Molecular Analysis of Idiopathic Subglottic Stenosis for Mycobacterium Species

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Objectives/Hypothesis: Idiopathic subglottic stenosis (iSGS) is an unexplained obstruction involving the lower laryngeal and upper tracheal airway. Persistent mucosal inflammation is a hallmark of the disease. Epithelial microbiota dysbiosis is found in other chronic inflammatory mucosal diseases; however, the relationship between tracheal microbiota composition and iSGS is unknown. Given the critical role for host defense at mucosal barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial pathogens.

Methods: Utilizing 30 human iSGS, 20 intubation-related tracheal stenosis (iLTS), and 20 healthy control specimens, we applied molecular, immunohistochemical, electron microscopic, immunologic, and Sanger-sequencing techniques.

Results: With unbiased culture-independent nucleic acid, protein, and immunologic approaches, we demonstrate that Mycobacterium species are uniquely associated with iSGS. Phylogenetic analysis of the mycobacterial virulence factor rpoB suggests that, rather than Mycobacterium tuberculosis, a variant member of the Mycobacterium tuberculosis complex or a closely related novel mycobacterium is present in iSGS specimens.

Conclusion: These studies identify a novel pathogenic role for established large airway bacteria and provide new targets for future therapeutic intervention.

Key Words: Mycobacterium, Mtb, idiopathic subglottis stenosis, tracheal stenosis, laryngotracheal stenosis, iSGS, ISS.

Level of Evidence: NA

INTRODUCTION

Idiopathic subglottic stenosis (iSGS) is a debilitating extrathoracic obstruction involving the lower laryngeal and upper tracheal airway. It arises without known antecedent injury or associated disease. Emerging study has demonstrated affected patients possess tightly conserved clinical demographics,1 histopathologic findings,2 anatomic injury,3 and physiologic impairment.4 Despite description of iSGS more than four decades ago,5 only recently has the inflammatory fibrosing phenotype been characterized at the molecular level. Data show highly upregulated activation of the inflammatory IL-17A/IL-23 pathway in the mucosal scar in iSGS, yet the mechanisms responsible for the characteristic demarcated airway inflammation are unknown.

In alternate pulmonary pathologies6–9 both structural and functional changes in the lung epithelium appear to be integral to fibrotic remodeling, occurring in the setting of chronic airway inflammation. Epithelial microbiota dysbiosis, with subsequent sustained host inflammation, is found in other chronic inflammatory mucosal diseases.10–18 Although the trachea is lined with respiratory epithelia, which readily support colonization by a diverse microbiome at other upper respiratory sites such as the oropharynx,19,20 to date nothing is
known of the composition of the resident microbiome of the large airway or its contribution to airway remodeling in idiopathic subglottic stenosis. Microbiological studies that rely on culture-based techniques underestimate the diversity of species present and offer limited detection of intracellular pathogens. The application of culture-independent approaches offers the opportunity to both provide a broader picture of tracheal microbiome composition and identify discrete pathogenic species associated with disease states.

Previously, work has demonstrated activation of the canonical IL-23/IL-17A pathway in the tracheal mucosa of iSGS patients, and has identified γδ T cells as the primary cellular source of IL-17A. Given the established role of γδ T cell IL-17A in host defense at mucosal barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial pathogens. Our unbiased molecular interrogation of the tracheal microbiota of iSGS patients provides detailed nucleic acid, protein, and immunologic evidence to demonstrate *Mycobacterium* species within tracheal scar. Together with our previous work, these studies offer new insights into the pathogenesis of iSGS. They suggest that human tracheal mucosal health is highly dependent on the composition of the resident microbiota, identify a novel pathogenic role for established large airway bacteria, and offer targets for future therapeutic interventions.

MATERIALS AND METHODS

This study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice, and was approved by the institutional review board (IRB) at Vanderbilt University Medical Center (IRB: 140429).

Patients

In all, 30 iSGS, 20 intubation-related tracheal stenosis (iLTS), and 20 normal control patients were utilized for experiments (Supp. Fig. S1). Each iSGS and immunoglobulin-like transcripts (iLTS) diagnosis was confirmed using previously described clinical and serologic criteria. The control population consisted of patients without known tracheal pathology, malignancy, or systemic infection. Tracheal scar or freshly isolated peripheral blood mononuclear cells (PBMC) was the source of all specimens from the iSGS and iLTS patients, and normal trachea or PBMC was the source for the control patients.

**Culture Independent Quantitative Polymerase Chain Reaction Profiling of Respiratory Microbiome**

**DNA Isolation.** Genomic DNA (gDNA) was extracted with the Qiagen DNAeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, with slight modification as previously described. The gDNA concentration and quality were confirmed using the Bioanalyzer 2100 system (Agilent, Santa Clara, CA). Human respiratory pathogen quantitative polymerase chain reaction (qPCR) array (Qiagen) was performed as per manufacturer’s instructions in a StepOnePlus instrument (Applied Biosystems, Foster City, CA). Expression analysis was performed using PCR array analysis software (Qiagen).

In Situ Hybridization for Mycobacterial Gene Product GyraseA

Paraflin-embedded iSGS and iLTS airway stenosis tissues and healthy controls (US Biomax Inc., Rockville, MD; # RS321) were pretreated and probed for Gyrase A (Advanced Cell Diagnostics, Hayward, CA, #436701) following a modified RNAscope 2.0 Assay’s HD Detection Kit (Red) (Advanced Cell Diagnostics) protocol. Tissue was digested with proteinase-K (1:100 dilution) (Sigma-Aldrich Co., LLC. St. Louis, MO) in 20 mM Tris-Cl (pH 8.0) for 5 minutes at room temperature. Experimental controls run in parallel included bacterial gene *DapB* as a negative control to assess background signal and *Homo sapiens* HS-PPRI B to assess positive signals and protocol efficacy.

Sanger Sequencing of Mycobacterial Species Molecular subtyping of Mycobacterial Species.

**Determination of DNA Sequence of Amplified Products**

The rpoB gene products were run on a 2% gel and purified the 360 bp band using the Qiagen QIAquick Gel Extraction kit (Qiagen) and sequenced directly on both strands in the Vanderbilt Cancer Center Core Sequencing Laboratory, Nashville, Tennessee. Alignments of the rpoB sequences were performed using Sequencher 5.3 software (Gene Codes Corporation, Ann Arbor, MI).

Immunogold Labeling

Human tracheal mucosal biopsies were obtained in the operating room and immediately fixed with chilled buffer (50 mM sodium cacodylate [pH 7.4]) containing 2.5% glutaraldehyde and 2.0% paraformaldehyde and placed in 4°C overnight. The samples were then prepared as previously described. Briefly, samples were blocked with 0.1% coldwater fish skin gelatin in 50 mM sodium cacodylate buffer and stained with rabbit polyclonal anti-*Mycobacterium tuberculosis* (Mtb) antibodies (LS-C72966, LSBio, Inc., Seattle, WA), followed by goat anti-rabbit IgG conjugated to 20 nm gold particles (Electron Microscopy Sciences, Hatfield, PA). Samples were washed three times with phosphate buffered saline containing 0.1% Tween 20 and analyzed with an FEI T-12 transmission electron microscope (FEI, Hillsboro, Oregon) equipped with a side-mounted digital camera. A total of 30 to 35 individual cells in each group were imaged to analyze subcellular architecture and presence of bacteria.

**Elispot**

Preparation of PBMC and 6-kDa early secreted antigenic target (ESAT-6) peptides and Elispot assay were performed, as described previously. The number of specific interferon gamma (IFN-γ) secreting T cells was calculated by subtracting the mean negative control value from the mean spot-forming cell (SFC) count for duplicate wells inoculated with peptide. Negative controls always had < 50 SFC per 10^6 input cells. A
positive response was defined as a concentration of at least 50 SFC/10^6 PBMC, which is at least three times higher than the background level. Research assistants were blind to the clinical diagnoses of the study participants throughout the analysis.

Statistical Analysis
Statistical significance was set at a P value less than 0.05, and a mean difference equal to or greater than two-fold change in expression levels. Normal distribution of the variables was tested using the Shapiro-Wilk test. Differences between the x and y groups were determined using the Kruskal-Wallis and Mann-Whitney tests for normal and nonnormal distributions, respectively. Data were expressed as median ± standard deviation for nonnormal distributed variables. All statistical analyses were performed with Prism version 6.0 software (GraphPad Software Inc., La Jolla, CA).

RESULTS
Culture-Independent Profiling of Respiratory Microbial Flora
Given the role of γδ T cell IL-17A in host defense against pathogens at epithelial and mucosal barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial species. All iSGS patients (10 of 10) demonstrated PCR positivity to Mycobacterium species (Fig. 1A), whereas only two of 10 iLTS patients were positive by PCR (P < 0.001). In contrast, among iLTS patients, 10 of 10 showed PCR positivity to Acinetobacter baumannii (an established ICU pathogen39), whereas only one of 10 iSGS patients showed a positive signal for this pathogen (P < 0.001).

Further confirmatory testing was preformed on an additional 10 iSGS, 10 iLTS, and 10 healthy controls with in situ hybridization probing RNA expression of the specific mycobacterial virulence factor DNA gyrase subunit A.40 Seven of 10 iSGS specimens tested positive, predominantly in the tracheal epithelium, whereas only one of 10 iLTS specimens and 0 of 10 healthy control samples showing detectable signal (P = 0.03) (Fig. 1B).

Localization of Mycobacterium Species Within iSGS Tracheal Scar
To further investigate for Mycobacterium species within tracheal scar tissue, we utilized Immunogold labeling and high-resolution transmission electron microscopy. This analysis revealed multiple structures with associated labels that exhibit typical size (500 nm–2 μm) and shape (coccos or bacillus) of Mycobacterium species within the extracellular matrix (Fig. 1D-E), whereas controls using secondary antibody alone (data not shown) or an unrelated antibody to Haemophilus influenzae (Fig. 1C) were negative. Digital quantification of gold labels per bacterial cell by computerized algorithm confirmed visual analysis of anti-Mycobacterium tuberculosis complex (MtbC) antibody binding in iSGS tissues (P < 0.005) (Supp. Fig. S2).

Systemic Immunologic Response to Mycobacterium Antigens in iSGS
After detection of nucleic and amino acid signal for mycobacteria within iSGS, we sought to assess the systemic immunologic response in iSGS. Utilizing EliSpot, we analyzed antigen-specific responses of peripheral leukocytes from iSGS patients to the mycobacterial ESAT-6. We chose ESAT-6 peptides due to prior reports of systemic cellular immune responses to these conserved MtbC virulence factors in sarcoidosis.28,31 Peripheral blood mononuclear cells from iSGS patients showed a mean IFN-γ spot-forming-cell (SFC) count of 165.9 (standard error of the mean [SEM] ± 42.4) compared with 27.4 (SEM ± 18.1) in normal controls (P < 0.0076) (Fig. 1F). This IFN-γ response suggests systemic immunologic memory to MtbC exposure and is consistent with a pathological role for MtbC in iSGS.

Mycobacterium Species Subtyping via Sanger Sequencing
It was not possible to subtype the Mycobacterium species based on the initial primers in our discovery assay; therefore, we utilized Sanger sequencing26 to further classify the Mycobacterium species in a subset of samples based on the rpoB gene sequence. The rpoB PCR yielded a product of 360 bp, which Sanger sequence analysis identified as MtbC in eight iSGS samples. Seven of the eight positive iSGS specimens showed consistent polymorphisms in the same locations (at 2,312 and 2,313 base pairs) (Fig. 2A). Whereas the predicted protein coding sequence from iSGS specimens was homologous to Mtb reference sequences (Fig. 2B), phylogenetic analysis of the amino acid sequencing suggests that rather than Mtb, a variant member of the MtbC or a closely related novel mycobacterium (Fig. 2C) is present in iSGS specimens.

DISCUSSION
We demonstrate through multiple distinct approaches the presence of Mycobacterium within tracheal scar of iSGS patients. Our prior findings suggest a major role for γδ T cells in the IL-17A-dependent tissue inflammation and fibrotic remodeling seen in the airways of iSGS patients.21 Given the established role for γδ T cells in the early production of IL-17A in MtbC infection,32 and the critical role for IL-17A in host clearance of pulmonary MtbC,33 our prior results are consistent with our current work demonstrating mycobacterial species within the airway scar of iSGS patients.

An early report describing iSGS as a clinical entity could not isolate bacterial species34 in routine microbiologic culture. Similarly, all iSGS patients included in our study were culture-negative. However, since this publication in 1993, culture-independent techniques have become an established alternate methodology for identification of infectious agents. Polymerase chain reaction was used to identify the etiologic agents of bacillary angiomatosis (Bartonella henselae)35 and Whipple’s disease (Tropheryma whippelii).36
Fig. 1. *Mycobacterium* species in iSGS patients. The qPCR results for panel of respiratory pathogens from 10 iSGS and 10 iLTS patients. (A) Yellow indicates positive PCR products; blue indicates negative result. Ten of 10 iSGS patients had detectable PCR products for *Mycobacterium tuberculosis complex* (*MtbC*), compared with two of 10 iLTS patients (two-tailed, chi-squared test with continuity correction; \( P < 0.001 \)). (B) Representative images from in situ hybridization for RNA of *Mycobacterium* gene product *gyraseA* (arrows depicting positive signal in iSGS specimen). Accompanying summary graph depicting seven of 10 iSGS patients with detectable in situ hybridization signal, compared with one 10 iLTS and 0 of 10 controls (two-tailed, chi-squared test; asterisk denotes \( P < 0.001 \)). Immunogold labeling with an anti-MtbC antibody and high-resolution transmission electron microscopy analyses revealed multiple structures with associated labels that exhibit typical size (500 nm–2 \( \mu \text{m} \)) and shape (coccoid or bacillus) of *Mycobacterium* spp. Treatment with secondary antibody alone (not shown) or an unrelated antibody to *Haemophilus influenzae* (C) revealed sparse labeling that was significantly less than the labeling achieved with the anti-Mtb treatment (D, E). Distribution of IFN-\( \gamma \) production from ESAT-6 stimulated peripheral blood mononuclear cells isolated from the peripheral blood of iSGS patients (red; \( n = 10 \)) or healthy controls (green; \( n = 10 \)). Bars represent the median (50th percentile), asterisk denotes significance (two-tailed, Mann Whitney test; \( P < 0.005 \)) (F). Ctrl = control; IFN-\( \gamma \) = interferon gamma; iLTS = iatrogenic laryngotracheal stenosis; iSGS = idiopathic subglottic stenosis; *MtbC* = *mycobacterium tuberculosis complex*; PCR = polymerase chain reaction; qPCR = quantitative polymerase chain reaction.
The use of antigen-specific immune responses to microbial antigens has also been utilized to identify novel infectious agents, including Sin Nombre virus in hantavirus pulmonary syndrome,\textsuperscript{37} as well as a previously unknown coronavirus in severe acute respiratory syndrome\textsuperscript{38,39} and \textit{Mycobacterium} in sarcoidosis.\textsuperscript{28,40} Peripheral blood mononuclear cells from iSGS patients that are stimulated ex vivo with mycobacterial virulence factor ESAT-6 demonstrate a pronounced IFN\textgreek{y} response. This finding suggests that, despite negative culture results from iSGS specimens, mycobacterial antigens induce T-cell-specific responses in the blood of iSGS patients at similar frequencies to those of tuberculosis subjects.\textsuperscript{28} The observation of a pronounced cellular immune response to \textit{Mycobacterium} EAST-6 antigens in all 10 iSGS patients tested strongly supports the results from our molecular and protein analysis of iSGS scar.

The inability to identify mycobacterial microorganisms by routine histologic staining or to culture microorganisms from pathologic tissues provides caution to the establishment of a causative role for infectious agents in iSGS pathogenesis. However, based on prior microbiological experience with fastidious mycobacteria, there are several explanations for the failure to detect microbial species in iSGS in the initial reports of the disease: The bacteria may be present in quantities below the detection of histologic staining.\textsuperscript{41} Alternatively, the agent may have an ultraslow growth pattern that necessitates incubation periods much longer than the standard 6 weeks that cultures are held for isolation of \textit{Mycobacterium} tuberculosis, which is similar to the time needed for isolation of \textit{M. ulcerans}.\textsuperscript{42} Conversely, iSGS pathogenesis may reflect an immune response to infectious antigens and might not be dependent on actively replicating organisms, which is similar to the hypersensitivity pneumonitis that is induced by \textit{Mycobacterium avium}.\textsuperscript{43}

An association between \textit{Mycobacterium} and iSGS immunopathogenesis is supported by the detection of
mycobacterial proteins and nucleic acids in iSGS scar, as well as local and peripheral cellular immune responses to mycobacterial antigens in iSGS subjects. However, it remains unresolved whether the identified mycobacterial constituents drive disease or whether inflammation per se creates a niche for the outgrowth of specific bacteria. It should be noted, however, that tracheal stenosis arising after intubation (ILT; which also possess an inflammatory tissue phenotype in the airway) appears in our cohort to have a much lower percentage of patients with detected Mycobacterium. Given the disease rarity, these results will require confirmation in larger cohorts pooled from multiple institutions.

The presence of Mycobacterium within iSGS scar is particularly striking in light of proven association of Mycobacterium with otherwise healthy, older white females (the Lady Windermere syndrome). The characteristics of these patients (women with immunocompromise or underlying chronic lung disease and proven pulmonary Mycobacterium infection) closely mirror the iSGS population. Lady Windermere patients are predominantly Caucasian (86%) women (81%) presenting in their mid-sixties. The dramatic demographic similarities of the two diseases (NTM pulmonary infection/Lady Windermere syndrome and iSGS) offers clinical precedent for a pathogenic role for Mycobacterium in the development or progression of iSGS.

Although our results demonstrate Mycobacterium species within the tracheal scar of iSGS patients, the role of host genetics to iSGS pathogenesis has not yet been explored. Interestingly, strong alternate evidence links host genotype to mycobacterial susceptibility via the IL-23/IL-17A axis. Molecular analysis of patients links host genotype to mycobacterial susceptibility via both the ligand and receptor (IL-12R) disease has implicated polymorphisms in both the ligand and receptor (IL-12R) disease.50–52 Alternatively, multiple drugs are available targeting the IL-17A pathway.50–52 The benefit of IL-17A inhibition in the absence of pathogen control is unclear; thus, future clinical trials could test the clinical response of immunomodulation in combination with antibacterial therapy. Therefore, the implications this work may extend beyond the confines of iSGS to other disease arising at the interface of pathogen and host inflammatory response.

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AUTHOR CONTRIBUTIONS
A.G. designed and performed experiments, analyzed data, and wrote the article; N.K. analyzed data; M.M. designed and performed experiments; D.N. designed and performed experiments; B.R. aided in experimental design; J.D., E.S.E., E., J.K., A.H., analyzed data, preformed critical scientific review; G.G. aided in experimental design; L.Y. analyzed data; J.L. conducted experiments; J.N. aided in experimental design; C.W. aided in experimental design; D.F. aided in experimental design, statistical analysis; C.S. conducted experiments; K.J. conducted experiments; T.M. aided in experimental design, data analysis, review of article; T.B. aided in experimental design, experiments, data analysis, review of article; J.G. conducted experiments; W.D. aided in experimental design, experiments, data analysis, review of article.

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