Published in final edited form as:

Laryngoscope. 2019 January ; 129(1): 177–186. doi:10.1002/lary.27321.

T-Helper 2 Lymphocyte Immunophenotype Is Associated With Iatrogenic Laryngotracheal Stenosis

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Abstract

Objective/Hypothesis: This prospective controlled human and murine study assessed the presence of inflammatory cells and cytokines to test the hypothesis that immune cells are associated with fibroproliferation in iatrogenic laryngotracheal stenosis (iLTS).

Methods: Inflammation was assessed by histology and immunofluorescence (IF), quantitative real-time polymerase chain reaction (qRT-PCR), and flow cytometry of cricotracheal resections of iLTS patients compared to normal controls. An iLTS murine model assessed the temporal relationship between inflammation and fibrosis.

Results: iLTS specimens showed increased inflammation versus normal controls (159/high power field [hpf] vs. 119/hpf, $P = 0.038$), and increased CD3 + T-cells, CD4 + cells, and CD3+/CD4 + T-helper (T_H) cells (all $P < 0.05$). The inflammatory infiltrate was located immediately adjacent to the epithelial surface in the superficial aspect of the thickened lamina propria. Human flow cytometry and qRT-PCR showed a significant increase in interleukin (IL)-4 gene expression, indicating a T_H2 phenotype. Murine IF revealed a dense CD4 + T-cell inflammatory infiltrate on day 4 to 7 postinjury, which preceded the development of fibrosis. Murine flow cytometry and qRT-PCR studies mirrored the human ones, with increased T-helper cells and IL-4 in iLTS versus normal controls.

Conclusion: CD3/CD4 + T-helper lymphocytes and the proinflammatory cytokine IL-4 are associated with iLTS. The association of a T_H2 immunophenotype with iLTS is consistent with findings in other fibroinflammatory disorders. The murine results reveal that the inflammatory infiltrate precedes the development of fibrosis. However, human iLTS specimens with well-developed fibrosis also contain a marked chronic inflammatory infiltrate, suggesting that the continued release of IL-4 by T-helper lymphocytes may continue to propagate iLTS.

Keywords

Laryngotracheal stenosis; fibrosis; T-lymphocytes; human study; murine model

INTRODUCTION

Laryngotracheal stenosis (LTS) is a fibrotic disease that causes narrowing of the laryngeal and/or tracheal airway resulting in dyspnea and dysphonia.^{1,2} The stenosis usually occurs within the larynx or proximal trachea, although it can occur more distally. When fibrosis forms in the subglottis, it creates a rough surface that leads to turbulent flow and dysphonia.^{1,3} Up to 30% of patients with LTS have significant dyspnea requiring a tracheostomy, compounding the communication disability, which in severe cases results in aphonia.⁴ In many cases, it is a chronic disease with an average healthcare cost above \$5,000/year per person (unpublished).

The principal etiologies of LTS are iatrogenic, idiopathic, and autoimmune.^{2,4} Iatrogenic laryngotracheal stenosis (iLTS) secondary to prolonged intubation occurs in 3% to 12% of adults and up to 11% of infants.⁵⁻⁷ iLTS occurs more frequently than other etiologies, with studies demonstrating that endotracheal tube sizes 7.5 or greater place patients at higher risk for iLTS.^{4,8-10} iLTS generally presents more distal to the vocal folds and has a greater length and severity of stenosis when compared with other etiologies.⁸ In addition, iLTS patients have greater comorbidities, including those that promote microvascular injury and dysregulated wound healing such as smoking, chronic obstructive pulmonary disease, and diabetes, as well as obstructive sleep apnea and hypertension.^{4,8} These comorbidities, combined with more advanced Cotton-Myer staging and associated tracheomalacia, are predictors of worse iLTS outcomes, specifically a higher rate of tracheostomy dependence.^{4,8}

Improving outcomes in iLTS treatment is dependent on addressing the knowledge gap with regard to the pathophysiology of iLTS. Fibroblasts represent the end effector cell. By the time that increased collagen deposition results in clinical stenosis, current medical therapies are unable to reverse the fibrosis, committing the patient to a crude therapeutic regimen of repeated surgical dilation or the excision of fibrosis. Treatment paradigms, both surgical and medical, primarily focus on scar tissue. Surgical procedures, including endoscopic excision and dilation, cricotracheal resection, and laryngotracheoplasty, enlarge the airway and/or excise the scar tissue and have remained generally unchanged over the last 50 years.^{11,12} Furthermore, medical therapies, including antibiotics, mitomycin C, and systemic and local steroids, have shown inconsistent evidence of efficacy.¹³⁻¹⁶

The current perspective of iLTS is that of a fibrotic disease with hyperproliferative fibroblasts, often associated with a myofibroblast phenotype. In iLTS, the initial mechanical injury is initiated by a combination of 1) mucosal injury from and 2) foreign body reaction to the endotracheal or tracheostomy tube.¹⁷ Although wound regeneration and repair is initially physiologic, wound healing becomes prolonged and pathologic when unchecked. In iLTS, this leads to considerable abnormal tissue remodeling of the laryngotracheal lamina propria, where the synthesis of new collagen and other matrix molecules exceeds the rate of degradation, replacing normal tissue and ultimately resulting in the formation of permanent scar tissue.¹⁸

Recently, there has been a shift with investigations focusing on dysregulated immune cells as potential effector cells underlying the development of iLTS.^{19,20} Animal models suggest that inflammatory cells and their mediators contribute to the fibrosis. These results suggest that B-and/or T-lymphocytes as well as macrophages mediate the fibroproliferative response in ILTS. Combined, these studies suggest that signaling between immune cells and fibroblasts is critical to the fibrosis seen in ILTS; however, specific lymphocyte subsets have not been identified.^{19,20} The objective of this study is to characterize the inflammatory cells and cytokines that mediate the development of fibrosis in iLTS and test the hypothesis that inflammation is associated with the initiation and propagation of fibrosis in iLTS.

Improved understanding of inflammatory cells, signaling pathways, and potential immunological mechanisms in iLTS will allow for identification of rational therapeutic targets that may be modulated to lessen fibroblast proliferation and collagen deposition. An inflammatory symphony is an apt analogy when considering the delicate balance and timing of immunological signaling that initiates, sustains, and suppresses the fibrotic process. In fibrosis, the system is no longer functioning in concert.²¹ Therapy that inhibits proinflammatory cytokines combined with those that promote immunosuppressive factors will be critical to restoring an inflammatory balance that allows for physiologic wound healing and maintaining airway patency in ILTS. Ultimately, it could translate to targeted, effective therapeutics for fibroproliferative diseases in general.²¹

METHODS

Experimental Design

A prospective controlled human and mouse study, using immunofluorescence (IF) as the primary outcome measure and with gene and protein expression as secondary outcome measures. Histology and IF of human specimens (n = 10) obtained from cricotracheal/tracheal resections performed on iLTS patients were compared with histology and IF of healthy laryngotracheal specimens (normal control). Protein expression was subsequently performed on mucosal secretions taken from patients (n = 6) with iLTS and compared with secretions obtained from normal patients (n = 6). Gene expression of laryngotracheal scar (n = 7) were compared with normal tracheal mucosa (n = 7) from the same iLTS patients. Mouse specimens for IF, flow cytometry, and quantitative real-time polymerase chain reaction (qRT-PCR) were obtained from a validated mouse model of iatrogenic laryngotracheal stenosis.^{20,22}

Human Tissue Sampling

Informed written consent was obtained from all participants in accordance with the Johns Hopkins University Institutional Review Board (NA_00078310). Histology specimens were fixed in 10% formalin for 24 hours and then embedded in paraffin. Swab samples of laryngotracheal mucosal secretions for protein analysis and brush samples for gene expression analysis were obtained and processed as previously described.²³

Human Histologic Analysis and Protein Analysis by Immunofluorescence

Immunofluorescence slides underwent primary antibody staining with rabbit anti-human CD 3 (lot #: ab5690, Abcam, Cambridge, MA), mouse anti-human CD4 (lot #: ab846, Abcam), rabbit anti-human CD8 (lot #: sc-7188, Santa Cruz Biotechnology, Dallas, TX), and rabbit anti-human CD20 (lot #: RPA11836, ReproKine Ltd, Rehovot, Israel) followed by secondary antibody (Life Technologies, ThermoFisher Scientific, Waltham, MA). Quantification of lymphocytes was performed by calculating the positive-stained cells at high magnification (400×) from three areas of intense inflammation identified at low magnification (100×).²⁴ Percentage of lymphocytes was determined by dividing positive-staining cells by total cells in the high-power field (hpf). Human gene primer (Integrated DNA Technologies, Coralville, IA) sequences are shown in Supporting Table S1.

Mouse iLTS Model

This study was approved by the Johns Hopkins University Animal Care and Use Committee (MO12M354). Seventy-two C57BL/6 (Charles River Laboratory, Germantown, MD) mice were used. Mouse laryngotracheal complexes were chemomechanically injured with a bleomycin-coated wire brush.²⁰ Mice were sacrificed at 1, 4, 7, and 14 days following injury for gene expression analysis and at 4, 7, and 10 days following injury for protein expression with IF and flow cytometry as previously described.²² Results were compared with normal mouse tracheas. Murine gene primer (Integrated DNA Technologies) sequences are detailed in Supporting Table S2.

Statistical Analysis

Differences between iLTS scar and normal trachea samples were compared using a nonparametric Wilcoxon matched-pairs signed-rank test. A Mann-Whitney test was used to assess differences between the iLTS and normal subgroups. Results are displayed as the mean fold change, and standard error. A type I error rate (α) of less than 0.05 was considered statistically significant. Data analysis was performed using Prism software (GraphPad Software Inc., La Jolla, CA).

RESULTS

Patient Demographics

Patient demographics for diseased specimens used in this study are included in Supporting Table S3.

CD4 + T-lymphocytic Infiltrate Is Present Immediately Adjacent to the Epithelium in Human iLTS.—The affected mucosa in the 10 human cricotracheal resections specimens demonstrated an inflammatory infiltrate located in the superficial lamina propria immediately adjacent to the epithelium (periepithelial) (Fig. 1B). Immunostaining revealed a predominantly CD4 + T-lymphocytic infiltrate (Fig. 1C–D). In contrast to normal specimens (Fig. 2A, 2C), the iLTS lamina propria (Fig. 2B, 2D) was significantly thickened with a dense inflammatory infiltrate. Immunofluorescent staining revealed that normal tracheas had little T-lymphocyte (CD3) (Fig. 3A) or T-helper cell (CD4) (Fig. 3C) presence relative to the dense CD3 (Fig. 3B) and CD 4 (Fig. 3D) staining in

iLTS specimens. Normal and iLTS specimens had no cytotoxic T-lymphocytes (CD8) (Fig. 3E–F) or B-lymphocytes (CD20) (Fig. 3G–H).

iLTS specimens showed increased inflammation compared to normal controls, with a greater mean density of total cells (159 cells/hpf vs. 118 cells/hpf, $P = 0.038$), CD3 + cells (31 cells/hpf vs. 8 cells/hpf, $P = 0.0031$), CD 4 + cells (31 cells/hpf vs. 12 cells/hpf, $P = 0.03$), and CD 3+/CD4 + T-helper cells (13 cells/hpf vs. 2 cells/hpf, $P = 0.0087$). In these dense areas of inflammation, 21% (vs. 7% of normal, $P = 0.014$) (Fig. 4A) of cells were T-lymphocytes, as shown by CD3 + staining, whereas 8% (vs. 2% of normal, $P = 0.012$) (Fig. 4B) were CD4 T-lymphocytes. There was no difference in cytotoxic T-lymphocyte percentage in iLTS versus normal specimens (Fig. 4C).

Increased Human IL-4 Protein and Gene Expression.—In comparison with normal controls ($n = 6$), iatrogenic specimens ($n = 6$) demonstrated significantly greater IL-4 protein (7.19 picogram [pg]/mg vs. 1.78 pg/mg, $P = 0.005$) (Fig. 5A) and gene expression (6.23-fold higher, $P = 0.047$) (Fig. 6A). Other T-helper (T_H) 1/ T_H2 / T_H17 cytokines assayed were not significantly different between groups (Fig. 5B–G and Fig. 6B–G).

Increased CD4 + T-Lymphocyte Presence in Murine iLTS.—Immunofluorescence demonstrated a thickened lamina propria with a dense inflammatory infiltrate that was prominent within the posterior aspect of the trachea of iLTS mice (Fig. 7A). The inflammatory infiltrate was noticeable at day 4 and peaked at day 7 (Fig. 7B) with a reduced presence at day 14. Immunofluorescent staining of iLTS mice demonstrated intense CD3 + and CD4 + staining (Fig. 7C–D). Normal tracheas had no inflammatory infiltrate and little T-lymphocyte (CD3) or T-helper cell (CD4) presence.

Cell surface protein expression with flow cytometry showed significantly elevated CD3 + lymphocytes at days 4 ($P = 0.027$), 7 ($P = 0.002$), and 10 ($P = 0.012$) in the experimental group compared with the normal control (Fig. 8A). CD3+/CD4 + (T-helper) cells were significantly elevated ($P = 0.035$), at day 4 in the mouse iLTS model compared with the controls (Fig. 8B). CD3+/CD8 + (cytotoxic T) cells were not different between groups (Fig. 8C).

Increased Murine IL-4 Gene Expression by qRT-PCR.—Mouse iLTS specimens demonstrated significantly greater IL-4 expression (6.23-fold higher, $P = 0.047$) (Supporting Fig. S1A) on day 4 relative to normal. Other cytokines (IL-2, IL-6, IL-10, IL-17a, tumor necrosis factor alpha, interferon gamma) were not different from normal controls (Supporting Fig. S1 B–G).

DISCUSSION

This study demonstrated a CD4 + T-lymphocytic infiltrate that precedes the development of fibrosis, and persists, even after the fibrosis forms. Through a combination of gene and protein analysis of human and mouse specimens, the proinflammatory cytokine IL-4 was consistently expressed at higher levels than normal controls, supporting a T_H2 immunophenotype in iLTS. These findings presented here expand on recent publications

investigating immune cell presence in murine¹⁹ and human iLTS.²⁵ Although Ghosh et al. implicated lymphocytes in murine laryngotracheal stenosis,¹⁹ the data presented here more specifically nominate CD4 + T-lymphocytes as the critical adaptive immune cells associated with fibrosis. To further to elucidate a T-lymphocyte immunophenotype in iLTS, the cytokine profile of the tracheal mucosal surface was sampled using gel foam swabs. In the human cohort, protein expression demonstrated a significant increase in IL-4 expression in iLTS specimens versus normal controls (Fig. 5), with no difference in other cytokines. The increased IL-4 protein expression was confirmed with increased IL-4 gene expression analysis in biopsies of human iLTS specimens (Fig. 6) and murine iLTS specimens (Supporting Fig. S1). The combination of increased IL-4 gene and protein expression supports previous findings in iLTS by Motz et al. demonstrating higher levels of IL-4 gene expression.²³ IL-4 is principally secreted by T_H2 cells and rarely natural killer T-cells, eosinophils, basophils, and mast cells.²⁶ IL-4 has been associated with fibroproliferative diseases, including in lesional biopsies in human systemic sclerosis,²⁷ in plasma specimens in patients with cardiac fibrosis,²⁸ and with progression of human idiopathic pulmonary fibrosis.²⁹ Additionally, IL-4 has been shown to promote myofibroblast differentiation, collagen deposition, and fibroblast proliferation.³⁰ Its detection in surface secretions and within periepithelial tissue biopsies of iLTS patients suggests it to be a collagen-producing factor in these patients' active disease process.

The temporal pattern of these immunologic findings provides insight into the pathophysiology of iLTS. Persistent periepithelial T_H2 inflammation was identified in human specimens collected 3 to 25 months after initial intubation injury. The mouse model of iLTS was then utilized to assess the timing of greatest T-lymphocytic infiltrate, which peaked at 7 days. An earlier publication using the same mouse model demonstrated fibrosis to develop 14 to 21 days after initial injury.²⁰ A subsequent publication studying macrophage inflammation in the iLTS murine model showed increased macrophage presence on days 4 to 10 following injury with a persistent M2 immunophenotype preceding the development of fibrosis.²² On day 4, IL-4 gene expression was also increased. The early presence of the T_H2 infiltrate, combined with previously reported M2 inflammation, provides evidence that a T_H2/M2 immunophenotype is associated with the subsequent fibrosis in a murine model.²² This immunophenotype and the timing of Th2-cell response is similar to that found in fibrosis in other organs. CD4 + T-cell dependent pathways are key regulators of fibrosis of the lung, kidney, and skin.^{31–35} Prolonged Th2 responses with upregulated IL-4 and/or IL-13 cause dysregulated wound healing and increased fibrosis.^{21,36–38} Conversely, increasing the T_H1 effector subset with IFN γ , usually a potent proinflammatory cytokine, when its release is timed correctly, can result in reduced tissue fibrosis.^{21,31}

In addition to the temporal pattern of inflammation, its anatomic location further contributes to our understanding of iLTS pathophysiology. When compared with normal tracheas, there were significantly more CD4 + T-cells, which were consistently identified just deep to the epithelium in iLTS specimens (Fig. 2). These active immune loci implicates the initial epithelial injury with the subsequent fibrosis within the lamina propria. This finding adds detail to Minnigerode et al.'s histologic study leading to the hypothesis that mechanical

injury leads to subepithelial inflammation with thickening of the lamina propria connective tissue.¹⁷

The architectural findings of T-lymphocyte inflammation in the periepithelial region supports the use of topical application of medical therapies. Given the heterogeneity of T-lymphocyte subtypes and the multiple mechanisms of stimulation or attenuation with fibroblasts in the trachea, it is unlikely that universal T-lymphocyte depletion would be therapeutically beneficial in patients with iLTS, irrespective of systemic side effects.³⁸ A more promising therapeutic avenue may be specific phenotypic modulation of the infiltrating T-cells toward antifibrotic phenotypes.³⁸ Therapies that could be delivered locally to the larynx and trachea may avoid some of the side effects of systemic phenotypic modulation. Reduction of periepithelial inflammation may explain why local therapies including serial subepithelial steroid injections and excision of stenosis with placement of split-thickness skin graft circumferentially around the excised scar have efficacy in reducing or preventing recurrence of fibrosis.^{39,40} In the future, injection of specific immunotherapy would allow for directed therapy at the site of peak inflammation without systemic side effects. Alternatively, a shortterm drug eluting stent that targets IL-4 and/or T_H2 cells to modulate immunophenotype could result in sustained localized therapy to prevent fibrosis.

The limitations of this study included tissue availability, necessitating the use of different cohorts for each study methodology. In humans, protein extraction of intraluminal secretions was not adequate to assess lymphocytes due to their presence in the mucosa but instead could be employed to quantify secreted cytokines. These cytokines, available on the epithelial cell surface, were presumed to be released by subepithelial T-lymphocytes. To quantify lymphocyte presence in the lamina propria in human specimens, IF allowed for a more comprehensive evaluation of proteins than a single cell suspension, which would rapidly exhaust the specimens. In contrast, in mice, flow cytometry could quantify T-lymphocyte presence because tracheas were harvested from multiple mice and combined into a single sample to assess for lymphocyte surface markers.

The focal geographic nature to the inflammation in iLTS also raises the possibility of sampling error. This would be most likely to affect the specimens acquired with biopsy forceps or sponge swabs, which are not capturing areas of inflammation. One method to consider in future studies is laser capture microdissection, which would allow for guided selection of the dense immune loci using a microscope, avoiding potential sampling error. Notwithstanding, the concordance of results between the different types of specimens demonstrate a strong association of T_H2 lymphocytes with iLTS in both mice and humans.

The translation of the protein extraction technique from animal to human tracheal secretions utilized in this study adds to recent advances in surveillance of surface gene expression in LTS patients of all etiologies.^{23,41} Small biopsies of ILTS specimens to assess gene expression in humans has been validated as an accurate method to extract sufficient RNA to assess surface inflammatory cytokines.²³ Whereas specimens were collected in the operating room, the next advancement in superficial tissue sampling would be performing specimen acquisition during office-based bronchoscopy. This would allow for a continual assessment

of inflammation, with the data generated potentially allowing for diagnostic identification of biomarkers for LTS etiology and/or as a predictive response to therapy.

Although the mouse is an acute iLTS model, its similar pathophysiology and immunophenotype validate it as a model to study the molecular alterations that initiate and promote laryngotracheal fibrosis. It also allows for further investigation into the effects of host immunity, the microbiome, and interaction with adjacent systems such as the gastrointestinal tract. Unlike human studies, the mouse model can study these factors independent of each other, taking advantage of genetically altered mice to isolate individual molecules or cell types. For example, the mouse model could be utilized to test the association of T_H2 lymphocytes with iatrogenic laryngotracheal stenosis demonstrated in this study by selectively deleting functional T-lymphocytes or T_H2-lymphocytes to assess the progression of fibrosis. Finally, the model will provide a platform for preclinical testing of therapeutics that could reduce or reverse the development of fibrosis.

CONCLUSION

iLTS is a CD4 T-lymphocyte-mediated fibroinflammatory disease. Human and murine data demonstrates significant IL-4 presence, that is, a T_H2 immunophenotype, which fits with known fibroinflammatory diseases. An increased CD4 + T-lymphocyte population was identified prior to the development of fibrosis in mice and was present in human cricotracheal resection specimens as long as 2 years after initial injury, indicating that although inflammation may precede the fibrosis, it persists and likely continually perpetuates it. IL-4 gene expression was detected within the human subepithelial tissue, and the protein was significantly increased within the luminal secretions of iLTS patients. In sum, the data presented here depicts a T_H2/M2 immunophenotype that is associated with the development of laryngotracheal fibrosis. It also suggests that local therapies, including subepithelial injection or a drug eluting stent, that modulate immunophenotype could more specifically target immune cells and cytokines to prevent or even reverse fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Research reported in this publication was supported by the National Institute on Deafness and Other Communication Disorders (NIDCD) of the National Institutes of Health (NIH) under award number 1K23DC014082 (A.H.). This study was also financially supported by the Triological Society and American College of Surgeons (A.H.).

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Fig. 1. Histologic and immunofluorescent assessment of human iLTS. Photomicrograph of (A) human iLTS trachea ($2 \times$ magnification) demonstrates circumferentially thickened lamina propria with irregular epithelium. The white box denotes the area sampled for confocal microscopy seen in (B) showing periepithelial inflammatory infiltrate within the lamina propria deep to the epithelium in the superficial aspect of the fibrotic lamina propria ($100 \times$ magnification, hematoxylin and eosin stain). (C) Immunofluorescent staining depicts a high density of CD3 (green chromagen) and CD4 (red chromagen) double positive cells at $100 \times$ magnification and (D) at $400 \times$ magnification. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.] iLTS = iatrogenic laryngotracheal stenosis.

Fig. 2.

Histologic images of periepithelial specimens from normal and iLTS human tracheas. (A) Photomicrograph of a healthy control trachea demonstrating a normal lamina propria width (blue brackets, between the epithelium and underlying tracheal cartilage, 50 × magnification) compared with (B) a specimen from a patient with iLTS demonstrating thickened fibrotic lamina propria and dense immune cell infiltrate just beneath irregular epithelium (50 × magnification). For immunophenotyping of immune cell infiltrate, please see Figure 4. Higher magnification (400×) of (C) normal and (D) diseased mucosa. All panels from hematoxylin and eosin-stained specimens. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

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Fig. 3.

Lymphocyte immunofluorescence of human normal and iLTS specimens. Photomicrographs depicting little (A) T-lymphocyte (CD3) or (C) helper T-lymphocyte (CD4) presence in normal tracheal mucosa compared with intense staining for (B) CD3 (green chromagen) and (D) CD4 (red chromagen), indicative of a dense CD4 + T-lymphocyte infiltrate within iLTS periepithelial mucosa. There was no evidence of (E, F) cytotoxic T-lymphocytes (CD8) or (G, H) B-lymphocytes (CD20) in normal or diseased specimens. All specimens 400 × magnification. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

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** = $p < 0.01$

* = $p < 0.05$

Fig. 4.

Human T-lymphocytes and T-helper lymphocytes are present in significantly greater numbers in iLTS specimens than normal subglottic specimens. Bar graphs comparing the cell density (cells/hpf) for mean total cells (A), CD3 + cells (B), CD8 + cells (C), CD3 + (D), CD3+/CD4 + cells (E), and CD3+/CD8 + cells (F) in areas of high-density inflammation in both iLTS and normal specimens. * $P < 0.05$; ** $P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.] hpf = high power field; iLTS = iatrogenic laryngotracheal stenosis.

Fig. 5.

Flow cytometry of human protein extraction from laryngotracheal mucosal secretions of patients with iLTS compared with laryngotracheal secretions from control patients. There was a significant increase in (A) IL-4 expression in iLTS. There was no difference in protein expression in other (B–G) $T_H1/T_H2/T_H17$ cytokines assessed. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

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Fig. 6.

Gene expression analysis of diseased laryngotracheal mucosa compared with healthy mucosa from the same patients with iLTS. There was a significant increase in (A) IL-4 gene expression in diseased mucosa. There was no difference in (B–G) other cytokines. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.] iLTS = iatrogenic laryngotracheal stenosis.

Fig. 7.

Immunofluorescence demonstrates a thickened mouse trachea with dense CD3 + and CD4 + staining. Photomicrographs taken on day 7 after injury in a murine iLTS model depict (A) thickened lamina propria and a dense posterior periepithelial CD3+/CD4 + infiltrate (50 × magnification). (B) Inset, 200 × magnification. (C) A second day-7 specimen also shows CD3+/CD4 + staining with (D) inset, 400 × magnification. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

CD3: green chromagen; CD4: red chromagen; iLTS = iatrogenic laryngotracheal stenosis.

Fig. 8.

Flow cytometry of mouse tracheas demonstrates a CD4 + T-lymphocyte predominance. Cell surface protein expression with (A) increased CD3 + expression at days 4, 7, and 10 in the mouse iLTS group when compared to controls. Subset analysis revealed the T-lymphocytes to be (B) CD3+/CD4 + T-helper cells with few (C) CD3+/CD8 + cytotoxic T-lymphocytes.

[Color figure can be viewed in the online issue, which is available at

www.laryngoscope.com.]

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