration of multi-potent adult stem/progenitor cells (MSCs). Ortiz et al. (30, 31) demonstrated that murine bone marrow-derived MSCs decreased inflammation and collagen deposition in lung by expression of IL-1R antagonist. Rojas et al. (39) reported that the murine bone marrow-derived MSCs localized to lung and assumed the phenotypes of lung cells, but they also suppressed inflammation and triggered production of growth factors. In other models of acute lung injury, MSCs were shown to suppress proinflammatory cytokines, edema, and the influx of neutrophils (18, 22). They improved clearance of bacteria (9, 23). The beneficial effects of MSCs were largely explained by the ability of the cells to modulate immune and inflammatory reactions via several different mechanisms (32–34). Similar beneficial effects of MSCs were also observed in other disease models, such as sepsis (27), myocardial infarction (13, 19, 25), and acute kidney injury (47, 48).

Accordingly, a common theme in recent reports is that MSCs produce their beneficial effects by being activated by signals from injured tissues to express genes that modulate inflammatory and immune responses (18, 32). Among the anti-inflammatory factors MSCs are activated to express is TNF-α stimulated gene/protein-6 (TSG-6) (19). TSG-6 was first discovered in the early 1990s and was shown to have multiple anti-inflammatory effects (24, 50). It cross-links pro-
inflammatory fragments of hyaluronan (2) and catalytically transfers a heavy chain from inter-α-inhibitor to hyaluronan, thereby inhibiting the cascade of proteases released by inflammation (2, 26). It also inhibits transport of leukocytes through endothelial cells (3). Administration of recombinant TSG-6 was shown to reproduce most of the beneficial effects of MSC administration in models of myocardial infarction (19), peritonitis (7), and corneal injury (29, 38). Also, Danchuk et al. (8) demonstrated that the beneficial effects of MSCs in a model of LPS-induced lung injury were partly explained by the secretion of TSG-6 by MSCs.

Here we tested the hypothesis that beneficial effects can be produced by administration of TSG-6 to target the early inflammatory phase of bleomycin-induced lung injury. In effect, administration of the protein during the early inflammatory phase of the injury might be more effective than administration of MSCs. Therapies with MSCs are complex because the cells undergo a lag period of 10–12 h before they are activated by signals from injured cells to express potentially therapeutic factors such as TSG-6, and after intravenous (iv) infusion they disappear from the lung after 24 h (19). We used a rapid and real-time assay for arterial oxygen saturation (SpO2) to follow the deleterious effects of bleomycin on gaseous exchange in the lung. The results demonstrated that administration of TSG-6 on 2 and 4 days after bleomycin exposure decreased the initial cellular infiltration into alveolar spaces and improved both the subsequent decrease in SpO2 levels and the survival of the mice.

**MATERIALS AND METHODS**

**Animals.** Animal use protocol was approved by the Texas A&M Health Science Center Institutional Animal Care and Use Committee at Scott and White Hospital. Female 6- to 8-wk-old wild-type (C57BL/6) mice and female transgenic mice with a CD44 gene knockout (B6.Cg-Cd44tm1Hbg/J) obtained from Jackson Laboratory were used for this study. The mice were kept on a 12-h light-dark cycle and fed and watered ad libitum.

Bleomycin-induced lung injury. Mice were anesthetized with 4% isoflurane in 100% oxygen for 4 min to reach the level of deep anesthesia and placed on the intubation stand facing upward at a 45° tilt by using sterile elastic string positioned under the animal’s front incisors. The tongue was retracted with forceps and the trachea was intubated with a 22-G plastic sterile iv catheter (Terumo). An external high-power light source was used to visualize the tracheal opening. Bleomycin sulfate from *Streptomyces verticillus* (Sigma-Aldrich) at 2.25 U/kg body wt in 0.9% sodium chloride (Sigma-Aldrich) or 0.9% sodium chloride alone (sham injury) was instilled through a catheter at volume 4 µl/g body wt in two sets. The dose per mouse varied from 0.036 to 0.047 µl. Mice were kept on the board for an additional 90 s with the continued 4% isoflurane in 100% oxygen anesthesia via facemask.

Fig. 1. Pulse oximetry in the bleomycin model of lung injury. A: dynamics of oxygen saturation (SpO2) levels in bleomycin-injured and uninjured (sham-injured with NaCl) mice over the course of 12 days following injury. Only bleomycin-injured mice dropped SpO2 levels below 90% (dashed line) that were significantly lower than in uninjured mice beginning on day 8. B: SpO2 curves extracted from Windaq files of bleomycin-injured and uninjured mice prior to the beginning of the experiment and 12 days after injury. Note visible changes in the SpO2 curve of injured animals (bleomycin day 12). C and D: although no significant changes from preinjury baseline recordings (day 0) were detected, heart rates of bleomycin-injured mice were significantly lower on day 12 and breath rates were significantly lower on days 4, 6, and 12 compared with uninjured mice. E: weight changes normalized to preinjury (day 0). Significant weight changes were noted on day 6 and constituted ~20% weight loss in injured mice. P values were calculated between uninjured and bleomycin-injured groups at each time point by 1-tailed t-test. *P < 0.05, **P < 0.01.
Intravenous administration of recombinant human TSG-6 (R&D Systems) was performed on days 2 and 4 after bleomycin administration. Mice were anesthetized with 3% isoflurane, the tail vein was visualized by warming the tail, and a 28-G needle was used to inject rhTSG-6 (50 μg in 50–150 μl of sterile PBS) or sterile PBS (50–150 μl).

**End point tissue, blood, and bronchoalveolar lavage fluid collection.** At the indicated time points after bleomycin administration, mice were anesthetized with 3% isoflurane in 100% oxygen for 3 min and euthanized by intraperitoneal injections of ketamine-xylazine solution at 80 and 8 mg/kg body wt, respectively. The rib cage was opened without damaging the lungs. Blood was collected from the right heart ventricle and placed in a tube containing activators of clotting (Terumo Medical, Somerset, NJ) for 20 min and then stored on ice until further processing. Care was used to prevent bleeding into the rib cage. Serum was separated by centrifugation at 1,500 × g for 10 min and stored at −80°C until further analysis. Immediately following the collection of blood, the trachea was cannulated with a 20-G plastic iv catheter (Exelint, Los Angeles, CA). Two fractions of BALF were obtained. The first fraction was obtained by flushing the lungs back and forth four times with a single volume of 800 μl of PBS containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). The second fraction was obtained by flushing the lung four additional times each with a volume of 800 μl of PBS without protease inhibitor cocktail. The first BALF fraction was centrifuged at 500 g for 10 min and stored at −80°C until further analysis. The supernatant was then spun again at 10,000 g for 10 min and stored at −80°C until further analysis. The supernatant from the second wash was obtained by centrifugation at 500 g for 10 min and combined with the cell pellet from the first fraction. To lyse erythrocytes, the combined pellets were incubated at room temperature with red blood cell lysis buffer (eBioscience, San Diego, CA) for 5 min, centrifuged at 500 g for 5 min at 4°C, resuspended with 10 ml of ice-cold PBS, and centrifuged again. The supernatant was discarded and the cells were then resuspended in PBS containing 1% bovine serum albumin (BSA) (Thermo Fisher Scientific), counted by use of disposable Improved Neubauer Hemocytometer.

**Total cells (BALF)** was counted with a disposable hemocytometer. **Protein concentration (Conc) in BALF** was determined by the BCA assay. **Macrophages and neutrophils** also increased temporally following bleomycin injury. However, on day 4, whereas total cells and macrophages were either similar to or lesser in number compared with uninjured mice, neutrophil numbers on those days were higher than the uninjured mice. **Fig. 2. Temporal changes in cell composition of bronchoalveolar lavage fluid (BALF) and histological changes in the lungs following bleomycin injury.** BALF was collected at 2, 4, 6, and 12 days following bleomycin injury and at 12 days from uninjured mice (Fig. 2A–D). To visualize the area of inflammation and fibrosis, Masson’s trichrome-stained lung sections from bleomycin-injured and uninjured mice from day 8 were collected. Lower row represents magnification of area inside orange frame in upper row. Area of inflammation and fibrosis is clearly visible in bleomycin-injured lungs. Values are represented as arithmetic means ± SE (n = 3 mice per group). P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. *p < 0.05, **p < 0.01, and ***p < 0.001.
ometers (INYCTO, Cheonan-si, Chungcheongnam-do, South Korea), and used later for flow cytometry analysis.

After obtaining BALF, the lungs were excised, washed once in ice-cold PBS and immediately frozen and stored in −80°C until further processing.

**Flow cytometry.** The cells from BALF were prepared as described in the previous section, counted, resuspended in 100 μl of PBS with 1% BSA, and incubated for 10 min at 4°C with anti-CD16/32 antibody at a concentration 0.5 μg per 1 × 10⁶ cells in 100 μl (eBioscience) to block nonspecific binding to Fc-receptors. After being washed once with PBS-1% BSA, the cells were incubated for 20 min at room temperature with both phycoerythrin-Cy7-conjugated anti-mouse F4/80 antibody (eBioscience) and FITC-conjugated anti-Ly-6G antibody (BD Pharmingen, San Diego, CA). The antibodies were used at a concentration of 1 μg per 1 × 10⁶ cells in 100 μl of PBS-1% BSA. Isotype-matching antibodies at similar concentrations were used as controls for the specificity of labeling. After two washes in PBS, the cells were again resuspended in PBS-1% BSA and analyzed by FC500 flow cytometer (Beckman Coulter, Brea, CA) to determine macrophage (F4/80-positive) and neutrophil (Ly-6G-positive) populations.

**Cytokine ELISA in BALF and lung tissues.** BALF was prepared as described in the previous section and frozen lungs were homogenized with RIPA buffer (ThermoFisher Scientific). Appropriate dilutions were used in the assays. Lung protein concentration was determined by using Micro BCA Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Cytokine concentrations were determined by using commercially available ELISA kits for detection of IL-6, TNF-α, IL-1β, CCL2/MCP-1, CCL3/MIP-1α, CXCL2/MIP-2, and CXCL1/KC (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Histology.** Mice were anesthetized with 3% isoflurane in 100% oxygen for 3 min and euthanized by intraperitoneal injections of ketamine-xylazine solution as described previously. The rib cage was opened and the blood flow was stopped by placing a permanent silk suture between atria and ventricles of the heart. The trachea was then cannulated with 20-G iv catheter and the lungs were excised, immediately washed with PBS, and fixed with 4% paraformaldehyde at pressure 20 cmH₂O for 24 h. The lungs were then dehydrated with graded ethanol solutions and embedded in paraffin. Five-micrometer sections were cut with Leitz 1512 microtome (Leitz, Germany) and stained with a Masson TriChrome Kit (Richard-Allan Scientific, Kalamazoo, MI). The bright-field images were taken with Nikon Eclipse 80i upright microscope (Nikon, Kawasaki, Japan), and linear adjustments were made in Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

**Pulse oximetry.** A portable mouse pulse oximeter (STARR Life Sciences) was used to monitor SpO₂ and other physiological parameters (heart and breath rates, pulse distention) in free-roaming non-anesthetized mice. The collars of experimental animals were trimmed of fur at least a day before the beginning of pulse oximetry monitoring. At indicated experimental time points, mice were anesthetized with 3% isoflurane in 100% oxygen for 2 min and an extra-small MouseOX collar clip was placed on the animal’s neck. Mice were allowed to recover from anesthesia (1–2 min) and pulse oximetry readings were recorded as a Windaq Waveform file at 15-Hz sample rate. After placing the collar clip, we observed two sequential behavioral states in mice: 1) an agitated state, characterized by frequent movements resulting in an unstable signal, and 2) a relatively calm state with improved signal quality. The measurements were continued to allow recording of 3–5 min of stable signal. Using Windaq Waveform Browser (DATAQ Instruments), we extracted data from only the calm state. An in-house Microsoft Excel (Microsoft) VBA-based script was then used to filter values associated with error-free signals implementing formulas provided by the manufacturer. With the same script, arithmetic means for each reading were obtained and later used for statistical analysis.

**Fig. 3. Temporal changes in cytokine expression of BALF in the bleomycin model of lung injury.** Cytokine expression levels on days 2, 4, 6, and 12 were assayed in BALF by use of ELISA. IL-6 (A), TNF-α (B), CCL2/MCP-1 (C), and CXCL1/KC (D) expression levels were the highest on day 2 and were significantly higher than those in uninjured mice. Expression levels of all the cytokines tested returned close to uninjured levels by days 6 and 12. Significant differences in cytokine levels between groups on the individual days tested are indicated. Values are represented as arithmetic means ± SE (n = 3 mice per group). P values were calculated by 1-way ANOVA with post hoc Bonferroni’s multiple comparison analysis performed between all the groups. *P < 0.05, **P < 0.01, and ***P < 0.001.
Statistical analysis. Unpaired t-test or one-way ANOVA with Bonferroni’s post hoc analysis was used to compare two or more groups, respectively. Null hypotheses were rejected at P values less than 0.05. Survival of animals between groups was compared by log-rank (Mantel-Cox) test. All statistical analyses were performed with GraphPad Prism software.

RESULTS

Functional oxygen saturation correlates with changes in cellular infiltrates and cytokines in BALF. To monitor in real-time the bleomycin-induced injury to the lung, we used a pulse oximeter to assay SpO2 in mice (Fig. 1, A and B). Placing a collar clip on the necks of the mice agitated them and distorted the reading. However, with care in handling, the mice became calm so that reliable values with minimum associated errors were readily obtained. Intratracheal administration of 2.25 U/kg of bleomycin produced a progressive decrease in SpO2 below 90% reflecting hypoxemia beginning on day 8 (Fig. 1A).

As expected, the decrease in SpO2 paralleled changes in the BALF. There were progressive increases in total cells and protein concentration (Fig. 2, A and D). Macrophages in the BALF showed an initial decrease and then increased on day 6 and day 12 (Fig. 2B). Neutrophils were more variable but showed increases vs. a control in the number of neutrophils from days 2 to 12 (Fig. 2C). As also expected, the major changes in cytokine and chemokine levels occurred earlier than the decrease in SpO2 (Fig. 3). Levels of the proinflammatory cytokines IL-6 and TNF-α in the BALF were increased on day 2.

Fig. 4. TNF-α-stimulated gene/protein 6 (TSG-6) dose response on SpO2 and cellular infiltrates. A: effect of TSG-6 dose response on the dynamics of SpO2 levels over the course of 8 days following bleomycin (Bleo) injury. A high dose (50 µg) and a low dose (12.5 µg) were administered on days 2 and 4 following bleomycin injury. B: dot plot represents the distribution of SpO2 levels on day 8. Significant differences between high dose of TSG-6 and PBS groups was observed on day 8. For the analysis of cellular infiltrates, BALF was collected on days 2, 3, 5, and 8 following bleomycin injury. From day 3 onward following injury of the lungs with bleomycin, total cells (C) and macrophages (D) in BALF increased in the low-dose TSG-6 mice compared with PBS control mice. F and G: the high dose of TSG-6 lowered these parameters compared with PBS-treated mice with statistically significant differences between these 2 groups observed only on day 5. E and H: the low dose of TSG-6 increased neutrophil numbers early on day 2, whereas the high dose of TSG-6 maintained the neutrophil infiltration lower than PBS mice at all time points. Values are represented as arithmetic means ± SE (n = 5–8 mice per group). For the SpO2 data, P value was calculated between PBS and TSG-6 groups by 1-tailed t-test. For the cellular infiltrates data, P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. *P < 0.05, **P < 0.01.

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and then decreased. Similarly, the chemokines CCL2 and CXCL1 were increased on day 2 and then decreased.

In addition, the decrease in SpO2 roughly paralleled a decrease in body weight (Fig. 1E) and pathological changes in lung morphology (Fig. 2E). There were more variable decreases in heart rate and respiration rate (Figs. 1, C and D).

**Intravenous administration of TSG-6 improved functional oxygen saturation and survival of bleomycin-injured mice.** We elected to test the potential effectiveness of TSG-6 in suppressing the early inflammatory phase in the bleomycin model. Day 8 postinjury was chosen for performing last end point assays in further experiments. This was based on the findings from the optimization of the bleomycin model (Figs. 1–3) in which SpO2 levels in bleomycin-injured mice were significantly different from uninjured mice on day 8 and the extent of inflammation paralleled SpO2 levels. Additionally, the bleomycin-injured mice were observed until day 21 for long-term survival.

A high (50 μg) and a low dose (12.5 μg) of TSG-6 were tested to determine the most effective dose. The functional SpO2 data on day 8 (Fig. 4, A and B) and the cellular infiltrates on day 5 (Fig. 4, C–H) suggested that the high 50-μg dose would be most beneficial in reducing early inflammation and improving function in the bleomycin lung injury model. Hence the high dose (50 μg) was used for the rest of the study. The TSG-6 protein (50 μg) was infused intravenously on day 2 and day 4 to target the early inflammatory stage. As expected, there was a decrease in early signs of inflammation by a decrease in total cells, macrophages, and neutrophils in BALF on day 5 (Fig. 5). There was also a decrease in the proinflammatory cytokines TNF-α on day 2 and IL-6 on day 3 (Fig. 6, A and B). However, IL-1β expression in lung tissues was increased in TSG-6-treated mice on day 2 (Fig. 6C). In addition, administration of TSG-6 increased the BALF levels of the chemokines CCL2, CCL3, CXCL1, and CXCL2 on day 2 (Fig. 6, D–G).

Protein concentration levels were not significantly different among the groups (data not shown). On day 5, there were more variable decreases in heart rate and respiration rates in TSG-6-treated mice compared with PBS-treated mice. This was based on the results from the optimization of the bleomycin model (Figs. 1–3) in which these parameters were significantly different from uninjured mice on day 5. Recovery was observed in TSG-6-treated mice on day 8 and the extent of inflammation was significantly different between PBS and TSG-6 groups (\(P < 0.01\)) compared with uninjured mice. Values are represented as arithmetic means ± SE (n = 5–8 mice per group). P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. P values represented in the larger panels only indicate significant differences between PBS and TSG-6 groups following bleomycin injury. P values represented in the insets indicate significant differences between all 3 groups. *\(P < 0.05\), **\(P < 0.01\).
injured mice (Fig. 7, A and B). Furthermore, there was a significant improvement in percent change of initial weight on days 7 and 8 following TSG-6 treatment (Fig. 7E), whereas there were no significant changes in heart and breath rates between groups (Fig. 7, C and D). However, there was no significant reduction in fibrosis in TSG-6-treated lungs as measured by hydroxyproline content in the lungs on day 8 (data not shown), suggesting that there was no increase in fibrosis at this time.

The effects of TSG-6 are not observed in mice that do not express CD44. Previous reports demonstrated that some of the anti-inflammatory effects of TSG-6 involved its interaction with CD44 either by directly binding to the receptor or in a complex with hyaluronan (7, 24, 51). Therefore we elected to test the hypothesis that the beneficial effects of TSG-6 observed in the bleomycin model would not be observed in transgenic mice that did not express CD44 (42). As expected, TSG-6 administration had no effect in bleomycin-treated mice on SpO2 (Fig. 8, A and B), or on BALF content of total cells, macrophages or neutrophils (Fig. 8, C–E). Also, administration of TSG-6 had no effect on the BALF levels of the cytokines IL-6, TNF-α/H9251, and IL-1β/H9252 (Fig. 9, A–C). Similarly it had no effect on the BALF levels of the chemokines CCL2, CCL3, CXCL1, or CXCL2 (Fig. 9, D–G) and protein concentration (Fig. 9H).

DISCUSSION

The assay of SpO2 with pulse oximeter provided a useful measure of the deleterious effects of bleomycin on the lungs of mice. The data are obtained in a rapid, quantitative, and real-time manner without the need to euthanize the mice.
Therefore this assay has many advantages both in following the natural progression of the injury and for testing potential therapeutics for lung injury.

The response of the lung to injury by bleomycin is complex and occurs in several relatively distinct phases (10, 11, 17, 41, 46). The beneficial effects previously observed with administration of MSCs may in fact reflect the plasticity of the cells in responding to injured tissues in a manner that is appropriate to the type and phase of the injuries (30, 31). For example, MSCs were shown to produce beneficial effects (4, 22, 32–34) in various injury models by expressing factors that enhance vascularization (VEGF and IL-6), enhance cell proliferation (TGF-β, KGF), modulate immune responses (indoleamine 2,3-dioxygenase in human MSCs, iNOS with mouse MSCs, CCL2, MMP-9), reduce reactive oxygen species and apoptosis (stanniocalcin-1), and are antibacterial (peptide LL-37).

The characterization of the bleomycin model gave us an exact timeline of cellular and morphological events that occurred after the injury. Hence it allowed us to target specific inflammatory and functional events to examine whether TSG-6 had positive effects on reducing cytokine storm and infiltration of inflammatory cells and finally whether these early reductions translated to functional improvement and improved survival. In the experiments described here, we elected to use iv administration of TSG-6 over intratracheal administration for several reasons: 1) clinical relevance, 2) increased risk of physical insult resulting from the repeated intubation required, and 3) oxygen-exacerbated damage to the lung during anesthesia (5, 11, 12, 36). Under the conditions employed in this study, we reached therapeutic effect in the lungs by iv infusion of 50 μg TSG-6 protein. This was demonstrated by an improvement in oxygen saturation, increased survival, and modulation of cytokines following bleomycin injury.

To test TSG-6 in the bleomycin model, we infused the protein during the early inflammatory phase marked by the appearance of neutrophils and proinflammatory cytokines (IL-6 and TNF-α) in the BALF (day 2 and day 4). The TSG-6 decreased the inflammatory phase as indicated by a decrease in BALF on day 5 of total cells, macrophages, and neutrophils. It also decreased proinflammatory cytokine TNF-α on day 2, and IL-6 on day 3. At the same time, administration of TSG-6 increased the levels in BALF of chemokines (CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, and CXCL2/MIP-2) that attract macrophages to resolve inflammation. The potential beneficial
bleomycin-injured and uninjured mice, although, in the absence of CD44, TSG-6 failed to improve SpO2 levels over the PBS-treated group.

2 days following bleomycin injury (representative of 2 independent experiments) in CD44 knockout mice. No significant differences between TSG-6 and PBS groups were observed on any of the days tested. Note that SpO2 levels of both TSG-6 and PBS groups remained slightly above 90% threshold (dashed line) through day 8. B: dot plot represents the distribution of SpO2 levels on day 8 following bleomycin injury. Significant differences were observed between bleomycin-injured and uninjured mice, although, in the absence of CD44, TSG-6 failed to improve SpO2 levels over the PBS-treated group. C–E: BALF was collected in a similar manner as described above for the wild-type mice. No significant differences were observed between total cells, macrophages, and neutrophil numbers between TSG-6 and PBS-treated groups. Values are represented as arithmetic means ± SE (n = 5–8 mice per group). *P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. **P < 0.05.

Fig. 8. CD44 partly mediates beneficial effect of TSG-6 in vivo. A: effect of TSG-6 (50 μg) treatment on the dynamics of SpO2 levels over the course of 8 days following bleomycin injury (representative of 2 independent experiments) in CD44 knockout mice. No significant differences between TSG-6 and PBS groups were observed on any of the days tested. Note that SpO2 levels of both TSG-6 and PBS groups remained slightly above 90% threshold (dashed line) through day 8. B: dot plot represents the distribution of SpO2 levels on day 8 following bleomycin injury. Significant differences were observed between bleomycin-injured and uninjured mice, although, in the absence of CD44, TSG-6 failed to improve SpO2 levels over the PBS-treated group. C–E: BALF was collected in a similar manner as described above for the wild-type mice. No significant differences were observed between total cells, macrophages, and neutrophil numbers between TSG-6 and PBS-treated groups. Values are represented as arithmetic means ± SE (n = 5–8 mice per group). *P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. **P < 0.05.

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Effects of TSG-6 treatment in our model could partly explain the overall beneficial effects that we observed such as resolution of inflammation and reduced neutrophil numbers due to either reduced recruitment of neutrophils or their increased phagocytosis, thus resulting in improved oxygenation and survival. In addition, decrease in levels of TNF-α by TSG-6 could have resulted in decreased levels of apoptosis in alveolar epithelium as indicated by Wang et al. (49), although apoptosis was not addressed in our study. Thus the cumulative effects of TSG-6 on the early inflammatory stage in part explained the subsequent increase in SpO2 and survival seen in treated mice compared with controls (Fig. 10). However, in this model, there was some mortality in bleomycin-injured mice treated...
with PBS, with the earliest occurrence beginning on day 6. Thus, had these mice survived to day 8, the average SpO₂ levels would have been much lower and accordingly the numbers of cellular infiltrates in BALF would have been much higher compared with the bleomycin/TSG-6 group than what is represented. Although not explored here, more frequent or larger doses on TSG-6 may have been even more effective.

TSG-6 has been previously shown to have multiple interactions that can be anti-inflammatory (2, 3, 24, 26, 50). Particularly, in a recently published study by Choi et al. (7), it was shown that at least part of the inflammatory action of TSG-6 in the peritonitis model could be explained by its direct or indirect interaction with CD44 on resident macrophages. TSG-6 bound to CD44, either directly or in a complex with hyaluronan, to dissociate CD44 from TLR2 and thereby limit TLR2-driven NF-κB signaling. In CD44 knockout mice, bleomycin induced an unrelenting inflammatory response in a TLR2- and TLR4-dependent manner (14, 45). TSG-6 was also effective in wild-type mice but ineffective in CD44 knockout mice employed in a mouse model for sterile injury to the cornea (28). In the present study, we employed the CD44 knockout mouse model, similar to that used in the study by Choi et al. In these mice, CD44 expression is constitutively knocked out in all cell lineages and not restricted to only macrophages. Also, on the basis of previous publications (7, 29) we hypothesized that resident macrophages with functional CD44 could be important in mediating the effects of TSG-6 in bleomycin-injured lungs. Since TSG-6 was not effective in reducing inflammation and improving bleomycin-induced injury in the CD44 knockout mice, the results were consistent with the conclusion that

Fig. 9. Temporal changes in cytokine/chemokine expression following TSG-6 treatment in CD44 knockout mice. No significant changes were observed in CD44 knockout mice after TSG-6 (50 μg) treatment in any of the proinflammatory cytokines examined such as IL-6 (A), TNF-α (B), and IL-1β (C), or in the expression of chemokines such as CCL2/MCP-1 (D), CCL3/MIP-1α (E), and CXCL2/MIP-2 (G), or in protein concentration (H) levels. However, TSG-6 treatment significantly decreased CXCL1/KC (F) levels only on day 5 compared with the PBS group. Values are represented as arithmetic means ± SE (n = 5–8 mice per group). P values were calculated by 1-way ANOVA with a post hoc Bonferroni’s multiple comparison analysis performed between all the groups. No P values are indicated on this figure since there were no statistical differences between the bleomycin-injured (PBS and TSG-6) groups in most of the cytokines/chemokines tested and at any of the time points examined.
TSG-6 decreased inflammation by interacting with functional CD44 on resident macrophages in a manner previously demonstrated in zymosan-induced peritonitis (7) and chemical injury to the cornea (29).

Toxic agents such as bleomycin trigger a cascade of destructive events in the lung. It seems unlikely that any single therapy can halt all of them. The results presented in this study demonstrate that the systemic administration of TSG-6 improved functional outcome and survival of bleomycin-injured mice by modulating inflammation at early stages. Hence these data suggest that it may be useful to target specific phases in the cascade with agents such as TSG-6 for the early inflammatory phase. Similar phase-specific strategies may be useful for other diseases of the lung.

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DISCLOSURES

D. J. Prockop is the chairman of the scientific advisory committee of Temple Therapeutics. None of the other authors have a conflict of interest.

AUTHOR CONTRIBUTIONS


REFERENCES
