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

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Foskett AM, Bazhanov N, Ti X, Tiblow A, Bartosh TJ, Prockop DJ. Phase-directed therapy: TSG-6 targeted to early inflammation improves bleomycin-injured lungs. Am J Physiol Lung Cell Mol Physiol 306: L120–L131, 2014. First published November 15, 2013; doi:10.1152/ajplung.00240.2013.—Previous reports demonstrated that bleomycin-induced injury of lungs in mice can be improved by the administration of murine multipotent adult stem/progenitor cells (MSCs) from the bone marrow. Recently some of the beneficial effects of MSCs have been explained by the cells being activated by signals from injured tissues to express the inflammation modulating protein TNF-α-stimulated gene/protein 6 (TSG-6). In this study, we elected to test the hypothesis that targeting the early phase of bleomycin-induced lung injury with systemic TSG-6 administration may produce therapeutic effects such as preventing the deterioration of lung function and increasing survival by modulation of the inflammatory cascade. Lung injury in C57Bl/6J mice was induced by intratracheal administration of bleomycin. Mice then received intravenous injections of TSG-6 or sham controls. Pulse oximetry was used to monitor changes in lung function. Cell infiltration was evaluated by flow cytometry, cytokine expression was measured by ELISA assays, and lungs were assessed for histological attributes. The results demonstrated that intravenous infusion of TSG-6 during the early inflammatory phase decreased cellular infiltration into alveolar spaces. Most importantly, it improved both the subsequent decrease in arterial oxygen saturation levels and the survival of the mice. These findings demonstrated that the beneficial effects of TSG-6 in a model of bleomycin-induced lung injury are largely explained by the protein modulating the early inflammatory phase. Similar phase-directed strategy with TSG-6 or other therapeutic factors that MSCs produce may be useful for other lung diseases and diseases of other organs.

Lung injury; inflammation; pulmonary gas exchange; mesenchymal stromal cells

BLEOMYCINS ARE A FAMILY OF antibiotics that are currently used as chemotherapeutic agents to treat germ-cell cancers, lymphomas, and malignancies of head and neck (6). Unfortunately, bleomycin chemotherapy is a frequent cause of interstitial pneumonitis, a complex lung disease that can progress into interstitial pulmonary fibrosis with a poor clinical prognosis 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 multipotent adult stem/progenitor cells referred to as mesenchymal stem orstromal 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-

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Bleomycin significantly lower on changes from preinjury baseline recordings (day 0) were detected, heart rates of bleomycin-injured mice were significantly lower than in uninjured mice beginning on day 6. 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 production of TSG-6 by MSCs.

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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 centrifuging the blood sample 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 at 4°C to obtain a cell pellet, which was later combined with the cell pellet from the second fraction. The supernatant was then spun again at 10,000 g for 10 min and stored at −80°C until further analysis. The cell pellet 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, and labeled with antibodies against macrophage (F4/80) and neutrophil (Ly6G) markers and analyzed by flow cytometry. Macrophages and neutrophils also increased temporally following bleomycin injury. However, on day 2, macrophage numbers on those days were higher than the uninjured mice.

Both total BALF cell number and protein concentration increased temporally. Both parameters were the highest by day 8 postinjury and were significantly higher than in the uninjured controls. Significant differences in total cells and protein concentration between groups were also observed at various time points as indicated in the figure. A and D: total number of cells in BALF was counted with a disposable hemocytometer. Protein concentration (Conc) in BALF was determined by the BCA assay. **P < 0.01, ***P < 0.001.

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.

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 (A–D); for histology, lungs were collected at day 8 (E). A and D: total number of cells in BALF was counted with a disposable hemocytometer. Protein concentration (Conc) in BALF was determined by the BCA assay. Both total BALF cell number and protein concentration increased temporally. Both parameters were the highest by day 12 postinjury and were significantly higher than in the uninjured controls. Significant differences in total cells and protein concentration between groups were also observed at various time points as indicated in the figure. B and C: the cells were labeled with antibodies against macrophage (F4/80) and neutrophil (Ly6G) markers and analyzed by flow cytometry. Macrophages and neutrophils also increased temporally following bleomycin injury. However, on days 2 and 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. E: representative images of Masson’s trichrome-stained lung sections from bleomycin-injured and uninjured mice from day 8. 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 (INCYTO, 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 obtained from the same manufacturers and single-color labeling 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) 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.

**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

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Uninjured</th>
<th>Time after injury (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td></td>
<td>2 4 6 12</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>2 4 6 12</td>
</tr>
<tr>
<td>CCL2/MCP-1</td>
<td></td>
<td>2 4 6 12</td>
</tr>
<tr>
<td>CXCL1/KC</td>
<td></td>
<td>2 4 6 12</td>
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**Table 1.** Significance levels on 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 a 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

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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 post hoc Bonferroni’s multiple comparison analysis performed between all the groups. *P < 0.05, **P < 0.01.
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 between TSG-6- and PBS-treated mice on day 2 (Fig. 6, H). In addition, administration of TSG-6 lowered the BALF levels of the chemokines CCL2, CCL3, CXCL1, and CXCL2 on day 2 (Fig. 6, D–G).

![Fig. 5. TSG-6 treatment reduces cellular infiltration in the lungs during the early inflammatory phase. BALF was collected at 12 h and on days 2, 3, 4, 5, and 8 following bleomycin injury. On days 2 and 4 alone, BALF was collected 4 h after TSG-6 (50 μg) administration. From day 4 onward following injury of the lungs with bleomycin, total cells (A), macrophages (B), and neutrophils (C) in BALF increased compared with uninjured mice. TSG-6 treatment lowered these parameters compared with PBS-treated mice with statistically significant differences between these 2 groups observed only on day 5. Insets represent changes on day 5 in individual mice from the 3 groups. Note that on days 2 and 3 following bleomycin injury, macrophages (B) were significantly lower in PBS and TSG-6 groups (\( \forall P < 0.01, \forall P < 0.001 \)) 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 and **P < 0.01.

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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 (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.
of inflammatory cells and finally whether these early reductions had positive effects on reducing cytokine storm and infiltration of inflammatory and functional events to examine whether TSG-6 occurred after the injury. Hence it allowed us to target specific exact timeline of cellular and morphological events that occurred after the injury. Therefore this assay has many advantages both in following the natural progression of the injury and for testing potential therapies 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
effects of the increase in chemokines is consistent with the observations by Liang et al. (20) that mice with lung-specific overexpression of CCL2/MCP-1 were protected from bleomycin-induced lung injury because of increased recruitment of cells capable of clearing apoptotic cells.

However, our data on day 5 demonstrate that the early increases in MCP-1 after TSG-6 administration only translated to a modest increase in macrophage recruitment between days 2 and 5 but that these increases were still significantly lower than the number of macrophages recruited in the PBS-treated group. To explain this discrepancy, we speculate here that the macrophages recruited to alveolar spaces in TSG-6-treated animals possibly contained a larger proportion of alternatively activated or regulatory M2 macrophages. This could result in decrease of inflammation and subsequent decrease in the recruitment of proinflammatory monocytes from the circulation.

In support of this theory, Roca et al. (37) have demonstrated that CCL2 (MCP-1) in the lungs can induce M2-type macrophage polarization. Furthermore, employing models of gastric aspiration and lung contusion, there is evidence for the protective effect of CCL2 and its receptor CCR2 in the pathogenesis of acute lung injury (ALI) by attenuating the inflammatory response, decreasing neutrophil infiltration, increasing macrophage recruitment, activation of the M2 polarized macrophages, and improved survival (35, 44a). In a pneumonia model of ALI, Amano et al. (1) also demonstrated that CCL2 was critical in decreasing neutrophil infiltration and increasing the phagocytosis of apoptotic neutrophils by alveolar macrophages. Thus this study might also help explain why there was decreased neutrophil recruitment on day 5 in our model following TSG-6 treatment despite elevated levels of MIP-2 and KC chemokines on day 2. We speculate that TSG-6 treatment of mice that were injured with bleomycin results in a complex interaction between different types of cells and their corresponding cytokines, which needs further investigation. However, the combined results from these studies lead us to conclude that the early elevated chemokine levels following 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 improves bleomycin-injured lungs

Fig. 10. Modulatory effects of TSG-6 in bleomycin-injured lungs. The beneficial effects of TSG-6 on modulating the early inflammatory phase in bleomycin-injured lungs is indicated by 1) a decrease in proinflammatory cytokines IL-6 and TNF-α (days 2 and 3), 2) an increase in chemokines CCL2, CCL3, KC, and CXCL2 (days 2 and 4), and 3) a corresponding decrease in total cells and infiltrating cells such as macrophages and neutrophils (day 5). These early modulatory events promoted by TSG-6 lead to functional improvement by preventing the drop of SpO2 levels below 90% (day 8) and decreased mortality (weeks 1–3).

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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