Dysregulated Macrophages Are Present in Bleomycin-Induced Murine Laryngotracheal Stenosis

Alexander T. Hillel, MD\(^1\), Idris Samad, MD\(^1\), Garret Ma\(^2\), Dacheng Ding, MD, PhD\(^1\), Kaitlyn Sadtler\(^2\), Jonathan D. Powell, MD\(^3\), Andrew P. Lane, MD\(^1\), and Maureen R. Horton, MD\(^4\)

Abstract

Objective. To define the inflammatory cell infiltrate preceding fibrosis in a laryngotracheal stenosis (LTS) murine model.

Study Design. Prospective controlled murine study.

Setting. Laboratory.

Subjects and Methods. Chemomechanical injury mice (\(n = 44\)) sustained bleomycin-coated wire-brush injury to the laryngotracheal complex while mechanical injury controls (\(n = 42\)) underwent phosphate-buffered saline (PBS)–coated wire-brush injury. Mock surgery controls (\(n = 34\)) underwent anterior transcervical tracheal exposure only. Inflammatory and fibrosis protein and gene expression were assessed in each condition. Immunohistochemistry served as a secondary outcome.

Results. In chemomechanical injury mice, there was an upregulation of collagen I (\(P < .0001\), \(P < .0001\)), TGF-\(\beta\) (\(P = .0023\), \(P = .0008\)), and elastin (\(P < .0001\), \(P < .0001\)) on day 7; acute inflammatory gene III/\(\beta\) (\(P = .0027\), \(P = .0008\)) on day 1; and macrophage gene CD11b (\(P = .0026\), \(P = .0033\)) on day 1 vs mechanical and mock controls, respectively. M1 marker inducible nitric oxide synthase (iNOS) expression decreased (\(P = .0014\)) while M2 marker Arg1 (\(P = .0002\)) increased on day 7 compared with mechanical controls. Flow cytometry demonstrated increased macrophages (\(P = .0058\), day 4) and M1 macrophages (\(P = .0148\), day 4; \(P = .0343\), day 7; \(P = .0229\), day 10) compared to mock controls. There were similarities between chemomechanical and mechanical injury mice with an increase in M2 macrophages at day 10 (\(P = .0196\)).

Conclusions. The bleomycin-induced LTS mouse model demonstrated increased macrophages involved with the development of fibrosis. Macrophage immunophenotype suggested that dysregulated M2 macrophages have a role in abnormal laryngotracheal wound healing. These data delineate inflammatory cells and signaling pathways in LTS that may potentially be modulated to lessen fibroblast proliferation and collagen deposition.

Keywords

trachea, laryngotracheal stenosis, subglottic stenosis, mouse model, airway epithelial injury

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Laryngotracheal stenosis (LTS) is a poorly understood fibroproliferative disease that is characterized by epithelial injury, inflammatory cell infiltrate, and cytokine-mediated fibroblast proliferation with subsequent collagen deposition.\(^1\) The 3 most frequent causes are iatrogenic, idiopathic, and autoimmune disease.\(^2\) While the underlying cause may be different, it ultimately results in triggering an abnormal wound-healing response. In LTS, the initial inflammatory phase consists of neutrophil, leukocyte, and macrophage infiltration with minimal fibrosis. This transitions to a pathologic fibroproliferative phase, which leads to active tissue remodeling and the formation of permanent scar tissue.

Recently, a number of authors have focused on dysregulated immune cells as possible effector cells for the airway scar tissue.\(^3,4\) Animal models suggest that inflammatory cells and their mediators may be responsible for laryngotracheal fibrosis in humans. A recent ex situ mouse model...
showed evidence of lymphocytes mediating the formation of tracheal granulation tissue. These results and others demonstrated a significant thickening in the subepithelial connective tissue, or lamina propria, with fibroblasts, angiogenesis, and a predominately lymphocytic infiltrate. In another study, investigators demonstrated that abnormal fibroblasts potentiate the ongoing chronic immune response in scarred vocal folds. This suggests that signaling between immune cells and fibroblasts may contribute importantly to pathologic scar formation.

Inflammation’s role in fibrosis is better established in the lower airway and other organ systems. It is initiated by lymphocyte-macrophage interactions that produce cytokines, growth factors, and proteolytic enzymes. These factors stimulate extracellular matrix deposition with persistent tissue remodeling and destruction of normal architecture. CD4+ T-cell–dependent pathways are key regulators of fibrosis of the lung, kidney, and skin. Similar to T-helper (Th1 and Th2) lymphocytes, macrophages have been recognized as having distinct states of activation, with polarization driven by T-cell phenotype and associated cytokines. Classically activated macrophages (CAM or M1) are activated by Th1 cytokines, such as interferon γ (IFNγ), while alternatively activated macrophages (AAM or M2) are induced by Th2 cytokines, including interleukin (IL)-4 and IL-13. M1 polarization is considered proinflammatory, mediating host defense against bacteria, viruses, and other microorganisms, while the M2 phenotype is classically thought to have anti-inflammatory functions and regulate wound healing. However, sustained M2 polarization has also been implicated in pathologic inflammation and fibrosis. Specific to the larynx, other investigators have demonstrated that M2 and fibroblast interaction promotes inflammation associated with vocal fold scarring.

Our laboratory developed an in vivo model of LTS by using a bleomycin-coated wire brush to create chemical and mechanical injury to the trachea, which resulted in pathologic wound healing and fibrosis at 3 weeks. In contrast, phosphate-buffered saline (PBS)–coated wire brush injury demonstrated initial injury and lamina propria thickening that was consistent with a physiologic wire brush injury demonstrated initial injury and lamina propria thickening that was consistent with a physiologic

**Methods**

**Experimental Design**

This study was approved by the Johns Hopkins University Animal Care and Use Committee (MO12M354). The experimental design was a 14-day prospective controlled in vivo situ animal trial. In total, 120 C57BL/6 (Charles River Laboratory, Germantown, Maryland) mice were used in this study. Outside of planned euthanasia time points, the experimental group (n = 44) had 10 deaths, control group 1 (n = 42) had 8 deaths, and control group 2 (n = 34) had 1 death. Mouse laryngotracheal complexes were chemomechanically injured with a bleomycin-coated wire brush. Mice were sacrificed at 1, 7, and 14 days for gene expression and 4, 7, and 10 days for protein expression. The primary outcome measures were gene expression and protein expression with secondary outcomes of immunohistochemistry (IHC). Results from the experimental group (chemomechanical injury) were compared with 2 control groups: (1) mechanical injury: mice that underwent tracheal injury with a saline-coated wire brush and (2) mock surgery: mice that underwent a tracheal cut down with no internal tracheal injury.

**RNA Extraction and Gene Expression Analysis**

Tracheal tissue from mouse specimens was homogenized in a Bullet Blender Homogenizer (Next Advance, Averill Park, New York). RNA was extracted with the RNaseasy Mini Kit (Qiagen, Valencia, California), and complementary DNA (cDNA) was synthesized with the iScript cDNA synthesis kit (Bio-Rad, Hercules, California). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out on the StepOnePlus Real Time PCR System (Life Technologies, Carlsbad, California) and monitored with the Power SYBR Green PCR Mastermix (Life Technologies). The cDNA samples (n = 3, 1 μL for a total volume of 25 μL per reaction) were analyzed for each gene of interest. The level of expression of each target gene was calculated as 2^{(-ΔΔCt)} as previously described. The denaturation step temperature was 95°C for 15 seconds, and annealing and extension temperature step was 60°C for 60 seconds for all primers. Error was calculated as the standard error of the mean of ddCt. Primers (Integrated DNA Technologies, Coralville, Iowa) used included αSMA, Col I, Elastin, TGFβ, IL1b, CD11b, Inos, and Arg1.

**Flow Cytometry for Protein Analysis**

Fresh dissected mouse trachea specimens were rinsed with PBS and placed in dissociation enzyme solution (Liberase 100 μg/mL [Roche 5401127001] and DNASE I 40 μg/mL [Roche 10104159001] in Dulbecco’s modified Eagle’s medium; Roche Diagnostics North America, Indianapolis, Indiana) at 37°C for 45 minutes. Extra tracheal tissue was dissected, and the remaining trachea was minced. A second enzymatic dissociation at 37°C for 60 minutes was performed to generate a single cell solution. After incubation to block nonspecific antibody binding, conjugated antibody staining was performed for 45 minutes at room temperature. Cells were centrifuged and washed twice with cold PBS and fixed with 4% paraformaldehyde. The stained cells were analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, California). Data analysis was performed with FlowJo software (FlowJo, Ashland, Oregon). Cell surface protein was expressed as the increase in mean fluorescence intensity over background or percentage of positive staining cells.
Immunohistochemistry

A laryngotracheal complex from days 7 and 14 were fixed in 10% formalin for 24 hours, and then each specimen was embedded in paraffin. Slides were made from 5-μm-thick sections cut through in an axial plane, which were then deparaffinized and stained with conjugated rat anti–mouse F4/80 (AbD Serotec, Raleigh, North Carolina) antibody for 30 minutes. After rinsing, slides were mounted with Prolong antifade reagent and incubated with DAPI (Cell Signaling Technology, Danvers, Massachusetts). Images were obtained on a confocal microscope (LSM 510; Zeiss, Oberkochen, Germany).

Statistical Analysis

Results for murine data were expressed as averages ± standard error of the mean. Statistical analysis was performed using Prism (GraphPad Software, San Diego, California) with a 2-factor analysis of variance followed by a Tukey multiple-comparison post hoc test to examine differences between groups. Statistical significance was assessed at an α level of P < .05.

Results

Upregulation of Fibrosis, Acute Inflammatory, and Macrophage Gene Expression in Chemomechanical Injury Mice

Fibrosis gene expression showed elevated collagen I, Tgf-β, elastin, and αSMA in the chemomechanical injury group (bleomycin and wire brush injury) compared with that in the mechanical injury (wire brush injury only) and mock controls (tracheal cut down but no brush injury) with the exception of Tgf-β and elastin on day 14 (Figure 1). Gene expression peaked on day 7 in collagen I (P < .0001, P < .0001), Tgf-β (P = .0023, P = .0008), and elastin (P < .0001, P < .0001) compared with mechanical and mock controls, respectively. The acute inflammatory response in the experimental group was significantly greater for Il1b (P = .0027, P = .0008) vs mechanical and mock controls on day 1, trended down at day 7, and normalized to mock control levels by day 14 (Figure 2A). Assessing gene expression for macrophage markers, there was a significant upregulation seen at day 1 for the mouse macrophage marker CD11b in the chemomechanical injury group (P = .0026, P = .0033) vs mechanical and mock controls on day 1, respectively (Figure 2B). The M1 marker Inos was significantly less (P = .0014) than the mechanical injury control at day 7 (Figure 2C); otherwise, it was not significantly different from mechanical injury or mock controls, while the M2 marker Arg1 was significantly higher than mechanical and mock controls at days 1 (P = .0089, P = .0207) and 7 (P = .0002, P = .0001) (Figure 2D).

Macrophage cell surface protein expression showed significantly elevated CD11b+/CD11c+ macrophages at day 4 (P = .0058) in the experimental group compared with the mock surgical control, while it was higher but not significantly different from the mechanical control (Figure 3A). CD86+/CD11b (M1) cells were significantly elevated (P = .0148, P = .0343, P = .0229) at days 4, 7, and 10, respectively, in the chemomechanical injury compared with the mock controls, although not significantly elevated against mechanical controls (Figure 3B). CD206+/CD11b+ (M2) cells were significantly increased for both the experimental group and mechanical injury control.
over mock surgery at days 4 \((P = .0041, P = .0034)\) and 7 \((P = .0102, P = .0387)\), with the chemomechanical injury group remaining elevated over the mock controls \((P = .0009)\) and significantly increasing \((P = .0196)\) over the mechanical control at day 10 (Figure 3C). Immunohisto-chemical staining (Figure 4) of chemomechanical injury mice demonstrated a high density of F4/80 macrophages (red staining in Figure 4B,D) in the lamina propria and epithelium at day 7 with a reduced presence at day 14.

**Discussion**

In this study, the “in situ in vivo” mouse LTS model demonstrated increased macrophage presence associated with fibrosis. The chemomechanical injury model revealed an initial acute inflammatory response followed by increased macrophage gene and protein expression preceding the significant increase in fibrosis gene markers at day 7 in a bleomycin-induced LTS, which is different from mechanical injury and mock controls. Macrophage immunophenotype demonstrated a proinflammatory M1 response by flow cytometry that was greater than the mock control but not significantly different from the mechanical injury alone. Furthermore, the M1 gene inducible nitric oxide synthase (iNOS) was not increased. In both the chemomechanical and mechanical injury cohorts, the M2 cell surface marker CD206 was elevated at early time points compared with mock controls.
mock controls, with the chemomechanical injury group remaining elevated at day 10 while the mechanical controls were not different from mock controls. Consistent significant gene elevation in Arg1 expression in the experimental cohort supports the finding of an increased M2 response.

Macrophages are immune effector cells that regulate the inflammatory response, phagocytize cells and debris, and stimulate fibroproliferation. Macrophages and their cytokine signaling contribute to a normal wound-healing response; however, dysregulated macrophages and cytokine release play a critical role in abnormal wound healing with resultant inflammation and fibrosis. Studies show macrophage immunophenotype to be associated with fibrosis in various organ systems. For example, renal and myocardial fibrosis are associated with increased M1 macrophages in some studies and increased M2 macrophages in others. The distal airway scar in interstitial pulmonary fibrosis is associated with increased M2 markers. These seemingly contradictory results suggest that the impact of macrophage immunophenotype on pathogenic wound healing may vary depending on the context, organ system, initiating event, or temporal variation. Figure 5 summarizes the mouse macrophage response, showing the expected increase in M1 macrophages in both chemomechanical and mechanical injury mice. While the M2 macrophage infiltration is similar at earlier time points, the chemomechanical injury group significantly increases above the mechanical injury mice at day 10. Pathological wound healing associated with M2 macrophages has been associated with increased Arg1 expression and reduced nitric oxide synthase (NOS) activity, as was also seen in this study. The proposed mechanism for this pathway is the arginase increase causing an upregulation of proline and subsequent increased fibroblast proliferation and collagen deposition. Further investigation into macrophage immunophenotype and its effect on human LTS may reveal potential druggable targets to reverse or slow the inflammation and fibrosis.

Current medical therapies are not successful in halting the development of laryngotracheal scar tissue. Specific immunomodulation may be more effective than current adjuvant therapies, such as steroids. Examples include transforming growth factor-β (TGF-β) inhibitors, which have been...
investigated in LTS, and tumor necrosis factor α (TNFα) inhibitors, which are effective in treating other inflammatory diseases.\textsuperscript{25,26} One advantage to the treatment of LTS over inhibitors, which are effective in treating other inflammatory diseases, investigated in LTS, and tumor necrosis factor α (TNFα) inhibitors, which are effective in treating other inflammatory diseases.\textsuperscript{25,26} One advantage to the treatment of LTS over inhibitors, which are effective in treating other inflammatory diseases.\textsuperscript{25,26} One advantage to the treatment of LTS over fibrosis in other organ systems, such as the heart, lungs, and kidneys, is the ability to both topically apply medications combined with mechanical dilation of the area of fibrosis. The application of a topical therapy targeting abnormal macrophages or other drivers of inflammation, perhaps via a drug-eluting stent, has the potential to prevent restenosis and halt the cycle of serial dilation. Furthermore, direct sustained local immunotherapy at the area of fibrosis avoids side effects associated with systemic therapy.

There are a few limitations to this study and the comparison between bleomycin-induced LTS and human LTS. The pathophysiology of mouse LTS is an acute process, accelerated by the chemotherapeutic agent, bleomycin, which may differ from patients with LTS, especially those whose fibrosis is more of a chronic process. While bleomycin-induced LTS may not exactly reproduce human LTS, bleomycin is a commonly used inducer in fibrosis models. A further benefit to this mouse model is the comparison between the experimental and 2 control groups. The mock control group, with its external injury, allows for assessment of transmural injury. The mechanical injury group may be considered a physiologic wound-healing group to compare with the sustained inflammation and excessive fibroproliferation seen in the chemomechanical injury group. Future studies may be used to further investigate differences between these groups and yield insight into why most patients who have long-term intubation or other risk factors do not develop LTS.

In summary, bleomycin-induced LTS demonstrated an increased presence of macrophages involved with the development of laryngotracheal fibrosis. Macrophage immunophenotype suggested that dysregulated M2 macrophages have a role in the abnormal laryngotracheal wound healing. The data presented here begin to delineate inflammatory cells and signaling pathways involved in LTS that may potentially be modulated to lessen fibroblast proliferation and collagen deposition. Further studies targeting these immune cells in the mouse model could help identify medical therapies for human clinical trials.

**Author Contributions**

Alexander T. Hillel, concept, drafting, approval, agreement; Idris Samad, acquisition, drafting, approval, agreement; Garrett Ma, acquisition, revising, approval, agreement; Dacheng Ding, acquisition, revising, approval, agreement; Kaitlyn Sadler, acquisition, revising, approval, agreement; Jonathan D. Powell, concept, revising, approval, agreement; Andrew P. Lane, concept, revising, approval, agreement; Maureen R. Horton, concept, revising, approval, agreement.

**Disclosures**

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**References**

9. Simonian PL, Roark CL, Diaz del Valle F, et al. Regulatory role of gammadelta T cells in the recruitment of CD4+ and
CD8+ T cells to lung and subsequent pulmonary fibrosis. *J Immunol*. 2006;177:4436-4443.


