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Myeloid Phenotypes in Tracheostomy-Associated Granulation Tissue

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Objective(s): Tracheostomy-associated granulation tissue is a common, recurrent problem occurring secondary to chronic mucosal irritation. Although granulation tissue is composed of predominantly innate immune cells, the phenotype of monocytes and macrophages in tracheostomy-associated granulation tissue is unknown. This study aims to define the myeloid cell population in granulation tissue secondary to tracheostomy.

Methods: Granulation tissue biopsies were obtained from 8 patients with tracheostomy secondary to laryngotracheal stenosis. Cell type analysis was performed by flow cytometry and gene expression was measured by quantitative real-time polymerase chain reaction. These methods and immunohistochemistry were used to define the monocyte/macrophage population in granulation tissue and were compared to tracheal autopsy control specimens.

Results: Flow cytometry demonstrated macrophages (CD45+CD11b+) and monocytes (CD45+FSC^{low}SSC^{low}) represent 23.2 ± 6% of the granulation tissue cell population. The M2 phenotype (CD206) is present in 77 ± 11% of the macrophage population and increased compared to the M1 phenotype ($p = 0.012$). Classical monocytes (CD45+CD14^{high}CD16^{low}) were increased in granulation tissue compared to controls (61.2 ± 7% and 30 ± 8.5%, $p = 0.038$). Eighty-five percent of macrophages expressed pro-inflammatory S100A8/A9 and 36 ± 4% of macrophages co-localized CD169, associated with tissue-resident macrophages. M2 gene expression (Arg1/CD206) was increased in granulation tissue (3.7 ± 0.4, $p = 0.035$ and 3.5 ± 0.5, $p = 0.047$) whereas M1 gene expression (CD80/CD86) was similar to controls ($p = 0.64$, $p = 0.3$). Immunohistochemistry of granulation tissue demonstrated increased cells co-localizing CD11b and CD206.

Conclusions: M2 macrophages are the dominant macrophage phenotype in tracheostomy-associated granulation tissue. The role of this cell type in promoting ongoing inflammation warrants future investigation to identify potential treatments for granulation tissue secondary to tracheostomy.

Key Words: granulation tissue, granuloma, laryngotracheal stenosis, M2 macrophage, macrophage polarization, monocytes, tracheostomy.

Level of Evidence: 3

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INTRODUCTION

Tracheostomy-associated granulation tissue is a common and recurrent problem that occurs in tracheostomy-dependent patients, attributed to chronic mucosal irritation secondary to the prosthesis. Tracheostomy-associated granulation tissue occurs in 4% to 80% of all pediatric tracheostomies[1] and can also develop in adult patients requiring tracheostomy for chronic ventilator dependence or laryngotracheal stenosis (LTS).[2,3] Acutely, tracheostomy-associated granulation tissue can lead to bleeding, upper airway obstruction, respiratory failure after decannulation, failure to wean from the ventilator, and failure to decannulate.[4] In the long term, granulation tissue at the site of the tracheostoma promotes circumferential wound contracture and can lead to fibrosis and tracheal stenosis that ultimately require intervention.[4,5]

Granulation tissue formation is part of the repair phase of wound healing, providing a scaffold for cell migration. Within granulation tissue, macrophages help to remove debris and release inflammatory cytokines that promote wound healing. However, in patients with tracheostomy and other airway prostheses, persistent inflammation and constant movement of the prosthesis against the tracheal mucosa leads to granulation tissue formation.[6] Additionally, tracheostomy tubes provide a potential surface for bacteria growth and biofilm formation, exacerbating the local inflammatory response and promoting immune cell recruitment.[6,7] Granulation tissue is composed of a localized aggregate of host immune cells around a pathogen or site of persistent irritation. Histologically, granulation tissue displays an increased population

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of monocytes and macrophages recruited to the site of injury and/or inflammation.[8]

Current treatment strategies for tracheostomy-associated granulation tissue rely on serial drug application and surgical debridement or excision in the operating room. Recurrence is common, requiring frequent surveillance, highlighting the poor understanding of disease pathogenesis and the need for targeted therapies.[9–11] Prior studies have demonstrated that airway granulation tissue can develop in response to foreign bodies (non-absorbable sutures, Teflon stents) and polymicrobial infections,[12–14] however the molecular mechanism promoting granulation tissue and recurrence in patients with tracheostomy still remains largely unknown.

Granulation tissue has been extensively studied in the context of inflammatory diseases such as rheumatoid arthritis,[15] tuberculosis,[16] and sarcoidosis,[17] but less is known about granulation tissue that occurs in patients with a tracheostomy. In sarcoidosis, monocytes and macrophages play a key role in granuloma formation. Monocytes and macrophages are innate immune cells observed in granulation tissue.[20] Monocytes can be divided into 3 subsets: classical, non-classical, and intermediate, based on the differential expression of CD14 and CD16, with classical monocytes being the predecessors to monocyte-derived macrophages. Macrophages also exhibit distinct phenotypes dependent on local immune mediators.[21] The most basic paradigm of the macrophage phenotype includes M1 proinflammatory (classically activated) and M2 anti-inflammatory (alternatively activated) macrophages. These subpopulations arise in tissue environments due to polarization from surrounding cytokines, growth factors, and external molecular stimuli.[20] There is evidence that indicates a shift toward M2 macrophage subsets in granuloma specimens.[18] In granulomas related to mycobacterial infection, M1 macrophages are prevalent during the early stages of the disease, whereas M2 macrophages predominate in the chronic stage of the diseases.[19] The nature and role of the macrophage population in tracheostomy-associated granulation tissue have not been delineated. The objective of this study is to define the macrophage population present in tracheostomy-associated granulation tissue biopsies using quantitative polymerase chain reaction (qPCR), flow cytometry, and immunofluorescence. It was hypothesized that M2 macrophages are the predominant macrophage subtype in granulation tissue, along with increased monocyte infiltration and inflammatory cytokine expression.

METHODS

Patient Cohort

Tissue biopsies (approximately 2 mm) from patients with non-infectious tracheostomy-associated granulation tissue ($n = 12$) and iatrogenic LTS (iLTS) scar ($n = 5$) were obtained during endoscopic dilation procedures following informed consent. Normal controls ($n = 3$) were obtained from deidentified normal human tracheal autopsy specimens procured within 24 h from subjects without a history of intubation or tracheostomy. Tissue was procured from the proximal tracheal mucosa, where a tracheostomy would typically be placed. All tissue samples were obtained in accordance with Johns Hopkins University Institutional Review Board (IRB00250531).

Gene Expression Measurement using qPCR

Human tissue biopsies from patients with tracheostomy-associated granulation tissue ($n = 8$), iLTS scar ($n = 5$), and autopsy controls ($n = 3$) were mechanically homogenized using metallic microbeads in RLT lysis buffer (1% ß-Mercaptoethanol). RNA was extracted and purified using the RNeasy Mini Spin Column as specified in the RNeasy Mini-kit (Qiagen, Valencia, California) following the manufacturer's protocol. cDNA was synthesized using Superscript III Reverse Transcriptase (Life Technologies, Thermo Fisher Scientific) following the manufacturer's protocol. RNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) (Data S1). Gene primers (Integrated DNA Technologies, Coralville, Iowa) used included macrophage M1 markers (*CD80* and *CD86*), macrophage M2 markers (Arginase 1 (*ARG1*) and *MRC1* (also known as *CD206*)), and inflammatory cytokines: interleukin 1-beta (*IL1B*), interleukin 4 (*IL4*), interleukin 6 (*IL6*), tumor necrosis factor alpha (*TNF*), transforming growth factor beta (*TGFB1*) (Table S1). Quantitative real-time PCR (qRT-PCR) was carried out in triplicate using SYBR Green primers and a StepOnePlus Real-time PCR System (Life Technologies, Thermo Fisher Scientific). Relative gene expression was calculated by the ΔΔCt method. Full details of gene expression analysis are detailed in Supplemental Material (Data S1).

Flow Cytometry Characterization of Immune Cells in Dissociated Granulation Samples

Fresh biopsies from patients with tracheostomy-associated granulation tissue ($n = 4$) were collected during routine endoscopic procedures, along with normal controls ($n = 3$), and immediately processed into single-cell suspensions. Biopsies were mechanically minced into 1–2 mm portions and subsequently digested with an enzyme solution (0.4 mg/ml DNA-ase (Sigma, St. Louis, MO), 1 mg/ml Liberase (Sigma), RPMI with 10% FBS) for 20 min at 37°C. Single-cell suspensions were cryopreserved at −180°C and later thawed for flow cytometry analysis, as previously described.[22] Single cells were split into 10^6 cells/well (V-bottom 96-well plate) and stained for 30 min at 4°C with primary monoclonal antibodies (mAbs): CD3, CD45, CD15, CD14, CD16, CD11B, CD80, CD206, CD169, S100A8, and Siglec8 (Table I). Figure 1 illustrates the gating scheme used to classify immune cells. The LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit was used to exclude dead cells. The samples were then subjected to fixation and permeabilization required for intracellular antibody staining. A detailed flow cytometry protocol is included as supplemental material (Data S1). Macrophages were defined as CD45+CD11b+ cells. Further characterization of the macrophage population was performed using CD169 (tissue-resident macrophage [TRM] marker), CD14 (infiltrating macrophage marker), and S100A8/A9 (pro-inflammatory marker) staining. In addition, subpopulations of monocytes were defined using differential expression of CD14 and CD16, including the classical subtype ($CD14^{high}CD16^{low}$), intermediate subtype ($CD14^{high}CD16^{high}$), and non-classical subtype ($CD14^{low}CD16^{high}$). Granulation tissue samples were compared with normal tracheal samples used as controls, to calculate differences in antigen expression. All analyses were performed using FlowJo Flow Cytometry Analysis Software (v10.8.1, 2019, Ashland, OR).

Immunofluorescence

Tissue sections from granulation tissue and normal tracheal autopsy controls were processed and stained with markers CD11b, CD86, and CD206 (Data S1). Briefly, all specimens were fixed in 10% formalin and embedded in paraffin. Slides were incubated overnight

TABLE I.
Macrophage Flow Cytometry Antibodies.

Antibody	Antibody clone	Antibody source	Fluorochrome	Marker type
CD3	OKT3	Biolegend	BV 421	Lymphocytes
CD15	W6D3	Biolegend	BV 421	Neutrophils
Live/Death	N/A	Thermo Scientific	Aqua	Live Cells
CD86	IT2.2	Thermo Scientific	SB600	M1 Macrophages
CD14	M5E2	Biolegend	BV 650	Monocyte Subtypes
CD45	HI30	Thermo Scientific	Qdot800	Leukocytes
CD68	Y1/82A	Biolegend	FITC	Macrophages
Siglec 8	7C9	Biolegend	PerCP-Cy5.5	Eosinophils
S100A	NJ-4F3-D1	Biolegend	PE	Pro-inflammatory Marker
CD169	7-239	Biolegend	PE/CYN7	Tissue-resident macrophages
CD206	15-2	Biolegend	APC	M2 Macrophages
CD16	B73.1	Biolegend	AlexaFluor 700	Monocyte Subtypes
CD11B	M1/70	Thermo Scientific	APCefluor780	Macrophage

with both anti-CD11b (Rabbit anti-human, ab133357 Abcam) and either anti-CD86 (Mouse anti-human ab270719; Abcam) or anti-CD206 (Mouse anti-human ab117644; Abcam). After primary antibody incubation, slides were washed and then stained with a corresponding secondary antibody for 45 minutes. Finally, slides were mounted using Gold antifade DAPI (ThermoScientific, Fremont, CA). A set of 20 images of the entire tissue specimen were taken using Zeiss AxioObserver.Z2 inverted microscope (Zeiss, Oberkochen, Germany) at both 20x and 40x magnification. Negative controls were used to establish background fluorescence and non-specific staining of the primary and secondary antibodies.

Statistical Analysis

qPCR results were presented as a mean relative fold change ($2^{-\Delta\Delta Ct}$) ± standard error of the mean (SEM). Flow cytometry results were presented as mean percent expressed ± standard deviation. Groups were compared using an unpaired t-test to detect differences in gene expression and cell populations. The significance criterion for all analyses was set at $p < 0.05$. Data analysis and visualization were performed using Prism software (GraphPad Software Inc., CA, USA).

RESULTS

Experimental Cohort

Patient demographics are presented in Table II and were similar between groups. No significant differences were noted in age, sex, patient BMI, or comorbidities. Among the granulation tissue patients, the majority (92%) of patients were tracheostomy-dependent secondary to iatrogenic laryngotracheal stenosis, whereas idiopathic subglottic stenosis was the cause of tracheostomy in 1 patient. All patients had a history of tracheostomy for greater than 6 months, with mean duration of 17.3 months and ranging from 9.8 to 21.2 months.

Flow Cytometry Immune Cell Phenotype Characterization

Flow cytometry of granulation tissue demonstrated 48.5 ± 13.7% of all live cells were leukocytes, defined as live cells staining positive for CD45. Monocytes (CD45+ $CD14^{high}CD16^{low}$, CD45+$CD14^{low}CD16^{high}$, CD45+ $CD14^{high}$ $CD16^{high}$) represented 16 ± 5% of the live cell population whereas macrophages (CD45+CD11B+) represent 7.3 ± 2.8% of the live cell population. T-cells (CD3+) and neutrophils (CD15+) represent 4.5 ± 2.3% and 10.5 ± 3.1% of all live cells, respectively (Fig. 2). A minority (12 ± 4%) of neutrophils (CD15+) co-localized S100A8/A9, a pro-inflammatory marker. Eosinophils (Siglec 8+) were the least common cell type, representing 2 ± 1% of the live cell population. Granulation tissue specimens had an increased combined macrophage/monocyte population compared to controls (23 ± 0.06% and 2 ± 1%, $p = 0.03$). There were no significant differences in the total macrophage population compared to controls (7 ± 2.8% and 1.6 ± 1.0%, $p = 0.17$), or total monocyte population compared to controls (16 ± 0.05% and 1 ± 1%, $p = 0.07$).

Predominant Monocyte Populations in Granulation Tissue

Monocytes (CD45+$FSC^{low}SCC^{low}$), the precursors to monocyte-derived macrophages, are the most abundant cell type, making up 16 ± 5% of the granulation tissue cell population. The relative CD14 and CD16 staining demonstrated that the monocyte population is comprised primarily of the classical subtype ($CD14^{high}CD16^{low}$) at 61.3 ± 7.3%, followed by 27.6 ± 11.6% intermediate subtype ($CD14^{high}CD16^{high}$), and 11.1 ± 4.6% non-classical subtype ($CD14^{low}CD16^{high}$). $CD14^{high}CD16^{low}$ classical monocytes were increased in granulation tissue compared with normal controls ($p = 0.039$), with no observed differences in $CD14^{high}CD16^{high}$ intermediate ($p = 0.14$) or $CD14^{low}CD16^{high}$ non-classical monocytes ($p = 0.75$) (Fig. 3A).

Predominant Macrophage Populations in Granulation Tissue

Analysis of the CD45+CD11B+ macrophage population in tracheostomy-associated granulation tissue revealed

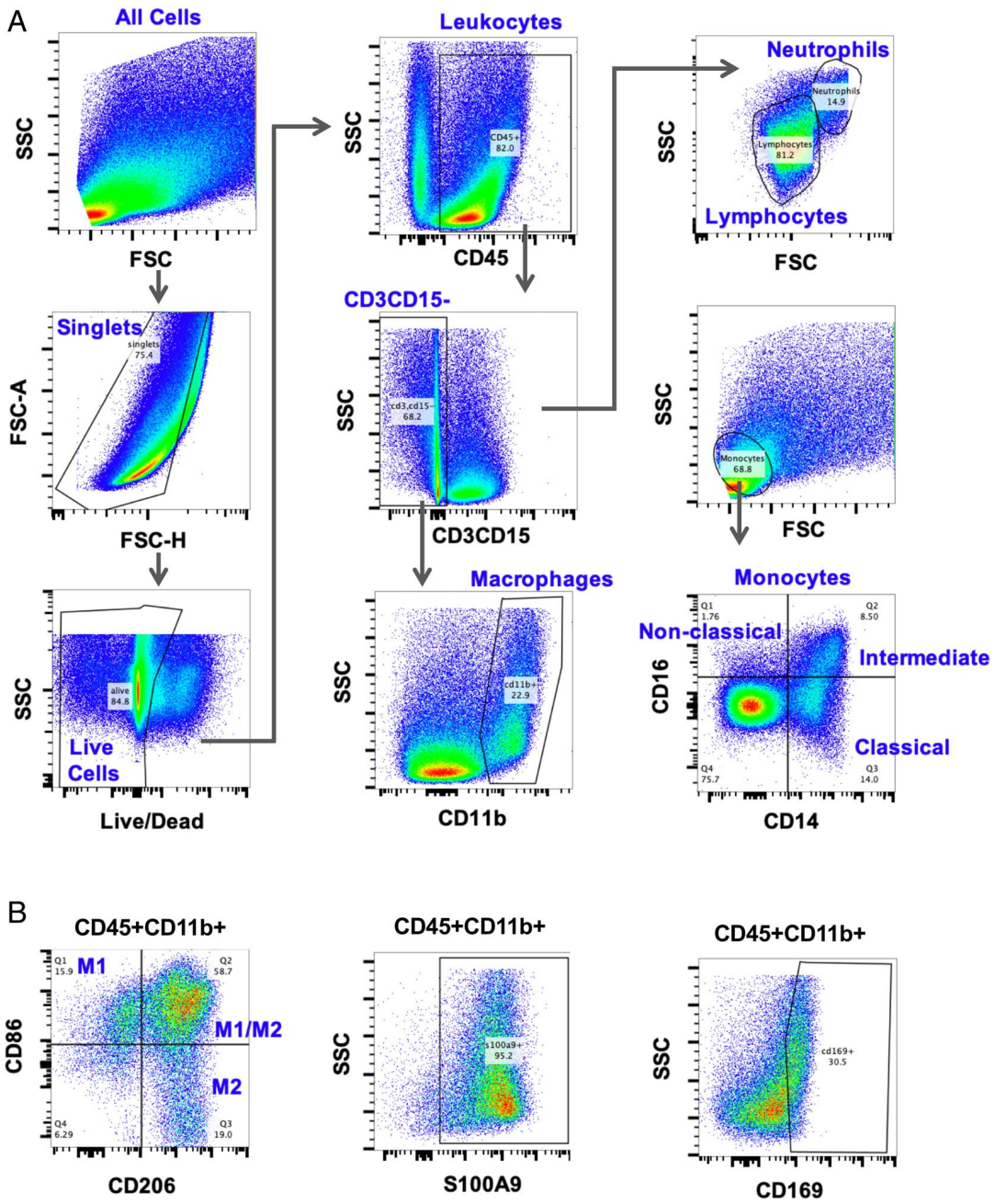

Fig. 1. Flow cytometry gating scheme for monocytes and macrophages. (A) CD45 was used to identify all hematopoietic cells, and macrophages were considered CD45 CD11b double positive. Other immune cells, including CD3+ T cells, CD15+ neutrophils, and Siglec8+ eosinophils were excluded. After identifying monocytes (CD45+FSC^{low}SSC^{low}) using forward and side scatter, CD14 and CD16 expression was used to identify non-classical, classical, and intermediate subtypes. (B) Macrophages were further categorized into M1 or M2 phenotypes using CD86 and CD206 respectively, as well as macrophages positive for pro-inflammatory marker S100A9 and tissue-resident marker CD169. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

that $36 \pm 4\%$ of all CD45+CD11B+ macrophages co-localized with the tissue-resident macrophage marker CD169,[23,24] compared to only $7 \pm 2\%$ in control specimens ($p = 0.002$) (Fig. 3B). Additionally, $80 \pm 9\%$ of the CD45+CD11B+ macrophages stained for the pro-inflammatory molecule S100A8/A9, compared to $54 \pm 18\%$ in the normal controls ($p = 0.21$) (Fig. 3C). However, in the CD45+CD11B+CD169+ TRMs, S100A8/A9 was only expressed on $16 \pm 9\%$ cells. There was no significant difference in CD14+ expression on CD45+CD11B+ macrophages in tracheostomy-associated granulation tissue when compared to controls. Analysis of the CD45+CD11B+ macrophage population revealed that $77 \pm 11\%$ were M2 macrophages (co-localizing CD206). In contrast, M1 macrophages (co-localizing CD86+) represented $23\% \pm 11\%$ of the CD45+CD11B+ macrophage population. When the percentage of M1 macrophages was compared to the percentage of M2 macrophages, there is a predominance of

TABLE II.
Patient Characteristics.

	Granulation tissue biopsies (qPCR)	Granulation tissue dissociations (FC)	iLTS biopsies (qPCR)
Enrollment	$n = 8$	$n = 4$	$n = 5$
Mean age (SD)	40.6 (10.2)	40.3 (11.6)	51 (10.2)
Sex, female (%)	6 (75%)	3 (75%)	5 (100%)
BMI (SD)	40.3 (11.2)	37.4 (15.5)	34.4 (8.6)
Race/Ethnicity (%)			
Caucasian	1 (12.5%)	0 (0%)	3 (60%)
African American	5 (62.5%)	4 (80%)	1 (20%)
Asian	1 (12.5%)	1 (20%)	1 (20%)
Other	1 (12.5%)	0 (0%)	0 (0%)
History of tracheostomy (%)	8 (100%)	5 (100%)	5 (100%)
Tracheostomy duration, months (SD)	16 (12.7)	21.2 (18.3)	9.8 (13.8)
Intubation duration, days (SD)	6.2 (2.7)	5 (0)	10 (6.1)
Charlson comorbidity index (SD)	0.75 (1.4)	0.75 (1.5)	3 (2.2)
Comorbidities (%)			
Hypertension	1 (12.5%)	1 (20%)	4 (80%)
Diabetes	1 (12.5%)	1 (20%)	2 (40%)
CAD	0 (0%)	1 (20%)	1 (20%)
Months followed[a] (SD)	16 (11.4)	18 (14.6)	9.8 (8.2)
Etiology (%)			
Iatrogenic, post-intubation	1 (12.5%)	1 (20%)	1 (20%)
Iatrogenic, post-tracheostomy	0 (0%)	1 (20%)	1 (20%)
Iatrogenic, post-intubation, and tracheostomy	6 (75%)	3 (60%)	3 (60%)
Idiopathic	1 (12.5%)	0 (0%)	0 (0%)

FC = flow cytometry; iLTS = iatrogenic laryngotracheal stenosis; PCR = polymerase chain reaction; SD = standard deviation.
[a]Months followed calculated from the time of first clinic visit to the procedure.

M2 macrophages ($p = 0.012$) (Fig. 4A). In normal controls, there was no difference in the percentage of M1 macrophages compared to the percentage of M2 macrophages ($p = 0.22$). In addition, when the ratio of M1 macrophages to M2 macrophages in granulation tissue is compared to that of normal controls, there is no significant difference in expression ($77 \pm 11\%$ and $67 \pm 16\%$, $p = 0.603$).

Gene Expression of Immune Cell Biomarkers and Inflammatory Cytokines

M2 biomarker gene expression (*Arg1* and *CD206*) was significantly increased in granulation tissue compared to controls (3.7 ± 1.2, $p = 0.035$ and 3.5 ± 1.5, $p = 0.047$). M1 biomarker gene expression (*CD80* and *CD86*) was similar in granulation specimens and controls ($p = 0.64$ and $p = 0.30$). Gene expression of M1 markers

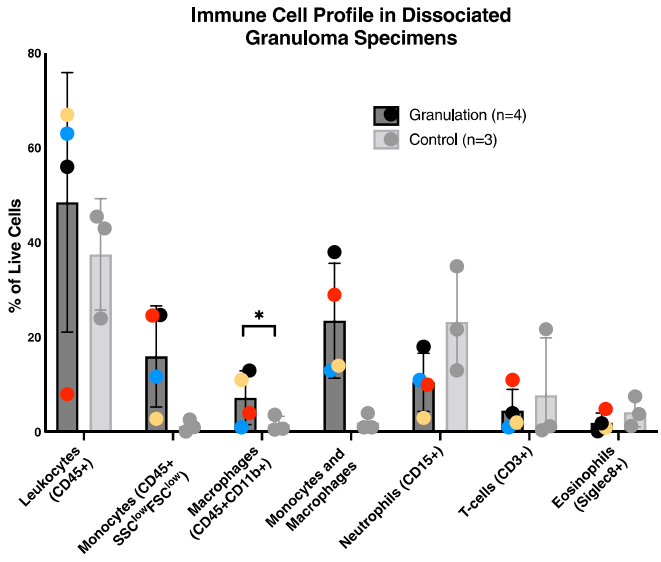

Fig. 2. Immune cell profile in dissociated tracheal granulation tissue. Dissociated granulation tissue and control samples were stained and analyzed using flow cytometry to identify leukocytes (CD45+), macrophages (CD45+CD11b+), monocytes (CD45+FSC^{low}SCC^{low}), including classical CD14^{high}CD16^{low}, intermediate CD14^{high}CD16^{high}, non-classical CD14^{low}CD16^{high}) distinguished using forward and side scatter properties, T-cells (CD3), neutrophils (CD15), and eosinophils (Siglec 8). Error bars signify standard error of the mean (SEM). [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

(*CD86*) and M2 markers (*Arg1* and *CD206*) of granulation tissue was increased compared to iLTS specimens ($p = 0.004$, 0.01, 0.02, respectively) (Fig. 4B,C). M1 or M2 biomarker gene expression in iLTS specimens was not significantly increased compared to controls. Finally, gene expression of inflammatory cytokines was not significantly elevated in tracheostomy-associated granulation tissue specimens (Fig. 5).

Immunohistochemistry

Tracheostomy-associated granulation tissue specimens demonstrated increased macrophage infiltration compared to normal controls, as indicated by CD11b positive cells. CD86 and CD206 staining revealed a large population of macrophages with the M2 phenotype (CD206+) compared to the M1 (CD86+) phenotype (Fig. 6).

DISCUSSION

This study demonstrates that markers associated with alternatively activated M2 macrophages are increased in tracheostomy-associated granulation tissue as determined by gene expression analysis of canonical biomarkers and cell surface antigens assessed by flow cytometry and immunofluorescence. Phenotypic assessment of the monocyte cell populations associated with granulation tissue reveals a predominant classical subtype. Collectively, these findings indicate that the monocyte and macrophage cell populations are increased in tracheostomy-associated granulation tissue compared

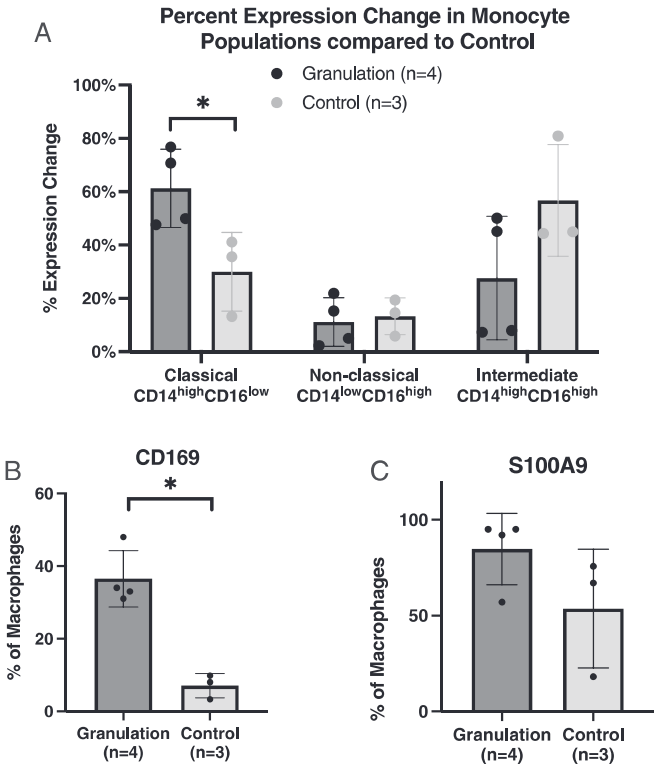

Fig. 3. Monocyte and tissue-resident macrophage subpopulations in granulation tissue. (A) Flow cytometry results designating intermediate, classical, and non-classical monocyte populations based on CD16 and CD14 antigen staining. (B) Tissue-resident macrophages are defined by the percentage of macrophages staining positive for CD169. (C) Percentage of macrophages staining positive for S100A8/A9. Values represent the percent of monocytes or macrophages present in the tissue population. Error bars signify standard deviation. *$p < 0.05$. n = number of samples.

to controls. Further, the associated macrophage subpopulation is primarily M2 in nature, representing a potential target for future therapies. Ongoing investigations to identify key signaling nodes that drive persistent immune cell recruitment and M2 differentiation in tracheostomy-associated granulation tissue are warranted.

The data presented in this study demonstrates that tracheostomy-associated granulation tissue has a disproportionate increase in M2 macrophages. Macrophage-mediated immune responses have been linked to persistent pathologic inflammation and poor wound healing in human and murine models of chronic diabetic ulcers.[25–27] Following acute kidney injury, a persistent M2 macrophage population, defined by F4/80 positivity along with M2 markers CD206 and Arginase 1, has been associated with persistent inflammation and the development of fibrosis in mouse models.[28] Mechanistically, this M2 macrophage population leads to increased production of TGFB1 and growth factors that promote the proliferation of myofibroblasts and subsequent fibrosis.[29,30] These findings suggest that the M2 macrophage population associated with tracheostomy-asssociated granulation tissue may be promoting granulation tissue recurrence and potentiating fibrosis near the tracheostomy; however, the mechanism for this remains to be elucidated.

Macrophages can originate from monocytes in the bloodstream that ultimately give rise to macrophages that reside in tissues (tissue-resident macrophages).[20] In contrast to monocyte-derived macrophages, which are actively recruited during inflammation,[31] tissue-resident macrophages have the ability to self-renew, maintaining their populations within the tissues.[32] Unlike monocyte-derived macrophages, TRMs originate from multipotent erythromyeloid progenitors that give rise to TRMs that carry out tissue-specific functions.[33] Fifty-eight percent of macrophages in tracheostomy-associated granulation tissue were monocyte-derived (CD14+). However, 36% of the CD45+CD11b+ macrophage population stained positively for the cell surface marker CD169+, a marker associated with tissue-resident macrophages. Several studies have shown significant alteration in the number of CD169+ macrophages in diseased tissues, such as cancer and autoimmune disease, suggesting that this cell type may play a role in disease regulation.[34,35] In particular, under inflammatory conditions such as systemic lupus erythematosus, atherosclerosis, and transplant rejection, monocytes/macrophages may be induced to express surface marker CD169. Mouse models in CD169 deficient mice have demonstrated that CD169 may exacerbate autoimmune encephalomyelitis by inhibiting regulatory T cells.[36] Unlike M1 and M2 macrophages, CD169+ macrophages can interact directly with T cells, B cells, and dendritic cells and participate in immune regulation.[37] Targeting this CD169+ cell population may impact the downstream regulation of lymphocytes and antigen-presenting cells and ultimately prevent granulation tissue formation. The specific role of CD169+ myeloid cells in tracheostomy-associated granulation tissue has not been well delineated and further studies are needed to define the role of this cell population in regulating the persistent inflammation driving pathologic granulation tissue formation.

Flow cytometry data demonstrate that monocytes ($CD45+FSC^{low}SSC^{low}$) are the dominant immune cell in tracheostomy-associated granulation tissue. Subgroup analysis reveals that this monocyte population is primarily comprised of classical monocytes. Monocytes are a major component of human peripheral blood, accounting for approximately 10% of all circulating leukocytes, with three main phenotypes based on the expression of CD14 and CD16, which encode for lipopolysaccharide receptor and low-affinity FC γ receptor, respectively. In human peripheral blood, classical ($CD14^{high}CD16^{low}$) monocytes are most prevalent (80–90%), followed by intermediate ($CD14^{high}CD16^{low}$) at 2%–5% and non-classical ($CD14^{low}CD16^{high}$) at 2%–10%. Classical monocytes are the predecessors to monocyte-derived macrophages and are primarily involved in phagocytosis and immune-cell migration.[38] In contrast, non-classical monocytes are involved in complement and Fc gamma-mediated phagocytosis and adhesion, whereas intermediate monocytes are the only subset to express CCR5 and express the highest level of antigen-presentation-related molecules.[39,40] The extent to which different infiltrating classical monocyte subpopulations contribute to tracheostomy-associated granulation tissue formation remains poorly understood. Changes in monocyte counts

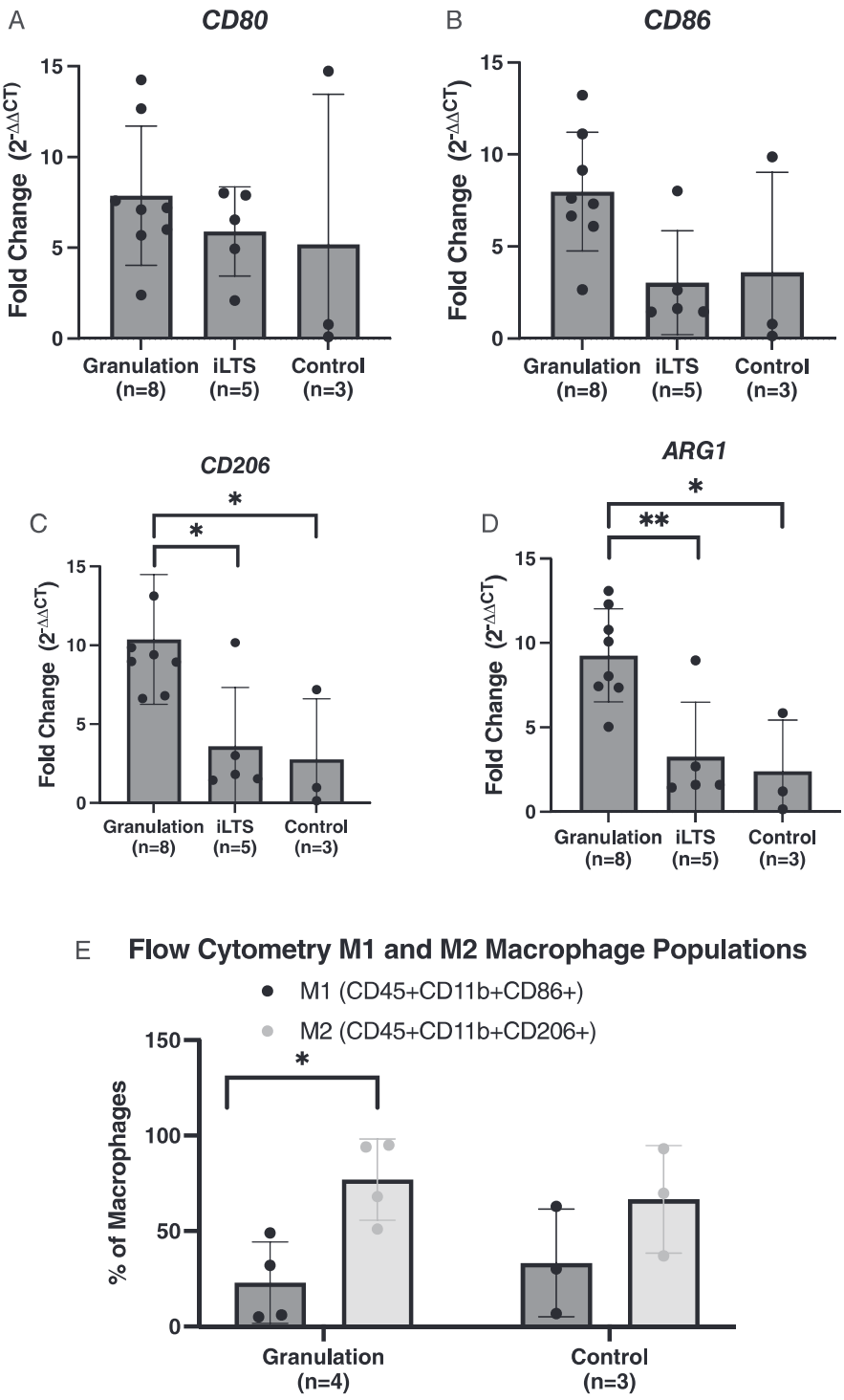

Fig. 4. M2 macrophages are predominant in granulation tissue. (A) Flow cytometry percentage of macrophages in M1 and M2 subpopulations, CD86 and CD206 (B and C) Mean gene expression of M1 (CD80 and CD86) and (D and E) M2 (CD206 and ARG1) macrophage biomarkers in granulation tissue and iLTS specimens compared to normal controls. *$p < 0.05$; **$p < 0.01$. ARG1 = Arginase 1. iLTS = iatrogenic laryngotracheal stenosis.

have been identified in the study of other chronic inflammatory disorders, including rheumatoid arthritis and chronic kidney disease, each demonstrating an increase in relative intermediate monocyte subpopulations.[41,42] These differential changes in monocyte subpopulations may directly affect disease outcomes but also impact the development of granulation tissue and persistent inflammation through their differentiation into macrophages. Further work delineating the role of monocytes in the pathogenesis of granulation may lead to the development of targeted therapies to limit monocyte infiltration and differentiation into monocyte-derived macrophages.

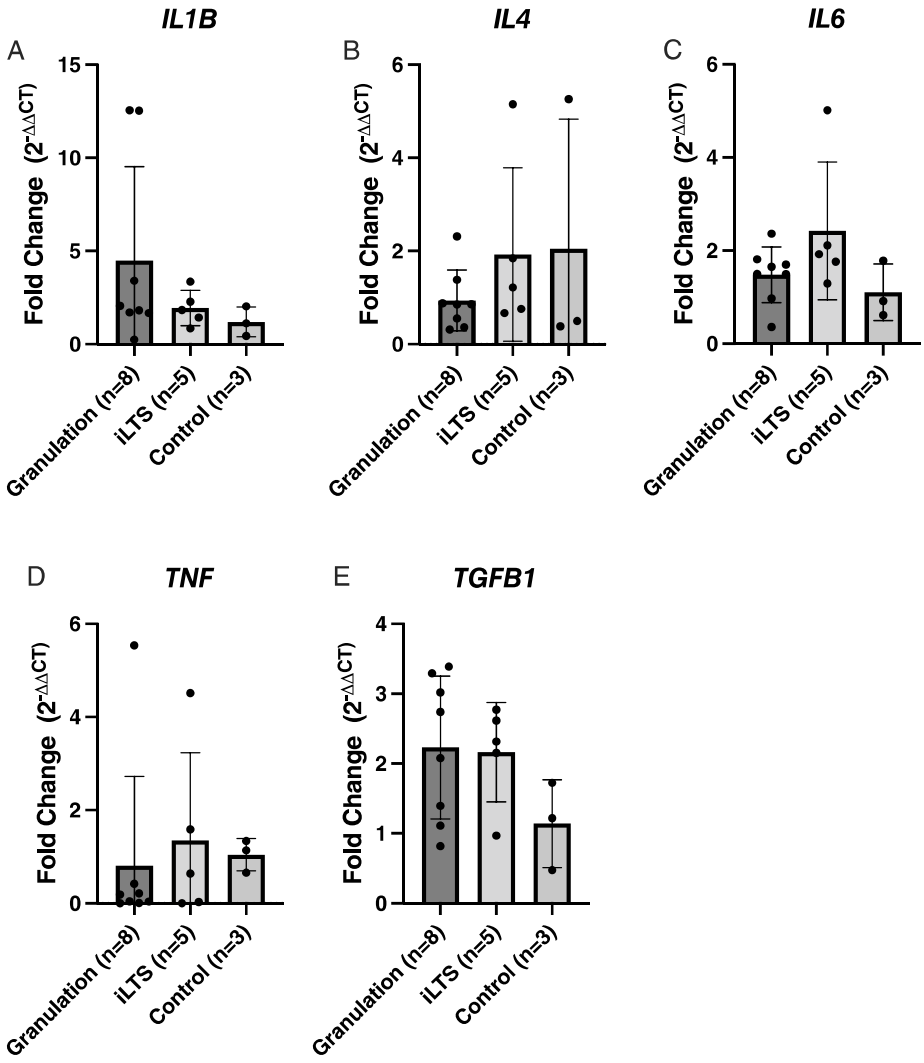

Fig. 5. Inflammatory cytokine gene expression in granulation tissue. Gene expression analysis was expressed in terms of relative fold change compared to normal control tissue for the genes (A) IL-1b, (B) IL-4, (C) IL-6, (D) tumor necrosis factor (TNF)-alpha, and (E) transforming growth factor (TGF)-beta. Error bars signify standard deviation. IL = interleukin; *n* = number of samples.

The majority of macrophages in tracheostomy-associated granulation specimens were also S100A8/A9 positive. S100A8/A9 is a Ca+ binding protein constitutively expressed in neutrophils and monocytes, which is often released during inflammatory processes.[43] In addition, S100A8/A9 is involved in myeloid differentiation and induces monocytes to secrete pro-inflammatory cytokines including TNFα and IL-1ß. During inflammation, S100A8/A9 stimulates leukocyte recruitment and induces cytokine secretion, and has been implicated as a prognostic biomarker and therapeutic target in several diseases involving chronic inflammation and fibrosis such as IPF,[44] renal fibrosis,[45] rheumatoid arthritis,[46] and osteoarthritis.[47] In addition, in the fibrotic disease adhesive capsulitis, it is demonstrated that S100A8/A9 expression was primarily localized to CD68+ macrophages.[48] In addition, in vitro studies, have shown that stimulation of M2 macrophages with S100A8/A9 results in the upregulation of pro-inflammatory cytokines typically produced by M1 macrophage (IL-1β, IL-6, and TNFα) without changing their surface protein M2 phenotype.[49] Thus, the elevated expression of S100A8/A9 may represent a pro-inflammatory nidus in tracheostomy granulation tissue. However, whereas this study did not observe differences in inflammatory cytokine expression, gene expression analysis may not accurately reflect the true cytokine milieu as IL-1β and other cytokines are regulated post-translationally.[50] Additionally, relatively low baseline expression levels of inflammatory cytokines may limit their detection threshold. As such, further investigation using protein-level assays is warranted.

Another distinct category of granulomatous lesions of the upper airway is laryngeal granulomas, which occur as contact and/or postintubation lesions in the posterior glottis commonly over the vocal process of the arytenoid cartilage.[51] Laryngeal granulomas are benign lesions that are less vascular and more focally localized than tracheostomy-associated granulation tissue.[52] Much like tracheostomy-associated granulation tissue, the etiology of laryngeal granulomas is unclear, however, the

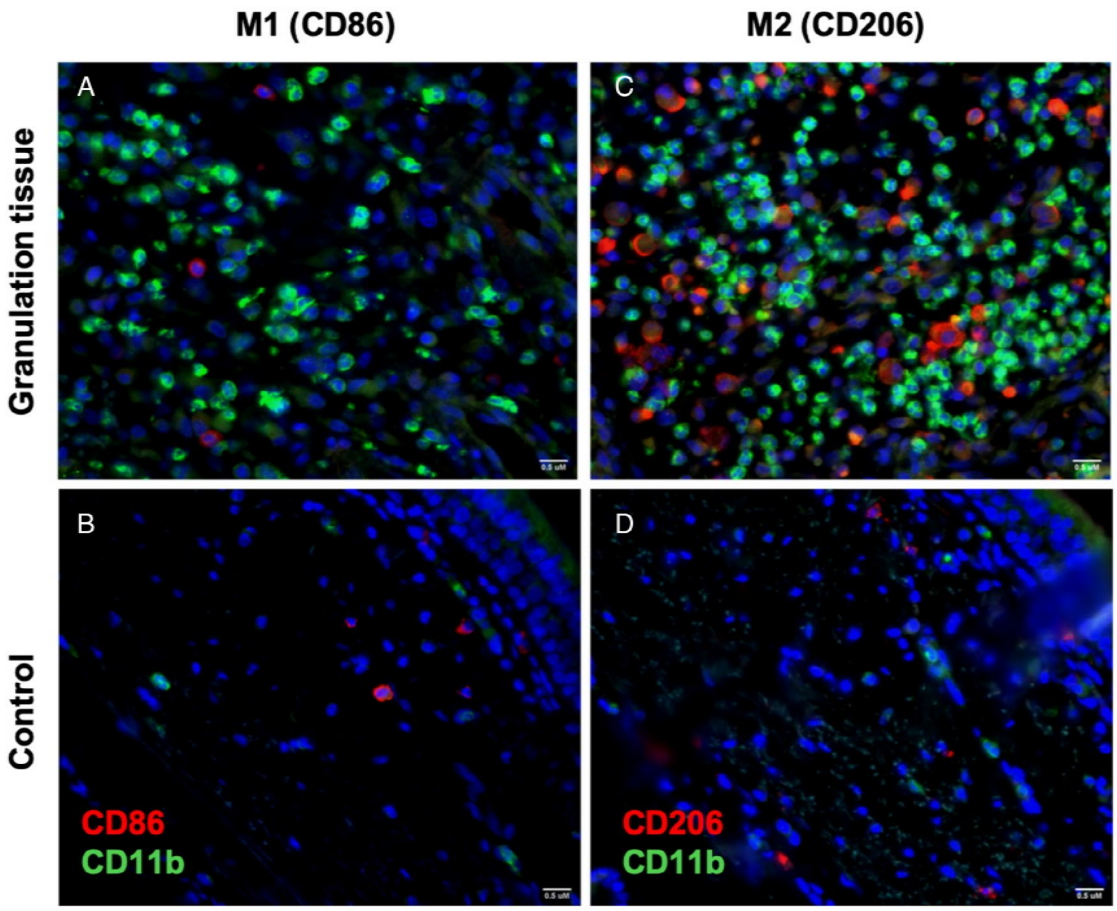

Fig. 6. Immunohistochemistry of macrophage phenotypes. Immunohistochemistry comparing M1 (CD86+) and M2 (CD206+) macrophage presence in granulation tissue (A and C) and control specimens (B and D). Staining with CD11b (green fluorochrome) and CD86 or CD206 (red fluorochrome). Scale bar = 0.5 uM [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

presumed pathogenesis involves irritation and possible underlying bacterial colonization.[52] DNA microarray gene expression analysis of vocal fold granulomas show clusters of upregulated genes involved in wound repair and inflammation (HIF1A, PRG1, SCYA2, HPS, LAPTM5), along with genes that play a role in remodeling the extracellular matrix (COL1A2, COL3A1, LUM).[22] Although the inflammatory cascade is presumed to be integral to their formation, the specific immune phenotype and cell populations have not been extensively studied.[53] As such, findings from this study may have relevance to vocal process granulomas but studies are warranted to define the immune cell population in this distinct entity.

Although this study is the first to define the innate immune cell population present in tracheostomy-associated granulation tissue and show M2 predominance among macrophages, there are several limitations. First, the dichotomous M1/M2 paradigm is not a full representation of the macrophage phenotypes, which have arisen in vitro and rely on the stimulation of macrophages in culture with defined cytokines. To account for this, the gating strategy of this study includes double positive staining CD86 and CD206 in both M1 and M2 macrophage subpopulations. Further, this study used multiple methods, including flow cytometry and immunohistochemistry to support the results of gene expression found using qPCR. Additionally, this study used immunohistochemistry from fresh biopsied tissue, which preserves the cell surface markers present in the granulation tissue microenvironment in vivo that may be cleaved during downstream tissue processing of flow cytometry. Our flow cytometry panel was limited by the number of antibodies permitted without fluorophore overlap. Further investigations with additional markers will elucidate the multifactorial role of dendritic cells and other immune cell phenotypes in the development of tracheostomy-associated granulation tissue. Finally, we anticipated TGFB1 and inflammatory cytokines would be upregulated, but this was not observed in granulation tissue specimens. This could be related to gene expression assays not reflecting protein level expression. Additionally, assessing TGFB1 activity relies on the quantification of the TGFB1 active form which is not reflected in gene expression assessments.[54] Future dedicated studies are warranted to assess TGFB1 and inflammatory cytokines in tracheostomy-associated granulation tissue. Finally, studies involving RNA-sequencing would help to contextualize these findings using gene expression profiles and cell surface markers to subset macrophages and their role in the granulation tissue microenvironment.

CONCLUSION

This study demonstrates that alternatively activated M2 macrophages are elevated in tracheostomy-associated granulation tissue. Additionally, monocytes represent the dominant immune cell population in granulation tissue with the majority being of a classical phenotype. The mechanisms promoting monocyte recruitment and macrophage differentiation represent critical signaling nodes that could be targeted to attenuate granulation tissue development. However, further investigation is needed to delineate the potential pathogenic mechanisms promoting this persistent cell population in tracheostomy-associated granulation tissue. In addition to our findings, this work provides a validated flow cytometry platform that can be applied for the assessment of the macrophage and monocyte populations within inflammatory conditions.

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