BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Protein Signatures of Remodeled Airways in Transplanted Lungs with Bronchiolitis Obliterans Syndrome Obtained Using Laser-Capture Microdissection

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Bronchiolitis obliterans syndrome, a common form of chronic lung allograft dysfunction, is the major limitation to long-term survival after lung transplantation. The histologic correlate is progressive, fibrotic occlusion of small airways, obliterative bronchiolitis lesions, which ultimately lead to organ failure. The molecular composition of these lesions is unknown. In this study, the protein composition of the lesions in explanted lungs from four end-stage bronchiolitis obliterans syndrome patients was analyzed using laser-capture microdissection and optimized sample preparation protocols for mass spectrometry. Immunohistochemistry and immunofluorescence were used to determine the spatial distribution of commonly identified proteins on the tissue level, and protein signatures for 14 obliterative bronchiolitis lesions were established. A set of 39 proteins, identified in >75% of lesions, included distinct structural proteins (collagen types IV and VI) and cellular components (actins, vimentin, and tryptase). Each respective lesion exhibited a unique composition of proteins (on average, $n = 66$ proteins), thereby mirroring the morphologic variation of the lesions. Antibody-based staining confirmed these mass spectrometry-based findings. The 14 analyzed obliterative bronchiolitis lesions showed variations in their protein content, but also common features. This study provides molecular and morphologic insights into the development of chronic rejection after lung transplantation. The protein patterns in the lesions were correlated to pathways of extracellular matrix organization, tissue development, and wound healing processes. (*Am J Pathol* 2021, 191: 1398–1411; <https://doi.org/10.1016/j.ajpath.2021.05.014>)

Since the first lung transplantations in the late 1980s, bronchiolitis obliterans syndrome (BOS) in the form of progressive airway obstruction has been recognized as the most important pathology associated with adverse outcome. The first definition and guideline for BOS was issued in 1993,¹ with revisions in 2002,² 2014,³ and 2019.⁴ The term BOS was defined as progressive airway obstruction causing >20% decline in forced expiratory volume in 1 second with no obvious reason, like infection or other macroscopic lesions. The pathologic correlate to BOS was identified as

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obliterative bronchiolitis (OB) lesions. These lesions were, however, small and patchy and could not be diagnosed reliably with radiology or transbronchial biopsies.³ BOS has been demonstrated in both single-center studies⁵ and the International Society for Heart and Lung Transplantation registry studies⁶ to afflict nearly all lung transplant patients. The median BOS-free survival is 3 to 4 years, and 10 years after transplantation, BOS, in some degree, is present in 90% of patients.

BOS was initially used synonymously to chronic lung allograft dysfunction, as the most common type of chronic lung allograft dysfunction. However, other subtypes are now recognized by distinct clinical parameters and histopathologic features, most prominently restrictive chronic lung allograft dysfunction/restrictive allograft syndrome.^{7–10} The present study focused on BOS lesions. Studies have suggested that immunologic processes in non-BOS lesions are different from BOS.^{11,12} The histopathologic correlates of BOS, the OB lesions, develop on remodeling of the small airways, although the events leading to the occlusions are not completely understood.¹³ Epithelial cell death has been suggested as a key event in the development of BOS¹⁴ and may trigger exaggerated wound healing, where mesenchymal cell activities, including buildup and turnover of extracellular matrix, subsequently obliterate the airway.¹⁵ In an innovative study, micro-computed tomography was used to demonstrate the patchy nature of OB lesions.¹⁶ The lesions were minute, affecting mainly small peripheral airways with a diameter of <2 mm. Furthermore, the lesions appeared to be heterogeneous, probably due to different developmental stages.

This study seeks to characterize these OB lesions in BOS exhibiting fibrotic occlusion of the small airways, by using a unique combination of laser-capture microscopy (LCM), mass spectrometry (MS), and antibody-based staining to combine state-of-the-art protein identification and spatial information on the tissue level. Thereby, it provides a snapshot view of ongoing events in OB lesions. Molecular profiling of the pathologic lesions in BOS is desirable to elucidate the cellular components and events initiating and driving the disease, with the aim to find new molecular tools to intervene.¹⁷ Thorough histopathologic studies have advanced the knowledge in this field by using complex imaging methods, protein analysis, and gene analysis.^{16,18–21} Characterization of these lesions is challenging because of their focal nature, scarcity in the lung, and the small amount of material available for analysis. Herein, we use a novel approach to increase the depth of MS protein identification and to describe the protein signatures of 14 pathologically remodeled airways in four end-stage BOS patients. This exploratory study, by exquisitely dissecting out the lesions and carefully excluding confounding tissue surrounding the OB lesion, identified cellular pathways involving extracellular matrix organization, tissue development, and wound healing

processes that warrant further investigation, with the aim of finding therapeutic targets.

Materials and Methods

Clinical Data

Lung tissue was obtained from four patients in two centers (Skåne University Hospital, Lund, Sweden; and Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark). The patients had end-stage BOS as a complication of lung transplantation. Lung tissue material was obtained from the explanted lungs after retransplantation.

Patient characteristics are shown in [Supplemental Table S1](#). Informed consent and ethical permit were obtained at the respective center (Lund dnr 2015-89), and data were obtained from Scandiatransplant (dnr 2007-58-0008). Every patient consented to the explanted lung to be used for research. Healthy lung tissue was obtained from Sahlgrenska Hospital, Gothenburg, Sweden (ethical permit, Gothenburg, dnr 2008/413), and informed consent was obtained.

Sample Preparation

The study is based on OB lesions identified in lung explant tissue. The formalin-fixed explanted lungs underwent thorough macroscopic examination, and the tissue was extensively sampled, typically with at least one central (containing bronchus) and two peripheral tissue blocks per lobe. Tissue samples were dehydrated and paraffin embedded according to routine pathology procedures. Using a microtome, tissue sections (4 µm thick) were produced and placed on routine glass slides (Menzel Superfrost Plus; VWR, Stockholm, Sweden) for hematoxylin/eosin (HE) staining, immunohistochemistry (IHC), immunofluorescence (IF), and LCM [in Auto-laser pressure catapulting (LPC) mode]; or polyethylene (PEN)-membrane slide (Zeiss, Stockholm, Sweden; Membrane Slide 1.0 PEN, 415101-4401-000) for LCM (in Robo-LPC mode). Before usage, PEN slides were activated by 30 minutes of UV irradiation (254 nm) using a Spectrolinker/Crosslinker XL-1000 UV (DOT Scientific Inc., Burton, MI).

Tissue blocks from the four patients were available for the research study. A large amount of tissue was processed, and submitted according to the protocol for macroscopic examination of explanted lungs. During the processing, tissue sections were placed alternating on routine glass slides or PEN-membrane slides ([Supplemental Figure S1](#)). Because of their high cost, it was not feasible to use PEN-membrane slides for all of the material. Depending on where OB lesions were identified in consecutive, HE- and elastica van Gieson-stained sections, OB lesion material was lifted during LCM from routine or PEN-membrane slides. According to our data and the instrument manufacturer's data (Zeiss), this does not affect the quality of the protein retrieval after LCM.

Identification of OB Lesions

OB lesions containing tissue blocks were identified by a pathologist at the respective center (H.B. in Lund and K.W. in Copenhagen). A total of 50 to 100 sections were produced from each tissue block, and a systematic screening for OB lesions was performed using HE-stained tissue sections in regular intervals at different depths of the specimen (Histolab, Askim, Sweden; Mayer's hematoxylin/eosin, according to manufacturer's instructions). Once identified, consecutive tissue sections were used for LCM, IF, and IHC (Figure 1). Identification of OB lesions during the screening procedure was done at the Lung Biology Group, Lund, and lesions were then reviewed independently by the two pathologists at the respective center (H.B. in Lund and K.W. in Copenhagen). Only lesions for which the pathologists' assessments were in concordance were included in the study. Lesions where precise delineation could not be performed by LCM because of technical difficulties were excluded. No further material was considered when a satisfactory number

of lesions was established. A satisfactory number was not reached for patient 4 (only one lesion included in the study) because of lack of well-delineated and suitable OB lesions.

Immunohistochemistry and Immunofluorescence

Consecutive sections to the ones used for HE and LCM were used for antibody-based staining of selected proteins of interest, as previously published.²² After deparaffinization, heat-induced epitope retrieval was performed on a PT Tissue Link system (Histolab) using citrate buffer. IHC for collagen α 1 and 2 (IV) chains and tryptase was performed using the EnVision Dual Link System (K4065; Dako, Glostrup, Denmark), according to manufacturer's instructions, including horseradish peroxidase–coupled secondary antibodies and counterstaining with Mayer's hematoxylin to visualize nuclei. IF for periostin, collagen α -1 (VI) chain, Ki-67, and α -smooth muscle actin (α -SMA; aortic smooth muscle actin) was performed by incubation with primary antibodies for 1 hour and with fluorochrome-coupled

Figure 1 Schematic overview of the experimental workflow. Selection and assessment of consecutive tissue sections for histologic identification of obliterative bronchiolitis (OB) lesions used for laser-capture microscopy (LCM) tissue extraction. Sample preparation and use of LCM/mass spectrometry for identification of protein targets and immunofluorescence (IF)/immunohistochemistry (IHC) for spatial tissue distribution of proteins of interest. BOS, bronchiolitis obliterans syndrome; HE, hematoxylin/eosin.

Table 1 Antibody Specifications for the Proteins Detected in Histology

Antibody	Description	Manufacturer, catalog no.	Dilution	Method	Secondary antibody
Collagen α -1 and α -2 (IV) chains	Basement membrane	Abcam (Cambridge, UK), ab6586	1:4000	IHC	DAB ⁺
Collagen α -1 (VI) chain	Structural protein	Abcam, ab6588	1:1000	IF	A-21246
Periostin	Glycoprotein	Abcam, ab79946	1:100	IF	A-21246
α -SMA	Cytoskeleton	Sigma (St. Louis, MO), C6198	1:2500	IF	Directly conjugated
Ki-67	Proliferation marker	Abcam, ab15580	1:1000	IF	A-21246
Tryptase	Mast cell	Dako, Kod M7052	1:100	IHC	DAB ⁺

A, Alexa Fluor (Thermo Fisher Scientific, Waltham, MA), at 1:200; DAB⁺, diaminobenzidine positive [Dako EnVision + Dual Link System-HRP (K4065)]; IF, immunofluorescence; IHC, immunohistochemistry; α -SMA, α -smooth muscle actin.

secondary antibodies for 45 minutes. Sections were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen, Carlsbad, CA) to visualize nuclei. Nonspecific staining of the secondary antibodies was assessed by omitting the primary antibodies (Table 1).

Image Acquisition

Images were obtained with a Zeiss fluorescence microscope or with a VS120 virtual microscopy slide scanning system (Olympus, Tokyo, Japan), in either bright-field mode (hematoxylin/eosin and IHC) or fluorescent mode (IF). From the scanned slides, representative images were acquired using the OlyVIA software 2.8 (Olympus). Exposure times, acquisition settings, and image brightness adjustments were used consistently for each respective staining, including negative controls, apart from the collagen VI and periostin stainings in healthy control subjects that were performed at a later time point.

Laser-Capture Microdissection

Tissue sections intended for LCM were deparaffinized (2× xylene for 5 minutes, 2× absolute ethanol for 2 minutes, 95% ethanol for 1 minute, 70% ethanol for 1 minute, and two changes of distilled water). OB lesions were previously reported to contain an abundance of hyaluronan; thus, hyaluronidase digest (0.2% bovine testicular hyaluronidase; Sigma, St. Louis, MO; type I-S) in acetate buffer (20 mmol/L sodium acetate and 150 mmol/L sodium chloride, pH 5.2) was performed for 1 hour at 37°C, to enable effective protein extraction from the microdissected material.²³ Pre-digested tissue sections were stained with Mayer's hematoxylin (Histolab) for 30 seconds to visualize nuclei. After three changes of water, sections were dehydrated (2 × 95% ethanol for 1 minute, and absolute ethanol for 2 minutes), air dried, and stored at −20°C in sealed containers. Before LCM, sections were allowed to equilibrate to room temperature, avoiding condensation. LCM was performed on a PALM MicroBeam system (Carl Zeiss MicroImaging, Jena, Germany) using the PALM Robo 4.6 Pro Software (Carl Zeiss MicroImaging), with a 10× objective. Depending on whether a suitable OB lesion was available on a PEN-membrane glass, Robo-LPC mode (defined larger structures were cut out and lifted in one piece

by the laser) was used (Supplemental Figure S1). For OB lesions on regular object glasses, Auto-LPC mode (small tissue fragments were lifted directly off the glass by discrete laser pulses) was used (Supplemental Figure S1). OB lesions were defined by submucosal fibrosis narrowing the bronchiolar lumen, and material within the smooth muscle ring was collected. Dissected material was catapulted into the lids of adhesive cap tubes (Zeiss Adhesive Cap tubes opaque 415190-9201-000; 500 μ L). Images of the slides were acquired using an AxioCamICc1 (Zeiss), and the CapCheck function was used to control successful collection of dissected tissue into the lid.

Mass Spectrometry Sample Preparation

Samples were prepared according to Braakman et al,²⁴ with the modifications of including 8 mol/L urea in the extraction buffer and using SP3 beads for peptide desalting. LCM material in the lids of the tubes was dissolved in 20 μ L/lid 0.1% RapiGest w/v (Waters, Milford, MA; 186001861) in 50 mmol/L ammonium bicarbonate with 8 mol/L urea and incubated at room temperature for 30 minutes. Subsequently, the lysate was centrifuged to the bottom of the tube (30 seconds/6082 × g; Biofuge pico, Heraeus; DJB, Buckinghamshire, UK). Tissue slices were sonicated in Bioruptor Plus (Diagenode SA, Seraing, Belgium) at 4°C for 20 cycles, 15 seconds on/off. Proteins were denatured at 99°C, 300 rpm, for 5 minutes, reduced by incubation with 5 mmol/L dithiothreitol at 60°C for 30 minutes, and alkylated with 15 mmol/L iodoacetamide for 30 minutes. Samples were diluted with 50 mmol/L ammonium bicarbonate to 1.6 mol/L urea concentration and digested with 1 μ g Lys-C/Trypsin mix (Promega, Madison, WI) overnight at 37°C, on an orbital shaker with a 3 mm orbit, 300 rpm. The digestion was stopped by addition of 10% trifluoroacetic acid to a final concentration of 0.5% (v/v), and RapiGest was degraded by incubation at 37°C for 60 minutes. The peptide samples were desalted using SP3 beads, as described by Hughes et al.²⁵ As the starting material was peptides, the peptide SP3 cleanup procedure was followed. Briefly, acidified peptide samples were supplemented with 4 μ L SP3 beads, and acetonitrile was added to a final concentration of >95%. After incubation for 8 minutes, samples were washed with 100% acetonitrile. Peptides were

eluted by incubation for 5 minutes in aqueous buffer with 2% dimethyl sulfoxide, followed by sonication. Peptide samples were mixed 1:1 (v/v) with 2× buffer A (4% acetonitrile and 0.4% formic acid), giving a final concentration of 2% acetonitrile, 0.2% formic acid, and 1% dimethyl sulfoxide.

Liquid Chromatography—MS/MS Analysis

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the Proteomics Identification Database partner repository (PRIDE, <https://www.ebi.ac.uk/pride/archive/projects/PXD014171>, publication date, May 19, 2021). Peptides were separated on an EASY-nLC 1200 high-performance liquid chromatography system (Thermo Fisher Scientific, Waltham MA) using a 50-cm EASY-spray PepMap RSLC C18 column (Thermo Fisher Scientific). For peptide separation, the following segmented gradient of solvent B (0.1% formic acid in 80% acetonitrile) over solvent A (0.1% formic acid) was used: 3% to 10% solvent B for 11 minutes, 10% to 30% solvent B for 87 minutes, 30% to 45% solvent B for 22 minutes, 45% to 80% solvent B for 4 minutes, followed by 100% solvent B for 10 minutes, using a flow of 350 nL/minute. Data acquisitions were performed on a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific). For data-dependent acquisition, full MS scans were acquired at resolution of 120,000 at 200 m/z, automatic gain control target 3 million, with maximum injection time of 100 milliseconds at mass range 350 to 1650 m/z. Each full scan was followed by MS/MS fragmentation of the top 15 most abundant ions with 30,000 resolution at 200 m/z, automatic gain control target 3e6, and maximum injection time of 50 milliseconds.

Data Analysis

Analysis and searches of raw files were performed in MaxQuant version 1.6.3.3 (<https://www.maxquant.org>, last accessed May 1, 2019) and Andromeda toward a reviewed UniProt human database with standard contaminants (downloaded November 17, 2015). Default settings were used in MaxQuant and the summed protein intensities were reported for all peptides identified for the respective protein group. Search parameters included trypsin with maximum two missed cleavages at 4.5 parts per million for precursors and 20 parts per million for fragment ions. Carbamidomethylation was set as fixed modification, and methionine oxidation was set as variable. A false discovery rate of 1% was used for both proteins and peptides. The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (<https://www.ebi.ac.uk/pride/archive>, last accessed June 7, 2019) with the data set identifier (PXD014171).²⁶ For classification of extracellular matrix proteins, we used the matrisome assignments described by Naba et al.^{27,28} All MS data are summarized in

Supplemental Table S2, presenting information of the number of proteins in each protein group; the number of peptides for each protein group; the summed intensity of the protein group for respective sample; and information regarding how many of the patient samples the protein group was identified in. If not otherwise indicated, data are shown for proteins, which were identified in more than one sample. A minimum CI score of 0.4 was used for the protein network illustration because of low numbers of proteins for these types of statistics. For classification of identified proteins into functional groups, this type of tool can describe potential cellular pathways involved in the pathology of OB lesions. Data were visualized using RStudio version 1.1.442 (RStudio, Boston, MA).

Results

Histologic Identification of OB Lesions

Fourteen OB lesions were identified in the pneumonectomy specimens by examination of HE-stained sections from tissue blocks. Seven different lesions were analyzed (OBa-g) from Patient 1 (P1; P1 OBa-g); 3 from Patient 2 (Patient 2 OBa-c); 3 from Patient 3 (Patient 3 OBa-c); and 1 from Patient 4 (Patient 4 OBa). Because of a clear delineation of the lesions and preferably cross-sectioned airways, these lesions were particularly suitable for LCM. Material was collected from each lesion during laser-capture microdissection from several tissue sections (4 µm thick). Consecutive sections of each OB lesion were used for LCM/MS and IHC/IF to characterize their protein content (Figure 1). The lesions exhibited substantial morphologic heterogeneity (Figure 2 and Supplemental Figure S2). No patient-specific histologic pattern was observed. Characteristics of previous studies were confirmed in the submucosal fibrosis with narrowing of the airway lumen, with complete obliteration in Patient 3 OBB. This study included three histologic subgroups: i) terminal bronchioles with OB-like features, ii) bronchioles with classic OB, and iii) bronchioles with suspected OB and muscular hypertrophy.

Proteome Description of the 14 OB Lesions

LCM was used to select the specific OB lesion regions and data-dependent acquisition MS, followed by data analysis in MaxQuant, to identify and describe the proteome composition of the 14 OB lesions. Proteome analyses were performed on minute amounts of sample, and mean LCM-collected area was 499,131 µm² from sections (4 µm thick) (Figure 3A). Variations in number of identified proteins (Figure 3B) did not depend on differences in LCM-collected amount of material (Figure 3A) or LCM type (Figure 3B). Spearman correlation plots were used to visualize correlations between identified proteins within the lesions (Figure 3C). No group cluster associations were found

Figure 2 Obliterative bronchiolitis (OB) lesion morphology. Histologic classification of OB lesions (hematoxylin/eosin) exhibiting different morphologic features. Patient samples were grouped into the following three subgroups: i) terminal bronchiole with OB-like features (**solid lines**), ii) bronchiole with OB (**dotted lines**), and iii) bronchiole with suspected OB, with smooth muscle hypertrophy (**dashed lines**), based on histologic classification. Scale bars = 100 μ m. P1 to P4, Patients 1 to 4.

within the analyses when evaluating LCM type used during microdissection, histologic subgroup of the OB lesions, or patient belonging. This suggests that these parameters do not drive the resulting proteome correlations within the OB lesions. The proteome correlations varied from 0.16 to 0.84 between the samples. A separate correlation matrix was constructed, excluding the single lesion analyzed from Patient 4 ([Supplemental Figure S3A](#)), finding a similar result.

For functional annotation of the identified proteins within the OB lesions, the protein network obtained with STRING database ([Figure 4](#)) was used to show the suggested protein-protein interactions. The biological process with the gene ontology term extracellular structure organization showed the highest representation, and proteins within this node are visualized in red. The nodes with gene ontology terms tissue development (green) and response to wounding (blue) were selected for visualization. The enrichment of proteins associated with extracellular structure organization in the OB lesions suggests this pathway to be of high importance. Excluding the single lesion from Patient 4 did not alter the protein network as none of the proteins was unique for this lesion. Validation and spatial distribution of proteins within this node are further evaluated in representative OB lesions with MS and histology staining.

Representative Proteins in OB Lesions

To further capture proteomic similarities within the OB lesions, sample presence was visualized by showing how often the same proteins were identified in each OB lesion and the proteins' log₁₀ abundance distribution ([Figure 5A](#)). From these analyses, the protein candidates identified in >75% of samples were selected and visualized in a heat map ([Figure 5B](#)). This cutoff was accepted as a threshold because this represented, after careful analysis of other cutoffs (such as present in more than one sample or present in 50% or 100% of the samples), the best-balanced representation of the data. These 39 proteins represent the selection of protein candidates descriptive for OB lesions. Analyzing the data by excluding the single sample obtained from Patient 4 showed similar results ([Supplemental Figure S3B](#)). A complete list with identified proteins in each OB lesion is presented in [Supplemental Table S2](#) (MS data).

Next, six structural extracellular matrix proteins were selected [collagen α -3 (VI) chain, collagen α -1 (VI) chain, collagen α -2 (VI) chain, collagen α -2 (IV) chain, collagen α -1 (IV) chain, and periostin ([Figure 5B](#))], and their spatial distribution was analyzed within the OB lesions using

Figure 3 Laser-capture microscopy (LCM)—mass spectrometry description of obliterative bronchiolitis (OB) lesions. **A** and **B**: For each OB lesion, the collected total area (**A**) and the number of identified proteins and LCM mode (**B**) were estimated. **C**: The Spearman rank correlations between samples take proteins into account that were identified in more than one lesion. LCM type used during collection of samples, histologic subgroup, and patient belonging are included in the legend. Spearman rank correlation is colored from red (high correlation) to blue (low correlation). P1 to P4, Patients 1 to 4.

immunohistochemical stainings. These proteins were chosen because of their importance in the basement membrane (BM) structure and how they are affected during pathologic tissue remodeling of airways, which in BOS may result in disruption of the epithelial barrier, thereby leading to disease progression.

Accumulation of Collagen Type IV, Collagen Type VI, and Periostin in OB Lesions

To characterize the fibrotic compartment of the OB lesions, collagen type IV, as identified by MS, was

selected and its histopathologic distribution was characterized by IHC/IF.

Collagen type IV, an exclusive BM component, was identified by both its α -1 and α -2 chains by MS (Figure 6, A and B) in 11 of the 14 lesions. Figure 6C shows smooth muscle and bronchiolar BM staining as expected (normal). Distinct variations in collagen type IV deposition, well mirroring the MS results, were seen in different lesions. P1 OBa showed extensive deposition of collagen type IV in the fibrotic protrusion, whereas Patient 2 OBa showed a thickened, positive bronchiolar BM. Patient 3 OBc had a relatively low deposition of collagen type IV (Supplemental Figure S4). Similarly, collagen type VI was detected by

Figure 4 Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database analysis of all proteins identified within the obliterative bronchiolitis lesions. Gene ontology group annotations are colored: red, extracellular structure organization; green, tissue development; and blue, response to wounding. The line thickness indicates the strength of confidence of data support, where the minimum required interaction score was set to 0.4 (medium confidence). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

MS in all lesions (Figure 7A). Strong deposition of collagen type VI in the fibrotic protrusions within the OB lesions was seen by IF in most lesions; however, this was not seen in the ones classified as suspected OB/smooth muscle hypertrophy (Figure 7C and Supplemental Figure S5). Similarly, periostin was identified by both MS (Figure 7B) and IF (Figure 7D) in selected lesions. Distinct periostin accumulation was found in the fibrotic areas protruding into the airway lumen, most prominently in sample P1 OBa and P1 OBb, as well as in generalized submucosal fibrosis in P1 OBc. IF staining of collagen type VI in healthy control bronchiolar tissue typically resided in basal membrane and is present throughout the subepithelial space inside the smooth muscle ring, whereas periostin staining was mainly visible outside the smooth muscle ring (Supplemental Figure S6).

Accumulation of α -SMA— and Tryptase-Positive Cells in OB Lesions

IF staining for α -SMA was used to detect the smooth muscle wall of the pulmonary airways surrounding OB lesions, but also identified an accumulation of positive cells with an elongated cell shape in the fibrotic protrusions (Figure 7, C and D). These likely represent myofibroblasts, according to morphologic features. Cells in the OB lesions were negative for the proliferation marker Ki-67 (Supplemental Figure S7), but some were positive for tryptase (Supplemental Figure S8A), indicating presence of mast cells. OB lesions with low levels of tryptase (Supplemental Figure S8B) looked similar to the healthy control (Supplemental Figure S8D), whereas high tryptase levels in the MS analysis, Patient 3 (Supplemental

Figure 5 Proteomic characterization of obliterative bronchiolitis (OB) lesions. **A:** Sample presence and log10 intensity distribution of identified proteins within OB lesions. **B:** Heat map with unsupervised hierarchical clustering of proteins representatively present in >75% of the characterized OB lesions. Terms marked in red show the structural extracellular matrix proteins used for further spatial tissue distribution. EGF, epidermal growth factor; EMILIN, elastin microfibril interfacier; NA, not applicable; P1 to P4, Patients 1 to 4.

Figure S8A), showed more prominent tryptase staining (Supplemental Figure S8C) compared with healthy control airways (Supplemental Figure S8D).

Discussion

This study shows a link between the morphologic appearance of OB lesions in remodeled airways in lungs from patients experiencing end-stage BOS and their respective

protein content. Protein signatures of the OB lesions correlate to extracellular matrix organization, tissue development and wound healing processes, and aberrant BM composition. Interestingly, these findings corroborate results from earlier studies from our group obtained in a larger patient cohort, where early extracellular matrix changes were shown to be associated with later development of BOS.²⁹ In line with this, previous findings demonstrate that the vascular endothelial growth factor/vascular endothelial

Figure 6 Collagen type IV immunohistochemistry (IHC). **A** and **B**: Corresponding signal intensities from laser-dissected regions analyzed by mass spectrometry for the collagen type IV α 1 (**A**) and α 2 (**B**) chain. **C**: IHC for collagen type IV, depicting areas of high intensity (a), low intensity (b and c), and bronchiolar basement membrane (b and arrows) staining of collagen type IV in selected obliterative bronchiolitis (OB) lesions as well as in an airway unaffected by fibrosis (normal). Positive collagen type IV staining, brown. Scale bars: 50 μ m (**C**, top row); 10 μ m (**C**, bottom row). E, epithelium; M, airway smooth muscle; P1 to P4, Patients 1 to 4.

growth factor receptor 2 axis is involved in early signs of OB lesions and may serve as a marker for bad prognosis.³⁰ Moreover, total collagen, collagen type IV, and biglycan were found at an early stage, 3 months after transplantation, and were predictive of later development of OB lesions, which was also confirmed in this study.²⁹ A possible mechanism may include the release of biglycan from the extracellular matrix on tissue stress, which contributes to increased fibroblast migration and release of transforming growth factor (TGF)- β , a prominent actor in fibrosis.^{31,32} Keratins 5, 9, and 14 are detected in the OB lesions (Figure 4). Keratin 5 is a marker for basal cells, and keratin 14 is expressed by basal cells during lung inflammation and is, as keratin 9, involved in intracellular intermediate

filaments, which have an impact on cell migration during lung tissue repair.^{33–35} Keratin 6c, also found in the OB lesions (Figure 4), is important for epithelial-mesenchymal transition and is involved in cell migration and proliferation in lung cancer.³⁶ These results point toward an intriguing role of the keratins in lung fibrosis, inflammation, and cancer.

Furthermore, in the analyzed OB lesions, a multitude of proteins (maximum, 89/lesion—minimum, 30/lesion) were identified, thereby dismissing the hypothesis that the airway lesions are composed of only a few characteristic proteins, which highlights the complexity of the disease and underlines the need to identify the earliest disease events and cells responsible for producing fibrogenic proteins.

Figure 7 Collagen type VI/ α -smooth muscle actin (α -SMA) immunofluorescence (IF). Corresponding signal intensities from laser-dissected regions analyzed by mass spectrometry for the collagen type VI (A) or periostin (B). IF staining for collagen type VI (yellow; C) and periostin (yellow; D) together with α -SMA (red; C and D) in selected obliterative bronchiolitis (OB) lesions. **Asterisks** indicate strong collagen type VI or periostin deposition; **arrows**, single α -SMA-positive cells; blue, nuclei; **white dashed lines**, area within the smooth muscle ring. Scale bars = 50 μ m (C and D). P1 to P4, Patients 1 to 4.

Furthermore, both MS data and histologic visualization of proteins, like collagen type VI and periostin, showed pathologic deposition. The BM protein collagen type IV showed an unusual distribution by being accumulated outside the usual epithelial BM, most likely evidence of a disturbed epithelial/mesenchymal balance in the OB lesion. Collagen types IV and VI are two of several proteins in the BM, and the loss of their integrity causes an abnormal lung architecture thought to promote fibrosis. Interestingly, these proteins are known to have both adhesive and antimicrobial properties, and act as key players in connective tissue innate immunity.^{37–39} In addition, periostin has been shown to

promote myofibroblast differentiation and collagen type I production, and studies to date suggest that periostin could be a useful biomarker for clinical progression in lung disease, resulting in various types of fibrosis.⁴⁰

Both hematologically derived mononuclear phagocytes and epithelial-mesenchymal transition have been proposed as sources of myofibroblasts that are responsible for the fibrogenesis in the OB lesions.^{41–43} Indeed, the examined lesions in this study contained a multitude of α -SMA-/vimentin-positive, elongated cells, with morphologic and immunohistochemical features of myofibroblasts. Interestingly, transgelin, a TGF- β inducible protein detected in the

OB lesions (Figure 5), is an early marker of myofibroblast differentiation, and has previously been shown to be involved in epithelial cell migration in idiopathic pulmonary fibrosis.⁴⁴

Fibrocytes may be recruited from the circulation into the OB lesion as producers of proteins commonly found in fibrotic tissue.⁴⁵ Moreover, macrophages of donor origin have been indicated to mediate fibrosis in OB lesions following bone marrow transplantation through the action of TGF- β .⁴⁶ More important, macrophages are often localized in close proximity to collagen-producing myofibroblasts and have been suggested to be master regulators of fibrosis.⁴⁷ Indeed, TGF- β promotes synthesis of collagen and other extracellular matrix molecules, such as hyaluronan and proteoglycans.^{46,48} TGF- β also induces tissue inhibitors of matrix metalloproteases that are involved in fibrosis by blocking extracellular matrix degradation. In fact, in bronchiolitis obliterans, the balance of tissue inhibitors of matrix metalloproteases and matrix metalloproteases is altered, and especially the matrix metalloprotease-8, matrix metalloprotease-9, and tissue inhibitor of matrix metalloprotease-1 that we found are mirrored in bronchoalveolar lavage fluid from patients and may serve as biomarkers for prognosis.⁴⁹ This may reflect a polarization toward profibrotic macrophages, which are characterized by their expression of tissue inhibitor of matrix metalloprotease-1, matrix metalloprotease-9, and TGF- β , among other molecules.⁵⁰ Another cell type that has been proposed to be involved in the fibrotic propagation in OB lesions is the mast cell.⁵¹ Interestingly, the mast cell protein tryptase was identified in all examined lesions. Mast cell abundance correlates to myofibroblasts expressing α -SMA in human pulmonary fibrosis,^{52,53} and the stiff extracellular matrix in OB lesions itself has an inductive effect on mast cells and their release of tryptase and chymase, which activate profibrotic TGF- β pathways.⁵⁴

The protein signatures of the OB lesions reported herein mirror the heterogeneity seen at the histologic level. This study found that the OB lesions differed within and among patients. Interestingly, two suspected lesions were identified in Patient 2, which differed by exhibiting smooth muscle hypertrophy and a deviating protein pattern (Figure 4). More efforts are needed to further subgroup these different subtypes and to understand the functional implications of the accumulated proteins. Functional annotations underline the importance of the identified proteins in the context of extracellular matrix remodeling and highlight pathways belonging to tissue development and wound healing, processes that are suspected to play a role in the development of chronic lung allograft dysfunction.^{29,55,56}

Although the low patient number and the inability to follow the progression of lesions over time are distinct drawbacks, this study gives a snapshot of ongoing disease activity, including cellular pathways involving extracellular structure organization, tissue development, and response to wounding. These results persist after analyzing the data,

excluding the single sample obtained from Patient 4, supporting the validity of the data. In line with this, the study presents an unbiased and idea-generating approach to identify key cellular events in the course of BOS and provides a unique window into the nature of remodeled pathologic airways after lung transplantation. Other studies have focused on predicting BOS in lung transplanted patients by analyzing the bronchoalveolar lavage proteome, which has yielded several potential predictive markers as well as suggestive cellular pathways indicative of future BOS development.^{57,58} In conclusion, this study adds to the understanding of the formation of OB lesions, and the identification of early processes, and preferably their triggering events, will facilitate future development of tools to counteract fibrosis before symptomatic manifestation.

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Supplemental Data

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