

Exploring the Context of the Lung Proteome within the Airway Mucosa Following Allergen Challenge

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The lung proteome is a dynamic collection of specialized proteins related to pulmonary function. Many cells of different derivations, activation states, and levels of maturity contribute to the changing environment, which produces the lung proteome. Inflammatory cells reacting to environmental challenge, for example from allergens, produce and secrete proteins which have profound effects on both resident and nonresident cells located in airways, alveoli, and the vascular tree which provides blood cells to the parenchyma alveolar bed for gas exchange. In an experimental model of allergic airway inflammation, we have compared control and allergen challenged lung compartments to determine global protein expression patterns using 2D-gel electrophoresis and subsequent spot identification by MS/MS mass spectrometry. We have then specifically isolated the epithelial mucosal layer, which lines conducting airways, from control and allergen challenged lungs, using laser capture technology and performed proteome identification on these selected cell samples. A central component of our investigations has been to contextually relate the histological features of the dynamic pulmonary environment to the changes in protein expression observed following challenge. Our results provide new information of the complexity of the submucosa/epithelium interface and the mechanisms behind the transformation of airway epithelium from normal steady states to functionally activated states.

Keywords: lung • mucosa • epithelium • laser capture microscopy • proteome • mass spectrometry • annotation identity • allergen

Introduction

The lung is a complex organ, which sustains gas exchange through an intricate set of narrowing channels that provide the structural framework for erythrocyte trafficking and gas diffusion. Cellular and molecular barriers of shielding meet the secondary function of the lung, which is to protect an immense surface area against insults from the environment. The normal lung, then, provides the means for day-to-day maintenance of the shielding mechanisms and the mechanical structures necessary for gas exchange. The diseases of the lung such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, Acute Respiratory Distress Syndrome (ARD), Interstitial Lung Disease (ILD), Sudden Acute Respiratory Syndrome (SARS), as well as exposure to particulate matter and to bacterial, fungal, and viral agents, typically present clinically

with complex pathologies involving cells of many derivations. The diseases of the lung, like many other diseases of our society, are of such complexity, that the development of effective treatments will likely require a new basis of understanding of protein function during the normal course of pulmonary activity and within an environment driving a specific disease process.

The measurement of protein expression within tissues, such as the lung, presents many challenges for establishing accurate descriptions of the contextual relationships of proteins and their function within a dynamic environment. *What to measure? When to measure? Where to measure?* How do we establish a meaning, then, to these measurements? The modern approaches for studying the molecular basis of disease typically compare functions such as activation, proliferation, repair, and growth under both regulated and un-regulated conditions of gene and protein expression.

In terms of global protein expression, the exact contributions of specific cells within specialized tissue compartments are often difficult to resolve in tissue composed of heterogeneous cell types varying in activation and differentiation states. Within any cellular compartment, the relative concentrations of individual proteins vary, and the gradients of protein concentrations occurring in extra-cellular compartments will have an important impact on their potential functional activities. In

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terms of assigning biological significance to specific proteins encountered in global protein surveys, we will need to establish confidence limits and specific cut off values for the *what-when-where* measurements we obtain.

To better understand the kinetics and range of the lung proteome, our studies have related the phenotype of global (whole lung) protein expression at steady state to the patterns of expression induced during an allergic response in the airways. Our strategy was to establish histological landmarks, which could be used to compare and relate the relative distributions and phenotypes of resident and inflammatory cells to frank changes in airway structures, and to the overall pattern of proteins detected within these compartments. In this study, we have analyzed an experimental model of bronchial/pulmonary hyper reactivity caused by allergen sensitization and challenge. This model produces profuse epithelial hyperplasia and mucus production within the conducting airways and a severe bronchial and vascular inflammation. Using 2D-gel electrophoresis for separation and isolation of the proteome, and subsequent identification of spots by mass spectrometry, we have identified and annotated the global protein expression profiles in the whole lungs of both normal control and allergen challenged animals.

We then addressed whether we could detect changes in protein expression occurring at and within the sub-mucosa of the airways following allergen challenge. Using laser capture microdissection (LCM) we obtained samples of conducting airway epithelium, mesothelium, and inflammatory cells bordering surfaces exposed to allergic challenge. This microcosm of inflammatory responses was first characterized histologically, LCM sampled, and then analyzed ending with MS/MS spot/peak identity analysis.

The goals of this study were as follows: (i) To establish the limits of detection of tissue-associated proteins using modern state of the art MS/MS technology, and (ii) To characterize the dynamic range of proteins, which are especially expressed in the lung during allergic challenge. Our results provide new information on the complexity of the sub mucosa/epithelium interface and the cellular processes associated with the transformation of the airway epithelium, between steady stationary growth phases and activated mucosal surfaces, in response to allergen challenge.

Methods

Mice. All animal studies were performed under protocols approved by the Malmö/Lund ethical committee for animal experiments (M254–99). Female BALB/C, mice, weighing 20–25 g, were purchased from Bomholtgaard, Denmark and housed in plastic cages with pine chip bedding (10 mice/cage). The animal room was maintained at 22 °C with a daily light-dark cycle (0600–1800 light) and fed with chow and water ad lib. The mice were 8 weeks old at the time of study.

Sensitization and Provocation. We have adapted a previously described model of allergic airway inflammation in this study.¹ Briefly, for OA allergen induction, on days 0 and 7, Ovalbumin (OA) at 25 µg/mL was injected intraperitoneal (i.p.), 0.3 mL/animal, in a solution together with aluminum hydroxide (Alum). (7.5 µg OA/animal and 1.5 mg Alum/animal). The mice were then exposed twice to OA by inhalation on day 14 and day 21 in boxes. The aerosol was generated by a Bird nebulizer (pressure: 4.0 bar, filling volume: 50 mL for each 30 min) for 1 h. The OA concentration in the nebulization solution was 10 mg/mL. The estimated OA dose to the lung (extracted from

earlier deposition studies) was approximately 20 µg/animal. Control animals were injected with PBS i.p. on days 0 and 7 only. Twenty-four hours after the last OA challenge, the OA mice and the controls were sacrificed by intraperitoneal injection of 0.15 mL sodium Phenobarbital (60 mg/mL, ATL, Apoteksbolaget, Sweden).

Lung Sampling. The lungs of five animals were sampled from each of the control and allergen challenged groups. The mice were inserted with a cannula into the trachea and the lungs were slowly inflated by injection with 0.7 mL of a 66% solution of Tissue-Tek O. C. T. (Sakura Finetek, Torrance California) in PBS pH 7.2 The trachea was tied off, and the whole lung was dissected out and the left lung lobe was carefully removed and placed immediately into a bath of isopentane on dry ice for snap freezing. The lungs were placed into storage vials and stored at –70 °C until sectioning.

Histological Staining. In some experiments, the O. C. T. sufflated lungs were placed into a vessel and fixed overnight with formalin, embedded in paraffin, and thin sections cut in the sagittal plane were placed onto glass slides. These slides were then stained using conventional methods with haematoxylin and eosin, Alcian blue-Periodic acid Schiffs stain, modified elastin-Van Gieson's stain, Masson's Tri-chrome, or May-Gruenwald-Giemsa stain. In other experiments, cryostat sections were placed onto glass slides, dried, fixed with 99% ethanol (4 °C) stained with haematoxylin and eosin, and then dehydrated with 100% ethanol and xylene. Slides were analyzed histologically using a key for pathological indices specific for the airways, vasculature, or parenchyma on a Leica DMXR microscope

Morphometric Measurements. In some experiments, quantitative morphometric measurements were performed using microscope images acquired from a dedicated LEICA DMRXA microscope (Leica, GmbH Wetzlar, Germany). Images of the lung were acquired into a computer in 8-Bit gray scale at a final magnification of 200–400×. The images were then analyzed using Qwin Program, thresholded, and then smoothed and inverted using in-house routines written in Quips. Morphometric assessment consisted of determining the mean relative airway epithelial area (measured as area under the curve per linear 100 mm distance, as measured between the basement membrane and the apical edge of the epithelial cell layer), the mean thickness of the basement membrane, and the mean thickness of the elastic lamina. For each section of lungs, 20–30 histological fields were evaluated at 200× final magnification and the mean of 70–100 measurements covering 1000 linear mm in each field were used to compute mean group values for each of the stated measurements.

Sample Preparation. For studies of global protein expression, whole lung was thin sectioned in the sagittal plane on a cryostat (10 µm thick) as central transverse biopsies and kept frozen at –70 °C until use. To solubilize the whole tissue, three whole sections were placed into Eppendorf tubes containing two hundred microliters IEF lysing solution, in 7 M urea, 2 M thiourea and 4% CHAPS and vortexed vigorously for 1 min. and then centrifuged before further use.

2-DE Gel Electrophoresis. Immobiline Dry strips (180 mm, pH 3–10 NL) were rehydrated in 350 µL of the solubilization solution containing 7 M thiourea, 2 M urea, 4% CHAPS, 10mM DTT, and 0.5% IPG 3–10 buffer, together with the fractionated samples (100µL)

The isoelectrophofocusing (IEF) step was performed at 20 °C in a IPGphor (Amersham Pharmacia Biotech, Uppsala,

Sweden) and run according to the following schedule: (1) 30 V for 10 h, (2) 500V for 1 h, (3) 1000 V for 1 h, and (4) 4000 V until approximately 45000 Vhrs were reached. The strips were equilibrated for 10 min in a solution containing 65 mM DTT, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 50 mM Tris-HCl pH 8.8. A second equilibration step was also carried out for 10 min in the same solution except for DTT, which was replaced by 259 mM iodoacetamide. The strips were soaked in electrophoresis buffer (24 mM Tris base, 0.2 M Glycine and 0.1% SDS) just before the second-dimensional gel electrophoresis. The strips were applied on 14% homogeneous Duracryl slabgel. The strips were overlaid with a solution of 1% agarose in electrophoresis buffer (kept at 60 °C). Electrophoresis was carried out in an Hoefer DALT gel apparatus (Amersham Pharmacia Biotech, San Francisco, CA) at 20 °C and constant 100 V for 18 h. All lung samples were run in triplicate.

Gel Staining. Gels were stained with silver according to Shevchenko.²

Spot Analysis. Gels were scanned using a Fluor-S Multi-Imager (Bio-Rad Laboratories, Sundbyberg, Sweden). Spot analysis was performed using the PDQUEST (version 6.1.0) two-dimensional gel analysis system (Bio-Rad discovery series, Bio-Rad Laboratories, Sundbyberg, Sweden).

Mass Spectrometry Identification. Mass Spectrophotometry was performed as previously described.³ Briefly, the MALDI-TOF instrument used was a Voyager DE-PRO (Perseptive Biosystems Inc., Framingham, MA) mass spectrometer. The instrument, equipped with a delayed extraction ion source, utilized a nitrogen laser at 337 nm and was operated in reflectron mode at accelerating voltages of 20 kV. The sample probes were made of polished stainless steel. Sample deposition of nanoliter fractions was made on stainless steel MALDI-target plates and on a 100-position stainless steel target plate (Perseptive Biosystems).

Microdissection. Epithelial cells from the airways were microdissected by LCM (Pixcell II, Arcturus Engineering, Mountain View, CA). The laser spot size used were 30 μ m in diameter (pulse power: 30 mW, pulse width: 5.0 ms threshold voltage: 250 mV).

Protein Expression Analysis of LCM Isolated Epithelium. The epithelial mucosa attached to the polymer cap were lysed by IEF lysing solution containing 7 M urea, 2 M thiourea and 4% CHAPS and vortexed vigorously for 1 min. The IEF lysing solution was then reapplied to another cap holding cells from the same microdissected case, and the procedure was repeated until each tube contained material from approximately 7000 shots. The solubilized epithelial cells were then equilibrated with the Immobiline strip overnight and subsequently run on the 2nd dimension SDS gel and then analyzed as above.

Protein Identification and Annotation. The proteins were annotated and identified in a two-step procedure as partly previously described.³ First, image analysis was performed by hierarchical differential analysis where all protein spots were annotated as expressed entities on the 2D-gels. These annotations were next quantified by mean intensity values from the respective animal group, where the lowest regulation factors defined in this study was set to at least 2.0. Protein spots of interest were excised by the use of an automated spot cutting instrument (Proteome Works Spot Cutter, Biorad, Hercules CA), and positioned in a 96-well plate. These 96-well plates were digested and micro-extracted as described previously.⁴ The second step protein identity was performed by applying both

MALDI-, and ESI–tandem mass spectrometry, using SwissProt and NCBI databases for MS-spectra query.

Results

The Lung. In our studies, the mouse lung in its tiny form represents much of the complexity of the structural and cellular elements found in the lungs of higher mammals such as man. Histologically, the compartments of the mature mammalian lung contain a wide variety of tissue types composed of several lineages of differentiated cells including epithelium, endothelium, mesothelium, nerve, and lymphoid cell types. The complex structure of the lung provides a means for not only for the intimate contact of cells with inhaled air but also for accruing and re-distributing these cells throughout the body. This is accomplished through an intricate series of narrowing channels of airways and blood vasculature, which functionally provide ventilation and perfusion. For example, in man, the conducting airways used for inhalation and exhalation can contain more than 20 branches or sub-divisions as bronchi and then bronchioles, before it reaches and forms the basic functional unit of respiratory gas exchange, the alveoli.⁵ Alveoli are thin walled “air spaces” composed of micro-capillaries, epithelial pneumocytes, smooth muscle, a basal lamina, and elastic connective tissue. Each human lung contains 300–400 million of these alveolar air sacs within the parenchyma, and in total, the alveolar subcompartments comprise over 90% of the lungs surface area.^{5,6} The airways of the bronchial tree are lined with an epithelium layer that provides the lung with a cellular barrier against the air born environment, which can include volatile chemicals, infectious agents, and allergens. The pulmonary epithelium also provides a means for maintaining moisture through mucous, serous, and surfactant secretions. In addition, mucous secretion and epithelial ciliary movement within the airways are important mechanisms for capturing and transporting away infectious agents and noxious substances. The whole lung tissue and the airway epithelium are the focus of this study.

The Allergic Airway Model. Although the biological processes we study in this and other genetically inbred models are only partially representative of the range of cellular and pathobiological responses which are found in humans, there are many features observed in this model that make it attractive for studying protein expression during an inflammatory response. The allergic airway model we have studied produces a rapid onset of vigorous inflammatory response within the lungs of animals sensitized over a three-week period and then challenged intra-tracheally with ovalbumin (OA) (Figure 1). Within hours after OA challenge, a number of changes to the airways and vasculature occur which can be observed and measured histologically. The model does not produce destructive lung disease resulting in emphysema. In contextual reference to the lung proteome in nonchallenged animals, the inflammatory response produces vascular leakage and plasma exudation around vessels, which involves diffusion of proteins into the airway lumen, but the model does not disrupt or shear vessels, which could result in haemorrhage of blood into alveolar spaces or the lumen of airways.

In Figure 1 are shown sagittal sections of the left lung lobe of an allergen naïve control lung (Figure 1a) and an OA sensitized lung 24 h after challenge (Figure 1b) for comparison. Seen centrally in both micrographs, are the major conducting airways and surrounding blood vessels. The OA challenged lung contains a conspicuous inflammatory cell component, which

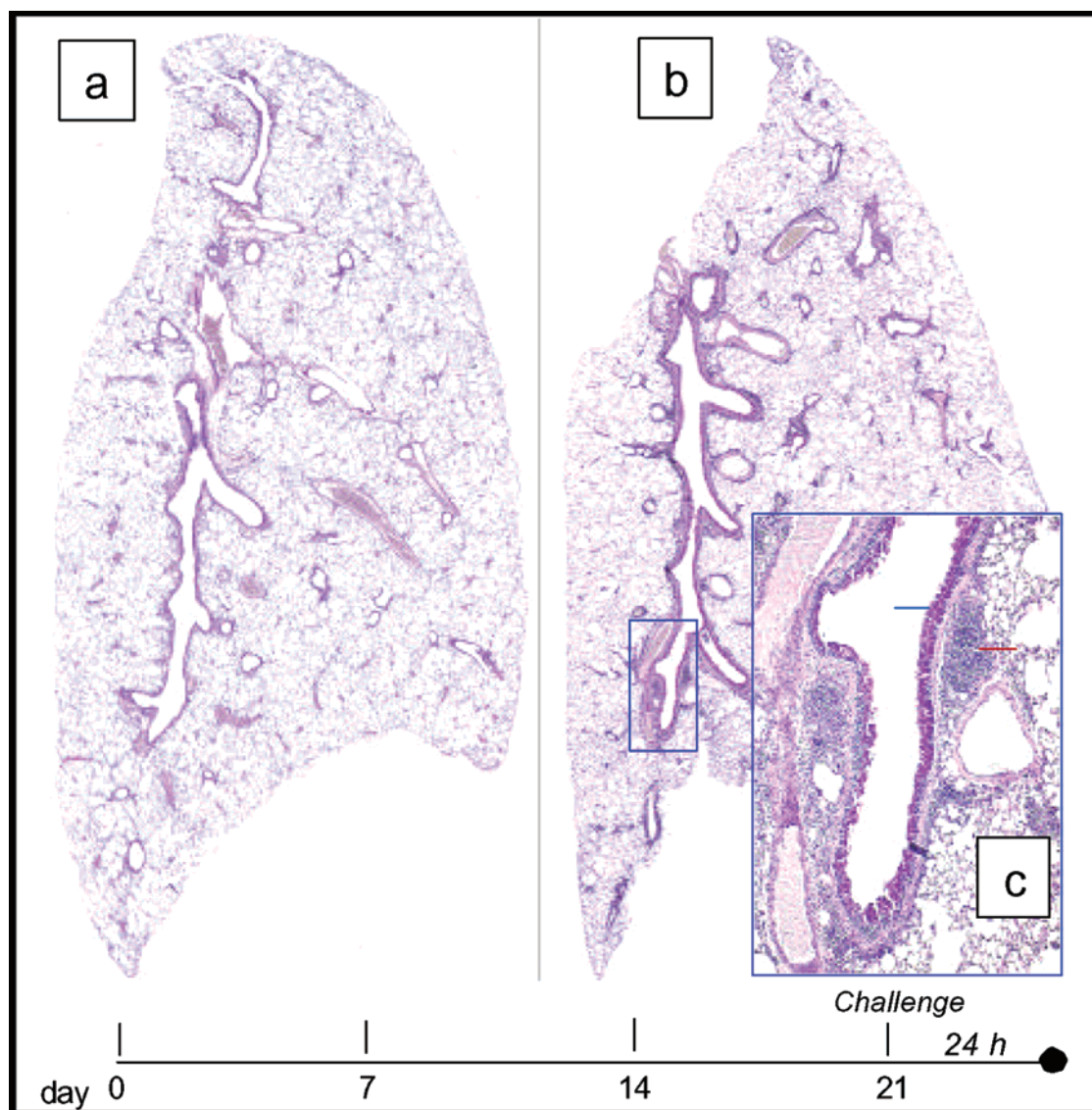


Figure 1. Histological features of the lung following allergen challenge. Animals were immunologically sensitized to ovalbumin with three injections at 7 day intervals, and then challenged with a single intra-pulmonary exposure to ovalbumin (OA) on day 21, and then sampled 24 h later. Seen in (a) is a central sagittal section of the entire left lobe from a healthy control and in (b) an OA challenged mouse exhibiting profuse peri-bronchial and peri-vascular inflammation. In the higher magnification insert (c), we see the lower aspect of the conducting airway and surrounding sub-epithelial mucosa. The violet stained epithelial layer (blue arrow) surrounding the luminal space is producing mucins. The dense blue pockets of inflammatory cells responding to allergen challenge (red arrow) are seen adjacent to surrounding blood vessels and beneath the conducting airways.

is seen peri-bronchial and peri-vascular as a thickened ring of cells around nearly every blood vessel. The expanded insert (Figure 1c) shows a portion of the lower airway in more detail. The distal parenchyma remains relatively intact without alveolar wall thickening or oedema. The prominent location of the majority of inflammatory cells around vessels indicates that cells are recruited from the blood into the tissue at sites near the airway lumen locations exposed to OA. The major cell component of the tissue inflammatory response is eosinophilia, however lymphocytes, monocytes, neutrophils, and resident pulmonary macrophages also are recruited interstitially. As shown in Figure 2 there are a battery of biological responses which occur at the mucosal inter-phase following allergen challenge including tissue destruction and repair, cell proliferation and apoptosis, chemotaxis and inflammation, and the secretion of mucus and extracellular mediators. The invasion of the eosinophils into the elastic and medial lamina, and

the mobilization of fibroblasts within the connective tissue, occur very near the epithelial sheath adjacent to the airway lumen.

In comparison to control airways (Figure 3a), the OA model also produces a profuse activation and proliferation of the epithelial cells lining the conducting airways and bronchioles (Figure 3b) which results in mucus hypersecretion. Mucus proteins form water-soluble homo-polymers or hetero-polymers with other mucins or proteins that are difficult to analyze in proteomic studies due to the high molecular weight of the complexes. In most histological staining procedures these mucin complexes are often washed away during the fixation and preparation processes. To preserve the natural layer of mucus within the airway lumen we used alcohol fixation and processing which allowed us to conserve the extensive production of mucus in the OA model as streams exuding from the apical tips of the epithelial cells lining the airway lumen.

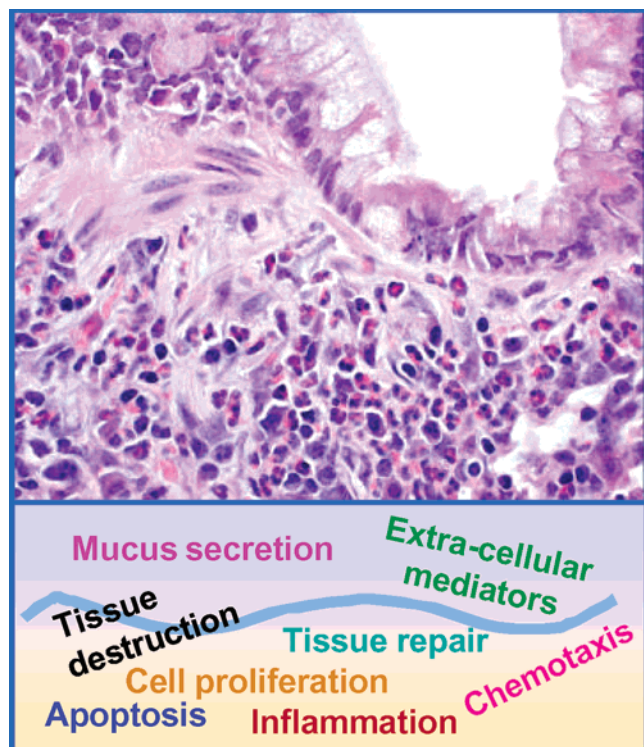


Figure 2. Lung sub-mucosa during allergen challenge. Twenty-four hours following allergen challenge, multiple immune and inflammatory processes are active within the submucosal compartment, involving both resident and inflammatory cells. Prominent invasive trafficking of the lamina by eosinophils, monocytes, neutrophils, and lymphocytes occurs in near proximity to the epithelial layer. In addition, fibroblasts, smooth muscle cells, myofibroblasts, and epithelial cells are activated, proliferate, and release soluble mediators and secretory products. The sub-basement connective tissue sheaths composed of collagen, elastin, and reticulin become enlarged and structurally disrupted. Overall there is a severalfold increase in the cellularity index within the central lung compartments compared to nonchallenged controls.

Overall, there is a severalfold increase in the “index of cellularity” or the absolute cell number, within intrapulmonary bronchiole compartments of allergen challenged mice in comparison to nonchallenged controls. Here, we can define the index of cellularity as a numerical function, which is proportional to the area within tissue that is occupied by nucleated cells in relation to the total area of tissue (cells, extra-cellular space, luminal space, and connective tissue). The index of the area occupied by cells is in turn related to cell size, and the dynamics of cellular recruitment and replacement.⁷ In summary, the model we have chosen provides a battery of inter-related and interactive biological processes which produce a dynamic proteome that reflects a variety of changes in (i) Cellular densities and activation states, (ii) Within structures comprising the specialized compartments of the lung, and (iii) Plasma proteins distributed within these tissue compartments.

Process for Identifying the Global Proteome. The study of total protein expression in the lung required that we form a working definition of the term “global” and a statistical approach for sampling equivalent compartments of total lung tissue. We can sample and study the entire pulmonary system following challenge (Figure 1). However, rather than analyzing the whole lung in its entirety, we chose to prepare samples of

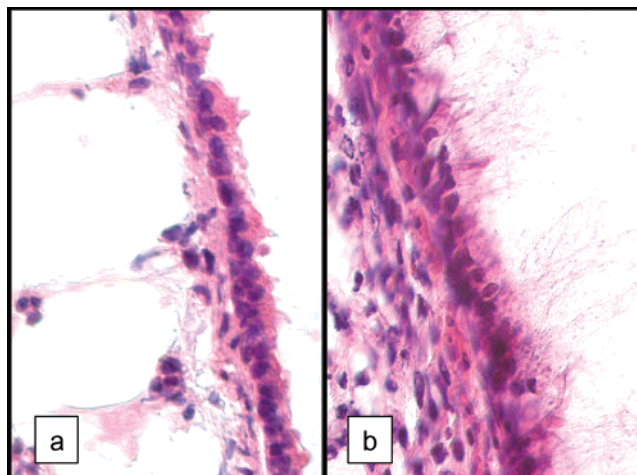


Figure 3. Epithelial activation and mucus secretion. The response to allergen includes a profuse hypertrophy and hyperplasia of epithelial cells lining the lumen of the conducting airways and bronchioles. Control epithelial cells are arranged in tight conjunction and are seen attached to the basement membrane with a polar orientation of their nuclei (a). Hypertrophic epithelial cells in allergen challenged airways show extensive production of mucus as seen in (b) streaming from the apical tips of activated columnar cells into the lumen.

the lung from analogous regions which allowed a finer contextual control over the analysis, due the ability to sample the lung and perform histological analysis simultaneously on adjacent sections. We prepared the left lobe of all of the study animals using a standardized procedure of sufflation, freezing and then sectioning. We then cut through the lungs in a sagittal plane in a slice-wise manner, to the level, which exposed the major conducting airway similar as shown in Figure 1. We estimate that each central 10 μm section represents approximately 1/300 of the total lung volume. [Footnote: Average lung section (at maxima $\sim 1200 \times 400 \times 10 \mu\text{m}$, approximate area 500 000 μm^2 , approximate area fraction of whole tissue occupied by hematoxylin and eosin stained elements = 60%)]. For global assessments of total protein and for optimal separation by 2-DE gels, we regularly applied three sections of lung per 2-DE gel and ran the gels in triplicate for each animal. In terms of cell numbers, the three slices of the lung totally represented approximately 75 000–100 000 whole cells and contained approximately 300 μg of total protein (data not shown).

The index of cellularity is an important factor when comparisons are made between experimental groups or between cellular compartments in protein expression studies. This became apparent to us in a recent study of the liver proteome in which we optimized the resolving capacity of 2-DE gel separation for MS/MS annotations with numbers of in put hepatic cells. We found that a maximal resolving capacity of the gel system was restricted by a limit amount of hepatocytes loaded³. However, the hepatocytes are relatively large cells (25 μm) in relatively tight conjunction with little interstitial space separating the cells. Whereas in this study of the whole lung, by definition, the alveolar luminal spaces which comprise a major volume of the lung are relatively sparse in cells and the average cell diameter overall was under 20 μm . Thus, to obtain high resolution gels of whole lung tissue, we were required to load nearly five times the amount of lung tissue as needed for liver.

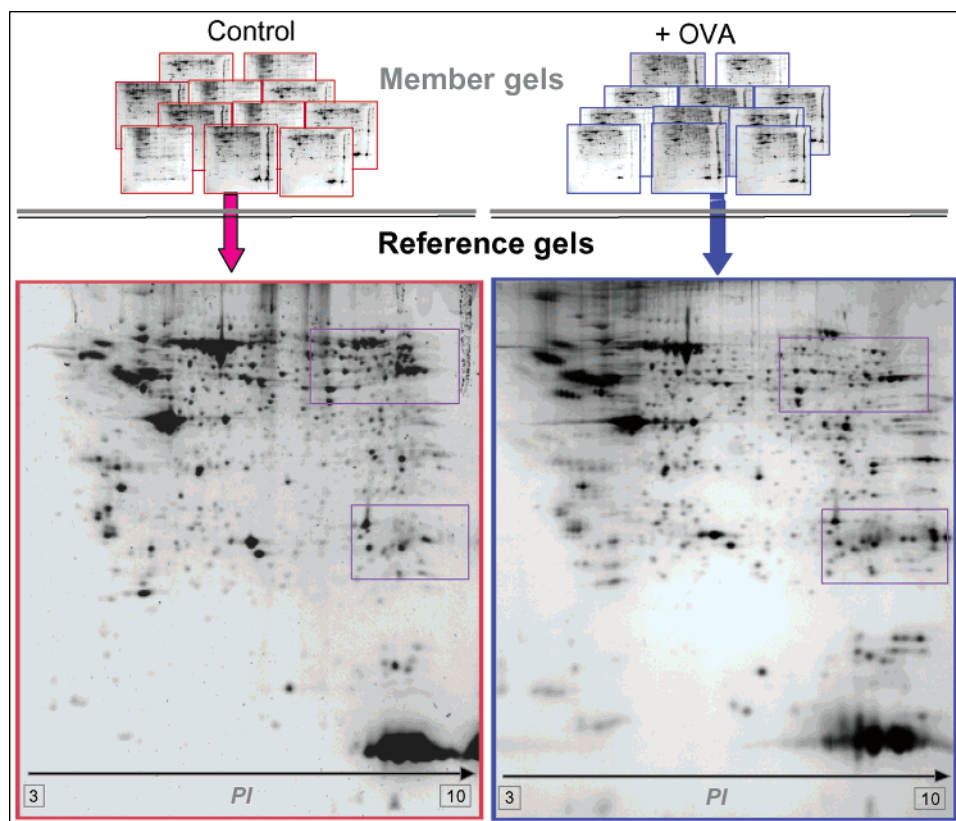


Figure 4. Global protein expression patterns of whole lung. Replicate member gels from control (red) and OA challenged (blue) groups were analyzed using a hierarchic image analysis procedure to create reference gels for the protein expression profiles of both the control lungs and the OA challenged lungs. We obtained good separation of both acidic and basic proteins and throughout a wide molecular weight range (10–150 kD). We localized 1400 spots on the ref 2-DE gels by image analysis and high match set analysis.

The ref 2-DE gels from control (red) and OA challenged (blue) groups are shown in Figure 4. To create the reference gels, triplicate member gels from each lung were scanned and spots were identified using hierarchical image analysis. (We use the name spots throughout the paper rather than protein. Many of the spots are irregularly shaped and contain multiple overlapping, concentric, proteins. We are unable to distinguish protein identity simply by spot identification alone but rather require MS spectral identity to assign protein identity.) Individual spot densities were measured, and a mean spot value was calculated for each lung sample. The reference gels were created using the weighed mean spot intensity values for each of the control and allergen challenged groups. The composite group reference images thus represent all of the protein expression information contained in member gels and can be used efficiently for higher match set comparisons. Both reference gels showed a distribution of proteins throughout a wide molecular weight range (10–150 kD), and pI range 3–10. We identified approximately 1400 spots on the 2-DE gels using image analysis and manual hierarchical matched set high level analysis. As shown in Figure 4, there were many similarities between the pattern of spots distributed on the gels of control and allergen challenged lung. We observed ~100 differences in spot/protein expression levels between the control and allergen reactive lungs at the level of high abundance spots. These differences encompassed both a relative up and down regulation of spots expressed in the controls and the allergen challenge groups (Figure 4 as shown in the boxed areas). We selected 800 of these spots from the control and OA allergic lung reference gels and spot picked examples for analysis from

the appropriate member gels for mass spectrophotometry identification. [The annotated protein spots from the gels were identified by mass spectrometry in accordance with the guidelines given by Jensen et al. (Jensen, O. N.; Podtelejnikov, A. V.; Mann, M. *Anal. Chem.* **1997**, 69, 4741–4750)]. Spots were excised from the gel, reduced and alkylated prior to enzymatic digestion and peptide maps analysis. Protein identifications were performed by peptide mass fingerprinting using MALDI-TOF MS analysis as well as follow up electro-spray ionization MS/MS sequencing. The resulting peptide map-, and sequence tables were BLAST analyzed against both in house and public sequence identification databases.⁸

Common Protein Family Groupings of the Global Lung Proteome. One of the goals of this study was to characterize the dynamic range of constituent proteins (10–150 kD) present within the local microenvironment of the lung under conditions of steady-state control (control lungs) and allergen provocation. It has been estimated that the dynamic range of individual proteins within serum can vary over 10 orders of magnitude⁹ and in cells¹⁰ can vary over 8 orders of magnitude. *What was the cut off level of detection in our studies?* We were able to obtain ~500 spot identities from this set. In Figure 5 are shown the respective proportional distribution of our identified protein set from the OA allergen challenged group using the Gene Ontology Consortium (GO) indexing system to classify the protein identities into common protein family groupings.⁸ Many of the proteins identified were present in multiple isoforms or processing intermediates. Among the set of protein peaks with previous recognized annotation identities, our study produced a rather broad coverage of protein classes including

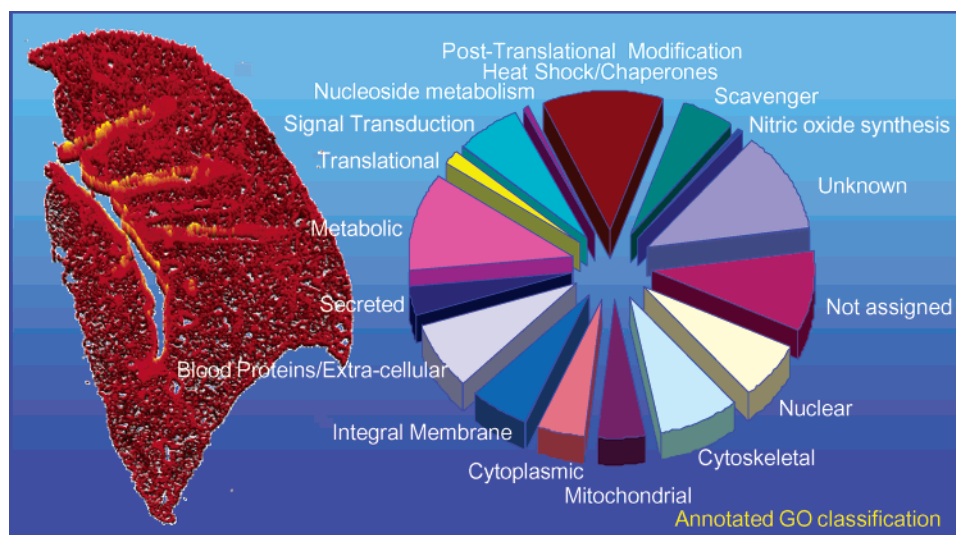


Figure 5. Common protein family groupings of the global lung proteome. A selection of 800 spots representing both high and low abundant proteins were picked, processed, and analyzed by MS or MS/MS. Approximately 500 of the spot MS spectra were identified within databases and included many examples of isomers and processing intermediates. Individual peak/protein identities were assigned to groupings of common protein families established using ontological association scoring from the Gene Ontology Consortium.⁸ The pie graph shows the distribution of proteins identified in lung tissue following allergen.

Cellular (~20%): nuclear, cyto-skeletal, cytoplasmic, integral membrane, mitochondrial; Pathways (~30%): nucleoside metabolism, signal transduction, translational, metabolic, nitric oxide synthesis, heat shock/chaperones, scavenger; and Soluble and extra-cellular proteins (~10%) including blood/plasma proteins. Many of the MS spectral peak identities were of uncharacterized proteins or of unassigned proteins (~20%). We also observed the change in isomeric forms of proteins including processing intermediates of pro-forms identified in the control lungs. Overall, we would set the cut off level at the medium abundance level with some low abundance proteins such as nuclear (i.e., U6 Sn RNA-Associated Sm-Like Protein 4, Human nucleic acid-binding protein, NuABP-34) and regulatory proteins (i.e., Adenosine Kinase (EC 2.7.1.20); Prohibitin (B-Cell Receptor Associated Protein 32) (BAP 32).

Our methodology likely is sensitive to the 10–50 fmol level of abundance.^{11,12} Even so, our approach did not identify some notable pulmonary proteins such as mucins, due to the size exclusion limits of our gel systems. It is interesting to note that the blood/plasma compartment of the samplings of both the normal and allergic lung did not contribute a large proportion of interstitial proteins. While some cytokines were detected at high abundance such as M-GSF and G-GSF we did not observe interleukins such as IL-4 or IL-5 which are associated with allergic models, which promote eosinophilia. However, immunoglobulins IgG, IgA, and IgE were identified.

The detection limit for identification of the “global proteome” in this study is dependent upon the dynamic range of protein expression within our samples. The amount of tissue mass, which we analyze, is in turn determined by the resolving capacity of the gel separation and mass spec identification systems we use. The tissue mass, which we load onto the system, was scaled to produce the highest level of annotation ready spots without smearing or overlap. From a sampling perspective, we identified proteins, which were gel friendly, and in abundance ranges that were detectable with the dedicated technology. Second, the time frame for sampling histologically observable changes in cellular activities and distributions versus the time frame of protein expression could be quite separated.

Some forms of protein expression such as interleukin expression could have simply preceded the time of sampling. The take home message that we present here is the mutual interdependence of histological evaluation with the surveys of protein expression.

The Process for Identifying a Local Lung Proteome: The Airway Mucosa. One of the key compartments responding to allergic provocation is the airway mucosa. Allergic provocation, such as the sensitization and challenge regimen used in this study, produces a dynamic microenvironment within the mucosa and sub-mucosa, which alters the steady state and drives activation, proliferation, and mediator release by resident cells. The mucosal surface of the luminal inner wall of the mouse conducting airway is composed of a layer of epithelial cells attached to a basement membrane which conjoins the elastic lamina containing smooth muscle, collagen, lymph ducts, and nerves that in turn surrounds the branches of the vascular tree. The normal day to day maintenance, repair, and cell replenishment functions of the epithelial layer are fostered by basal cells, columnar cells, and Clara cells which populate the air/airway barrier. The epithelial cells are loosely but directly connected to one another at points known as tight junctions; however, they also maintain intimate contact with other cells populating the laminar matrix through their common joinings to the basement membrane. The epithelium is permeable for the selective passage of soluble proteins from the interstium into the lumen. The airway epithelium also functions as a secretory engine producing and exporting mucins, Clara Cell secretory proteins, as well as growth factors and cytokines. All in all, the air/airway barrier is defined by distinctive structure–function relationships, which require the continuous production of proteins.

The morphology of the airway epithelial surface undergoes dramatic changes in response to allergic activation. The histological changes seen at the airway mucosal interface following OA allergen are pleotropic: epithelial proliferation, hyperplasia and metaplasia; extensive production and secretion of mucins; thickening of the basement membrane; expansion of the elastic and medial lamina including fibroblast and

smooth muscle proliferation, myofibroblast formation, collagen deposition and edema; invasion of the lamina by eosinophils, granulocytes, monocytes, and lymphocytes; diapedesis of the epithelial layer by inflammatory cells into the airway lumen; and the local expression and/or secretion of many types of cytokines, chemokines, integrins and inflammatory mediators.

Overall, the epithelial layer thickness increased by 3-fold following challenge and the elastic lamina doubled in thickness. In Figure 6 are shown examples of the morphological changes occurring within the epithelial mucosal of the airways. Shown at the same magnification (400 $\times$) are representative matched pair examples of control (Figure 6a–d) and allergen challenged (Figure 6e–h) bronchiole epithelial cells stained with a variety of histochemical staining methods. The histological assessment revealed dramatic differences in mucus production, cell morphology, and cell distributions within the epithelial layer. The changes in the submucosa were also observed in the sub basement elastic lamina and in the connective tissue elements supporting the epithelial surface.

LCM Isolation of Epithelium and Protein Characterization.

The histological evaluation provided a clear distinction between the control and activated epithelium on several levels but it was the clear differences seen in mucus production and cellular morphology which gave us confidence that we could statistically sample the appropriate cell compartments by LCM for comparison. To obtain the appropriate orientation within the lung sample for LCM, we stained a few of the central lung sections to determine the location of mucin producing epithelial cells within conducting airways (Figure 7a, insert). We then converted micrographs of these lung sections to 3D images (Figure 7a) to measure relative local density differences of mucin production, seen in the 3D as the white spike-like protrusions surrounding most major bronchioles but not present in vessels. This allowed LCM sampling of exactly those local areas, which represented a mature allergic response. Similarly, to establish some quantitative indices of the sampling, we also stained individual landmark sections with DAPI to detect and count cells within the respective airway compartments. A representative sampling showing the distribution of single cells within a local region of airway mucosa responding to allergen challenge is shown in Figure 7b where we see centrally the orange hemisphere of a vessel, with migrating cells attached to it's walls, surrounded by dense clusters of inflammatory cells which are seen to spread out below the epithelial surface. The airway epithelium in the control mice was also mapped out in a similar manner using DAPI staining of sentinel slides. Since the controls lungs showed little detectable mucin production we could only use traditional histological landmarks to map out appropriate airway areas for dissection.

The LCM procedure we used for isolating the airway epithelium is summarized in Figures 7c–f. Before dissection the laser was positioned over the dissection cap covering the intact tissue section by centring a pinhole light source seen in the middle of the micrograph (arrow). The 30 μm laser was then fired which melted the plastic and fused the underlying cells to the cap in a concentric circular area (Figure 7d). This exact field of cells now attached to the cap is shown using DIC microscopy (Figure 7e) or stained with DAPI (Figure 7f). In practice, the lifts of the entire airway were achieved by manually moving the sample under the laser source to an adjacent field following each laser burst (Figure 8). Following preset maps of the intended dissection, as described above, we typically required 5–8 sections of whole lung to cut through

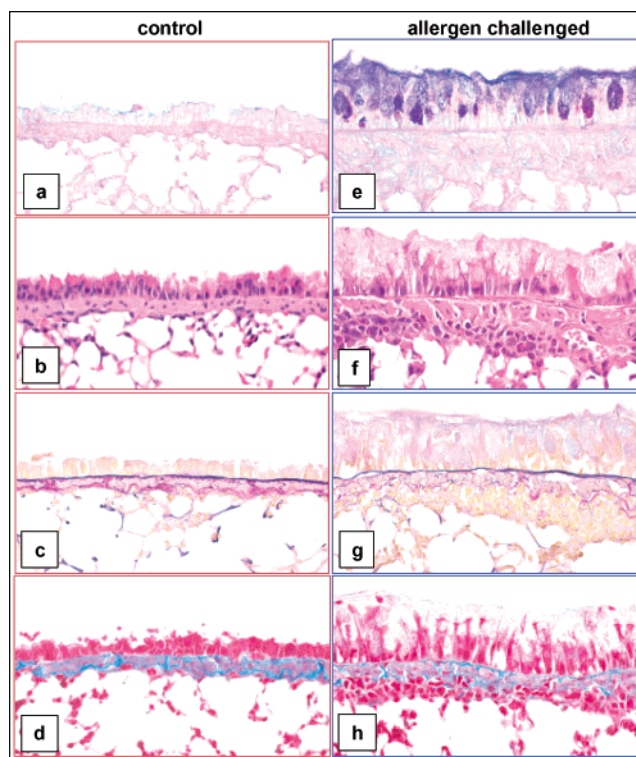


Figure 6. Morphological features of airway epithelium responding to allergen. In addition to mucus hyper-secretion, the response to allergen also modifies the structure and morphology of the outer, sub-mucosal, and connective lamina layers of the bronchial wall. Examples are shown of the morphological changes which occur in activated airway following allergen challenge. Shown at the same magnification (400 $\times$) are matched pair examples of control (a–d) and allergen challenged (e–h) airway luminal epithelium (higher cellular plane), basement membrane (middle position), elastic and medial lamina (mid to lower position), and parenchymal alveolar walls (lowest plane) differentially stained using standard histological methods: (a and e) Periodic acid Schiffs-Alcian blue (neutral mucins: purple staining within and on surface of apical columnar epithelium; acidic mucins: red granules within cells near basement membrane), (b and f) hematoxylin and eosin (H&E) (nucleus: dark blue; cellular features: pink); (c and g) elastin-Von Geisen (basement membrane: dark purple, elastic filaments and fibers: dark purple); (d and h) Massons Trichrome (connective tissue fibers: light blue, nuclei and cytoplasmic granules: red). Overall, the epithelial layer thickness increased by 3-fold following challenge and the elastic lamina doubled in thickness. Morphometric measurements were performed to determine: (i) The height of the epithelial layer: measured between the basement membrane and the apical surface of cells facing the lumen (control: $23.2 \mu\text{m} \pm \text{sem } 1.8$, range 14.7–33.3; allergen $60.7 \mu\text{m} \pm \text{sem } 4.2$ range 43.5–83.4); (ii) The thickness of the epithelial layer: measured as the area under the curve between the basement membrane and the apical epithelial surface as measured over 100 μm linear fields (control: $4529 \mu\text{m}^2 \pm \text{sem } 342$ range 2824–6921 μm^2 ; allergen $24106 \mu\text{m}^2 \pm \text{sem } 1728$ range 18026–34303 μm^2) (iii) the thickness of the elastic lamina measured between the upper boundary of the basement membrane and a line following the lower boundary of collagen identified in the Tri-chrome stainings: (control: $195.1 \mu\text{m} \pm \text{sem } 0.8$, range 187–198 μm ; allergen $393 \mu\text{m} \pm \text{sem } 2.0$ range 300–630 μm).

the central conducting bronchial tree. In preliminary experiments (data not shown) we determined that the minimum cell/

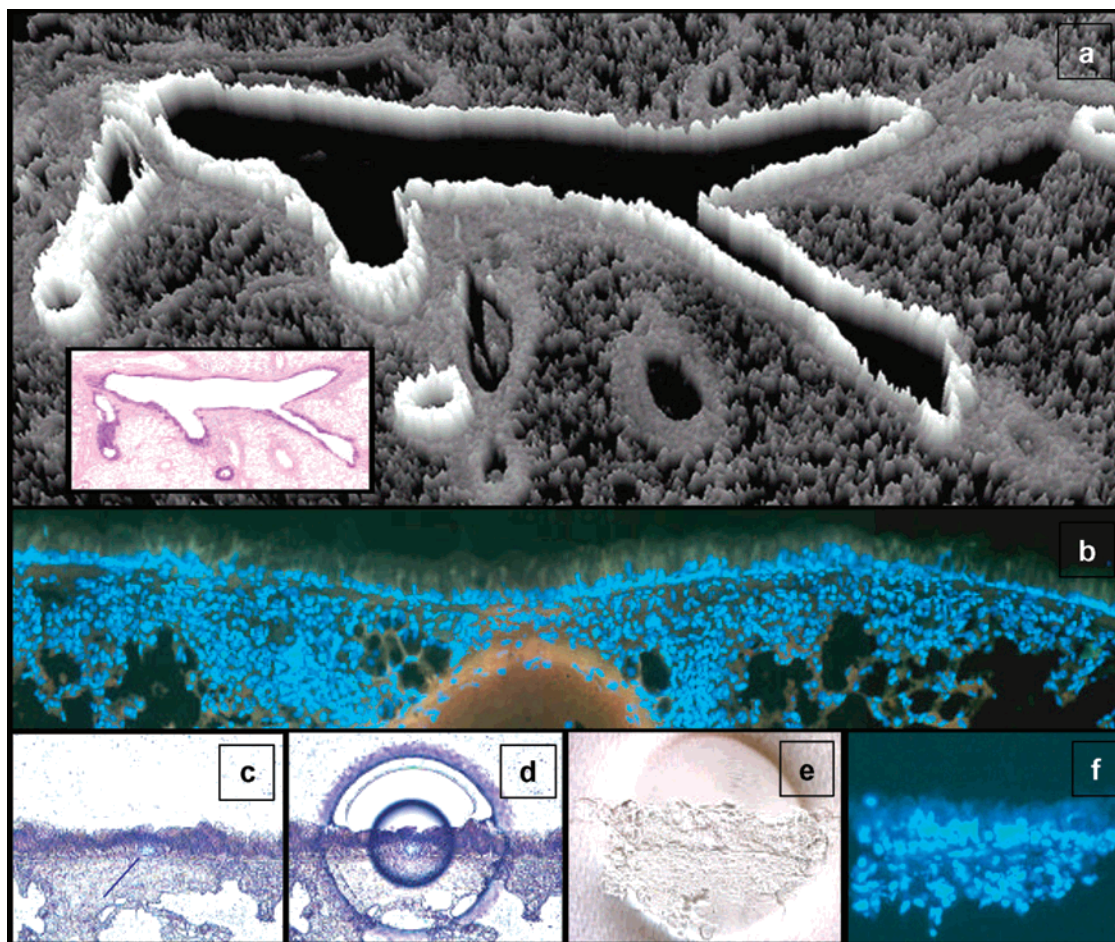


Figure 7. Identification and Isolation of the Airway Mucosal Compartment. To identify areas of lung containing bronchioles with developed responses to the OA allergen challenge, we stained sentinel sections of the lungs with a histochemical stain for mucus production (insert). Using 3-D rendering we converted micrographs of these sections into density images (a) to determine local differences in expression of mucin and simultaneously identify areas of epithelium for the LCM isolation. We also established quantitative indices of the cell distributions and cell numbers within local regions of bronchioles responding to allergen challenge. The panoramic section of the airway submucosa responding to allergen (b) shows inflammatory cells attached on the inner walls of the vessel, surrounding the vessel, and distributed beneath the epithelial layer. Laser capture microdissection (c–f) was accomplished by focusing the beam (arrow) on an area of interest (c); firing of the 30- μ m diameter thermal laser onto the section, melted the overlying plastic, which fastened the cells to the cap (d); the same cells now attached to the cap in the circumscribed area resulting from the pulse (e); the same cells localized using DAPI to show the relative distribution and frequency of the isolated cells (f).

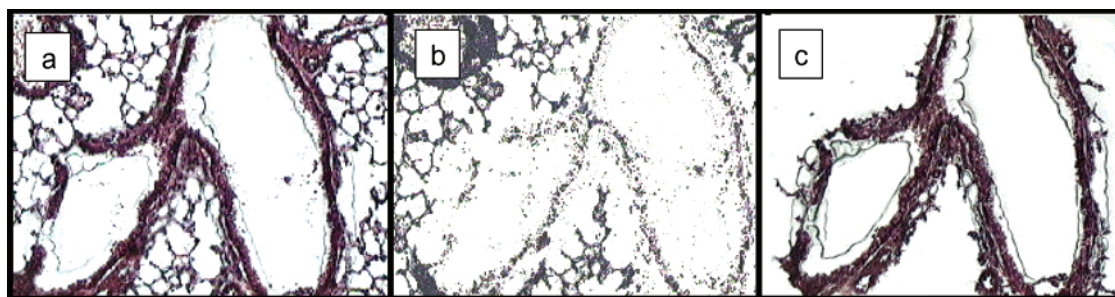


Figure 8. Preparative laser capture micro-dissection. Bronchioles identified on each section were systematically isolated by LCM. Shown here are a representative example of a section with two bronchioles before (a), and after dissection (b). The isolated intact bronchioles (c) from individual lungs were pooled and solubilized for study. In preliminary experiments we determined that ~7000 laser shots were required per gel to achieve high quality 2-DE separations.

LCM shot numbers required to obtain high quality 2-DE gel separations was ~7000 shots/member gel (data not shown). We ran triplicate 2-DE gels from each LCM sampled lung to create group member gels which were scanned and image analyzed to map spot positions and densities. The mean group

spot density values from three mice in each group were then used to produce the reference gels shown in Figure 9. The 2-DE reference gels of LCM isolated epithelium from control tissue (red) contained 574 spots and the gel of allergen challenged (blue) which showed 821 spots. The control and allergen

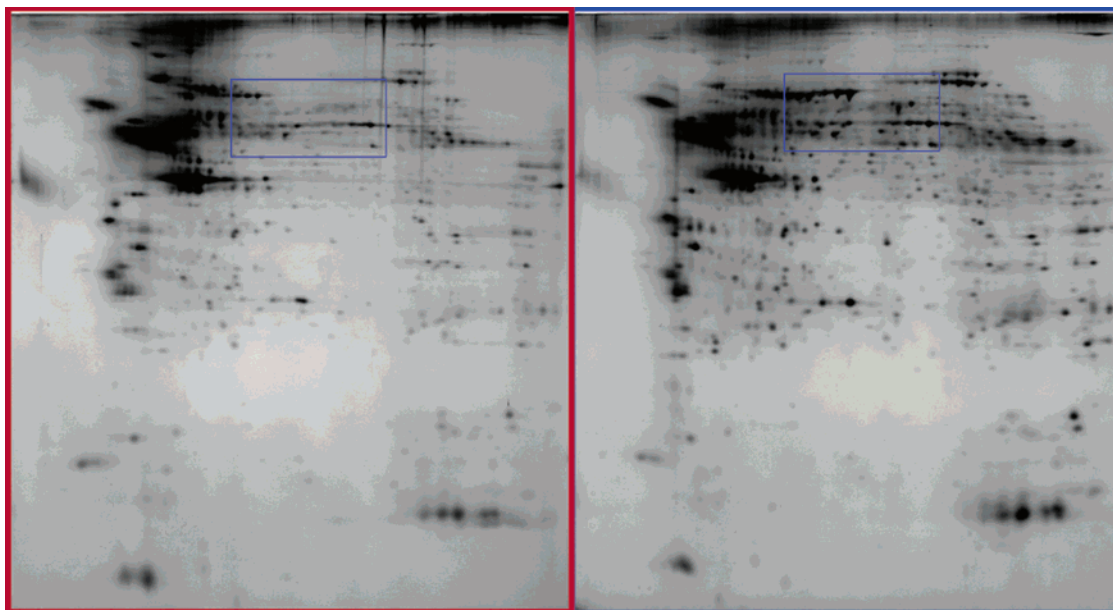


Figure 9. Protein expression patterns of LCM isolated epithelium. Reference 2-DE gels of LCM isolated epithelium from control (red) and OA challenged (blue) groups. We localized 574 spots on the reference gels of control epithelium and 821 spots from the allergen challenged epithelium. The gels were analyzed by high match set image analysis to compare differences in protein expression between the groups. Highlighted in the boxed area is a region of the gel showing differentially regulated protein expression. The acidic areas of the gels shared more common identities that did the basic.

challenged LCM isolated airway epithelium samples commonly shared many spot identities. However, we also observed relative differences in spot densities which were particularly evident in the basic regions of the gels, but also occurred in the neutral region of medium molecular weight proteins. Since the subset of epithelium analyzed here was also present in the samples of whole lung analyzed earlier, it was interesting to begin by comparing the overall expression patterns on the two sets of reference gels (compare Figure 4 and Figure 9). The acidic portions of the gels from both the whole lung and the isolated epithelium showed striking similarities, however, the basic regions were less prominent on the epithelium samples than on whole lung sample.

Using spot picking and subsequent MS analysis we identified ~120 spots on control and ~140 spots for the OA challenged samples. In Figure 10a is shown a map of the annotated spots which were identified by MS analysis on the reference gel of the allergen group epithelium. We did not use metabolic labeling of the proteins in this study and therefore cannot assign absolute levels of abundance or up–down regulation in expression, to the spots on the epithelium gels. The protein we find in tissue could be produced anywhere within or outside the organ. Rather, our results suggest relative differences in the presence and absence of specific spots in several regions of the gels.

As an example of the changes observed in the proteome of the LCM isolated epithelium we show in Figure 10b (control) and Figure 10c (allergen) high match set image analysis of the regions boxed in Figure 9. The protein identities of these spots are listed in Table 1. In this example of 10 proteins resolved in this gel region, the OA allergen challenged epithelium showed higher relative quantitative values in spot density than the control epithelium. We also found spots showing present–absent discordance (i.e., spots 2.A03 and 2.A07: absent in controls). The comparisons of relative abundance of spots was

facilitated by yet another level of analysis for performing the high match set comparisons, the use of 3-D rendering of the image pixels to determine the relative neighborhoods of pixel densities associated with individual spot identifications (Figure 10d). Furthermore, by applying color scaling of the absolute gray scale values (0–255) of individual pixels, we achieved an additional mapping of relative density. The density quotients for singular spots could then be matched between gels and groups.

The careful histological analysis of this airway epithelial compartment allowed us to provide a contextual framework around the patterns of protein expression profiles, whether as singular molecular species or as interrelated members of larger groups of regulated proteins. The histology also helped us to understand the possible contributions, both quantitatively and qualitatively, of different cell types present within our samples. Interestingly, using standard measurement tools, the profound histological changes seen following allergen challenge were only matched with apportioned changes in the overall patterns of protein expression at this point in sampling. Further studies are underway to increase our sensitivity in the detection and identification of “allergic” proteome.

Discussion

The lung is an organ rich in protein composition. The lung proteome is produced by a host of resident and circulating cells, as well as from the continual passage of plasma components through its capillaries. The lung is never in a resting phase and the cellular activities required to maintain the structural and metabolic features of perfusion and ventilation are in continuous cycles of regeneration, repair, replacement, and response. The model we used stimulated an allergic response within the conducting airways, and the submucosa and vasculature, in near proximity to these airways. The allergic response generated

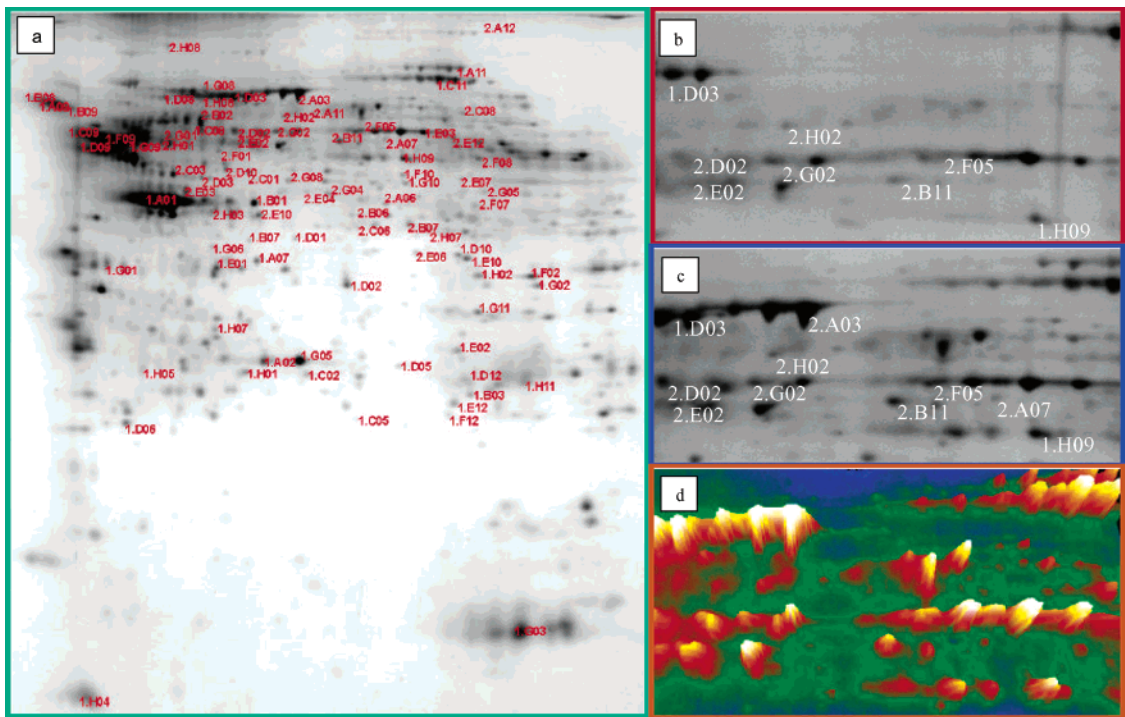


Figure 10. Identification of differentially regulated proteins of airway epithelium. Spots were picked from member gels, processed, and analyzed by MS. The mass spectra were then matched to annotation databases to identify the proteins. We identified 120 spots of control epithelium and 140 spots on the allergen challenged gels. A map of the annotated proteins identified on the allergen challenged epithelial gel is shown in (a). Using high match set image analysis we quantified the densities of specific proteins. The boxed region shown in Figure 9 is seen with annotation details that highlight differences between control (b) and allergen challenged (c) epithelium in one gel region. Density mapping of the differentially regulated proteins observed on the gels was accomplished by 3D rendering which identified pixel density difference between spots closely neighboring one another (d). The data represents only one time point in a dynamic complex process with inductive, active, and declining phases of response.

Table 1. Annotation Identities of Proteins Identified in Pulmonary Epithelium^a

ID	name	mol wt	relative density control ^b	relative density allergen	ratio ^c
1.D03:	SERUM ALBUMIN PRECURSOR	70700	249	832	3.3
1.H09:	ALDEHYDE DEHYDROGENASE, MITOCHONDRIAL PRECURSOR (EC 1.2.1.3)	57015	187	340	1.8
2.A03:	HEMOPEXIN (FRAGMENT)	51255	absent	1087	
2.A07:	CDNA FLJ20303 FIS, CLONE HEP06676	59884	absent	60	
2.D02:	60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR (HSP60)	61088	257	950	3.7
2.B11:	CORONIN-LIKE PROTEIN P57 (CORONIN 1A)	51627	97	202	2.1
2.F05:	TRYPTOPHANE ASPARTATE CONTAINING COAT PROTEIN	109159	201	375	1.8
2.E02:	HYPOTHETICAL 108.3 KDA PROTEIN	51641	313	337	1.1
2.G02:	SIMILAR TO ER-60 PROTEASE	57099	194	239	1.2
2.H02:	T-COMPLEX PROTEIN 1, ε SUBUNIT (TCP-1 ε) (CCT-ε)	60042	40	60	1.5

^a Spots on 2-DE gels were identified by hierarchal image analysis, picked, and analyzed by MS and MS/MS. The identified peak mass spectra were then matched to database records to assign exact protein identities. Shown in Table 1 are 10 identities of spots localized in one gel region. ^b The relative density measurements are weighed mean scores for each of the control and allergen challenged groups which were derived from nine gels each. ^c The ratio of relative density allergen/relative density control.

dramatic shifts in the activation level and numbers of epithelial cells, smooth muscle cells, inflammatory cells such as eosinophils, and in the elastic matrix of connective tissue within these compartments.

This study has identified a broad pattern of protein expression within whole lung tissue and in selected mucosal cell populations present within control and allergen activated lungs. Among the ~500 spot identities which were obtained were members of many common protein family groups including Cellular (~20%): nuclear, cyto-skeletal, cytoplasmic, integral

membrane, mitochondrial: Pathways (~30%): nucleoside metabolism, signal transduction, translational, metabolic, nitric oxide synthesis, heat shock/chaperones, scavenger; and Soluble and extra-cellular proteins (~10%) including blood/plasma proteins. We were thus able to establish a “first cut” understanding of the relative levels of abundance of proteins within the set of ~1400 spots resolved and identified by image analysis.

It was interesting to note that “pure” plasma proteins did not represent a major family within our GO annotated identities. Recent studies of the human plasma proteome have

clarified the definition of plasma in terms of dynamic range and common family groupings of proteins identified in plasma and serum.^{13–15} These plasma studies and similar studies of serum have indicated that a major component of plasma are in fact cellular proteins including nuclear, cytoplasmic and kinesi complex proteins.¹⁴ The 10 most abundant proteins from serum account for 90% of its mass. Within the remaining 10% are 3700 protein components of the plasma proteome which can be identified using technology similar to that which we have employed.¹³ It is not so surprising then to see that the proteome of tissue and plasma actually share many components in proportional alignment.

Is there a signature “allergic proteome” which can be identified within tissue? In this study, we have provided a glimpse into the composition and identity of proteins and protein families that are detectable in global surveys of protein expression. This study provides a starting platform for mapping out the inductive, active, and resolving phases of allergic inflammation. A comprehensive mapping will require kinetic studies over multiple time phase points in experimental models tested on genetically distinct backgrounds. The mapping exercise would also need to include a variety of approaches for isolating proteins and for creating peptide libraries from these proteins and for separation using multiphase liquid chromatography platforms. The results from these studies could then be compared to the identities of proteins obtained using alternative approaches such as tissue imaging by direct MS ionization of coated tissue.^{16,17} Last, to assign identities to the “allergic proteome” we will require reference peptide database annotation capabilities which are only beginning to be developed and utilized.

Each of the lungs specialized compartments and sub-compartments produces specialized sets of proteins which provide the lung with functions such as gas exchange, circular trafficking, or the clearance of substances attached to matrix connective tissue or mucosal barriers. Today, we know only few reports from studies which have investigated the proteome present in whole lung tissue. Even fewer reports have considered the proteome of lung epithelial cells [reviewed in ref 18]. A major focus of study today are attempts to characterize the protein component of differentiated and undifferentiated cancerous lesions and cells within the lung.^{19–23} These studies have resulted in the establishment of molecular fingerprints which distinguish histologically different tumors from another and have identified several groups of proteins that hold potential for being predictive indicators of disease.²² There are also well done studies in experimental models to determine patterns of changing expression profiles within the lung following exposure to irritants such as jet fuel^{24,25} and experimental asthma.²⁶ Both in terms of levels of abundance and relative indices of dynamic changes within the lung proteome, these latter studies provided good comparisons to the study we undertook to characterize the lung proteome during a dynamic response by resident and inflammatory cells following allergic challenge with ovalbumin.

We anticipated that comparative analysis of naïve and allergen challenged lung tissue would provide a ready index of landmark inflammatory allergic mediators up-regulated at the peak of cellular infiltration. The OA model we investigated produces high levels of classic inflammatory and Th2 cytokines such as TGF- β family members, eotaxin, IL-4, IL-5, and IL-13 which can be regularly measured in lung tissue using immunohistochemistry.^{27,28} Why then were we not able to detect

these cytokines as major constituents of the allergic lung proteome at the site of their production? We believe that this is explainable to a great degree by simple calculations of the dynamic range of proteins present in the lung samples we analyzed. To begin, it has recently been reported that the concentration of most cytokines existing in plasma is at the very lowest level (<pg/mL) of the detectable proteome (dynamic range of total proteome: 8–10 orders in magnitude = sub pg/mL–50 mg/mL).¹⁴ In our studies, we have loaded ~400 μ g of total protein/gel. If the proteome of tissue reflects the dynamic range of proteins present in plasma and/or cells, then we would expect that cytokines would exist in tissue at ranges of abundance which are proportional to their starting abundance levels and at maximal levels of no more than 5 orders of total magnitude compared to other proteins present in the same local compartment. If so, then the amount of cytokine present in 400 μ g sample before separation would be present at most 40 ng at 5 orders of abundance and 40 pg at 8 orders of reduction in dynamic range. These small amounts of protein could not be separated or identified using 2-DE gel analysis.²⁹

Histology as a discipline, is undergoing a renaissance of interest and activity within the life sciences community. Histological analysis plays a key role in interpreting studies describing biological processes within tissue by providing a common definition of the contextual relationships between structure, function, and cells within tissue. The proteome of many of the cell types identified in the airway, bronchioles, vascular, and parenchymal compartments sampled in this allergic challenge model have been studied in detail elsewhere, including eosinophils, lymphocytes, monocytes, macrophages, fibroblasts, and myofibroblasts.^{30–38} These studies have also investigated the response of these cells to mediators and cytokines associated with inflammation. The exact contribution of each of these cell types to the proteome present within the lung at any particular point in time will vary depending upon their activation state, numbers, and frequency of replacement. Our fuller understanding of the significance of protein expression patterns occurring within the local tissue compartments during reactions to allergens will require that we learn much more about the daily lives of resident cells and their relationships with other components within this environment.

We investigated whether specialized cell populations, such as the epithelial surface of airways, could be sampled directly in situ, analyzed, and then annotated to determine levels of relative protein abundance. We used laser capture technology to specifically isolate this specialized microenvironment of the mucosal lining, at the peak of an allergic activation, and compared these cells to control epithelium of nonchallenged mice. Since its introduction in 1997, Laser capture microdissection (LCM) has become a standard laboratory method for addressing first gene and then protein expression in tissue.^{39–41} We have adapted the method specifically to perform pan-protein identification studies. LCM allows for statistically significant samplings to be made from tissue sites such as the lung, which are difficult to approach with other micro-sampling methodologies. Few published studies have applied LCM to surveys of nonneoplastic epithelial cells isolated from the bronchial tissue of either human or experimental model origin.⁴² In our study, we were able to sample not only the epithelial layer alone, but rather in association with the neighboring micro-environment that provided the biological context influencing these cells. We used a strong allergic provocation to maximize the biological effect at the local sites

responding to challenge by the activation of secretory pathways and the recruitment of inflammatory cells into the affected regions. With this driver in place, we then set out to measure the depth and breadth of the dynamic proteome present within the mucosal space. Our results confirmed the validity and utility of LCM for studies characterizing the proteome of sites within tissue of medical significance.^{3,43–49} The coupling of LCM with high level histological characterization presents a powerful approach for assigning true context to the molecular and cellular basis of disease.

The proteome of normal bronchial epithelial cells has not been described in final detail. This is related to problems in attaining sufficient amounts of material for study. As mentioned earlier, there is an active interest in determining the proteome of epithelial tumor cells isolated from primary bronchial sites.^{19,20} In addition, the proteome existing at the surface of bronchial epithelial cells, the epithelial lining fluid, has prompted a great deal of study in the form of clinical samples known as bronchial washings, bronchial lavage, and bronchole-alveolar lavage (BAL). These samples typically are obtained by fiberoptic bronchoscopy and include in addition to the epithelial lining fluid, cells, and soluble interstitial proteins which have been transported to the airway lumen. The cell free proteome of BAL samples has been characterized in a variety of pulmonary disorders including interstitial lung disease, sarcoidosis, smokers in comparison to never smokers, cystic fibrosis, and various tumors presenting in the airways.^{50–55} The published BAL proteome patterns are much different in both distribution and abundance levels than the reference images that we have developed of whole lung tissue in this study. Notable is the high albumin content seen in BAL but not in this study material. Studies are currently underway to develop methods to deplete albumin in the BAL samples. In a recent study, we were able to show that the albumin not only masked smaller (<60 kD) and less abundant proteins, but that also albumin was a carrier for several smaller molecular weight protein species which could be eluted from albumin that was isolated from analytical scale BAL preparations (<300 μ L).⁵⁰

The study presented gives an insight into strategies of analyzing the protein expression within a complex organ such as the lung. We have progressed the objectives of the study by breaking it down into biological compartments, going to micro-environments, and further to dedicated target cells related to pulmonary diseases. Upon isolation of these entities, we have been able to give an overall view on processes whereby protein regulations successfully can be analyzed within the airway mucosa following allergen challenge.

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