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ANALYSIS OF PULMONARY COMPLEMENT PROTEIN EXPRESSION FOLLOWING ORGANIC DUST EXPOSURE

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ABSTRACT

Organic dust, as found in agricultural farm work, is ranked among the highest occupational exposure hazards by the CDC. Agricultural dust containing endotoxins, pesticides, mold, and other chemicals, contributes to increased rates of respiratory diseases among these workers. Human bronchial epithelial cells (HBEC), which line upper airways, are frequently exposed to pathogens. Understanding the role of HBEC in inflammation following dust exposure (DE) is necessary to understand the mechanisms underlying inflammatory diseases. The complement system, a nonspecific and non-adaptable defense mechanism, is composed of circulating proteins that promote inflammation by attacking the cell membranes of pathogens and recruiting immune cells that secrete mediators of inflammation. We characterized complement protein expression in DE-treated HBEC using previously generated SWATH proteomics data and Western blotting. Western blotting identified that DE treatment in HBEC mediates the release and activation of C3, while data identified via SWATH-MS proteomics indicated significant upregulation of CD59—a regulator of complement activation. These data suggest that DE-HBEC may regulate complement activation and aim to elucidate the mechanisms by which HBEC promotes the complement system, and thus induce pulmonary inflammation in the presence of organic dust.

KEYWORDS: *Human Bronchial Epithelial Cells; Dust Exposure; Complement System; Inflammation; C3; CD59*



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Sarah Ibrahim is a fourth year Biology Major. Her research focuses on studying the effects of dust exposure on bronchial epithelial cells. She has gained experience in biomedical laboratories since her first year and wants to continue pursuing research in the future. Sarah is also interested in addressing health care disparities in underserved communities and intends to pursue a career in medicine.



FACULTY MENTOR

Tara Nordgren, *Assistant Professor in the Department of Biomedical Sciences*

Dr. Tara Nordgren is an Assistant Professor in the School of Medicine, Division of Biomedical Sciences. She received her PhD and performed postdoctoral training at the University of Nebraska Medical Center, and is broadly trained in lung biology, immunology, lipid signaling, and toxicology. Dr. Nordgren's research interests involve identifying how environmental factors impact inflammation, injury, and repair in the lungs. In particular, she is interested in identifying

how agricultural dust exposures elicit lung inflammation, and is exploring the role of bioactive lipids derived from omega-3 fatty acids in promoting inflammation resolution and tissue repair.

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INTRODUCTION

The Importance of Identifying Complement Activation

Through participation in everyday activities, lungs are constantly exposed to dangers from dust particles which can result in damaged cells. Lungs have a defense mechanism to help remove dust particles from the respiratory tract; however, repeated exposure to dust can eventually result in lung disease (“What Are the Effects of Dust on Lungs?”). This project investigates the inflammatory effects of dust on human bronchial epithelial cells (HBEC) to better understand the mechanisms of how lung disease occurs. We studied two specific complement proteins, C3 and CD59, to verify whether the complement system, a function of the immunity defense mechanism, is active in the presence of dust. We hypothesize that exposing HBEC to dust will activate the complement system and cause an upregulation of CD59 and proteolytic cleavage of C3 as a defense mechanism to dust toxins. Understanding the immune system’s response through complement activation can help other scientific studies further their knowledge in signaling pathways activated in a cell to provide a framework for critical pathways affected by pulmonary diseases.

Background on the Pulmonary System

The pulmonary system, which consists of lungs and airways, mediates respiration. The lungs and airways directly participate to excrete waste products of cellular metabolism by gas exchange. The process begins as oxygen is brought into the body from the atmosphere. Through a series of branching tubes, such as the bronchus and bronchiole (**Figure 1**), the lungs release carbon dioxide through gas exchange. During the initial process of respiration, not all the particles entering the body’s system will reach the lungs primarily because of the airway’s function to act as a filter. These air

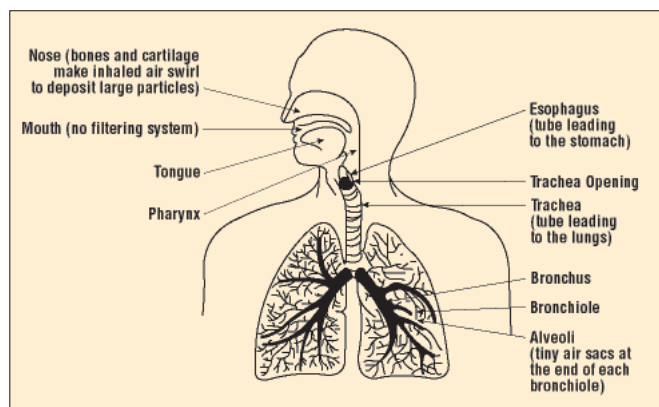


Figure 1. Image taken from the Canadian Centre for Occupational Health and Safety showing the pathway dust enters through the air and to the lungs

tubes are lined by bronchial epithelial cells that produce mucus to trap passing foreign particles, including dust. Lungs have efficient mechanisms to monitor foreign particles in the respiratory tract. One of the body’s mechanisms utilizes tiny hairs, called cilia, that cover the cells of the air tubes, to move the mucus up the throat. The collected particles exit the body’s system through coughing or sneezing. Even though large particles that reside in the nose may be removed via bodily protective mechanisms, smaller particles can successfully pass through the filters, reach the windpipe, and enter the air tubes that lead to the lungs

The Complement System

Although dust particles that extend to the alveoli and lower part of the airways are captured by specialized white blood cells of the immune system called macrophages, the lungs also have another system that functions to remove dust. The complement system utilizes proteins produced from the lungs to bind and neutralize dust particles (“What Are the Effects of Dust on the Lungs?”). This system is composed of complement proteins that activate inflammation and is one of the most vital mechanisms for protecting the body against pathogenic agents. Complement proteins are found in the lining fluid of the respiratory tract and bind to the surfaces of microorganisms. These proteins mediate opsonization by identifying specific pathogens that are already marked by antibodies used in recognizing antigens. During this process, molecules, microbes, or apoptotic cells are chemically modified to have a stronger attraction to the cell surface receptors on phagocytes to facilitate phagocytosis, and by direct lysis of microorganisms, can activate complement. Additionally, complement fragments, such as CD59, have important immunomodulatory and inflammatory effects including cell activation, chemotaxis, bronchoconstriction, increase of endothelial permeability, and production of pro-inflammatory mediators. Complement proteins are heavily localized in the blood and only a small percentage are activated simultaneously. Accordingly, dysfunctional activation leads to host cell damage. Complement activation is strictly regulated by surface-bound regulators that can accelerate the decay of the convertase CD55, act as a cofactor for the degradation of C3b and C4, or prevent the formation of membrane attack complex (MAC) structures, which permeabilize the membrane through CD59. CD59 is commonly known as a MAC-inhibitory protein, or protectin. The protein’s expression by cells is upregulated in response to stimulants, this includes cytokines which are secreted in the presence of inflammation (Morgan). In addition, C3, a complement component of the complement system, has been shown to not only clear pathogens, but also be used for tissue regeneration and synapse pruning to clear debris and control tumor cell progression (Ricklin). The activation of the complement system through the upregulation of CD59 and the proteolytic cleavage of C3 into its alpha and beta fragments leads to a cascade of functions to promote inflammation.

METHODS

Human Bronchial Epithelium Cell Culture

To study cells in serum-free culture conditions, we used primary human bronchial epithelium cells isolated from human patients in the sequential windowed acquisition of all theoretical mass spectra (SWATH-MS) experiments, and the human immortalized BEAS-2B bronchial epithelial cell line for C3 activation studies. Cells were purchased commercially from American Type Culture Collection. They were grown on 10 mL culture flasks pre-coated with a coating medium containing 30 µg/mL purified collagen (Vitrogen-100; Collagen Corporation, Palo Alto, California, USA) and incubated with coating medium (5 mL per flask) for 30 minutes in a humidified CO₂ incubator at 37°C. The coating medium was then aspirated. Cells were grown to 80% confluency for each exposure study (experiment repeated 4 separate times) in BEGM complete medium (primary cells) or LHC-9 (BEAS-2B). Treatments for cells included a control (medium alone) and a 6 hour treatment of 5% hog barn dust extract (HDE) alone was used to induce airway inflammation. Previous research in our lab indicated that this 5% of HDE dose response is enough to create an inflammatory response without killing the cells.

SWATH-MS Collection

Cells treated as outlined above were rinsed with 1X PBS and collected for SWATH-MS by scraping in a triton-X lysis buffer that contains protease inhibitors. Protein in lysates (cells from two wells pooled = 600 µL) were quantified by NanoDrop and delivered to the proteomics core laboratory at the University of Nebraska Medical Center.

SWATH-MS Statistics

Sequential Windowed acquisition of all theoretical mass spectra (SWATH-MS) is an optimal proteomic strategy to identify vital proteins involved in HBEC signaling pathway and progression in an unbiased approach. The experimental scheme of this study is shown in **Figure 2**. HBEC dust-treated and HBEC untreated samples were compared by SWATH-MS to identify differentially expressed proteins that are upregulated and downregulated in the presence of dust exposure. To avoid individual differences and detect true HBEC-related proteins, samples were analyzed by choosing proteins from both groups to determine a quantitative expression ratio between HBEC dust-treated and HBEC untreated tissue groups based on total ion intensity normalization. The targeted identification of peptides in SWATH-MS datasets requires the calculation of z-scores to assert up- and down-regulated proteins. The raw intensity of each protein was transformed by taking the natural log of the intensity followed by a calculation of z-score (Equation 1) in which x is the experimental value, μ is the mean of all experimental values and σ is the standard deviation of all

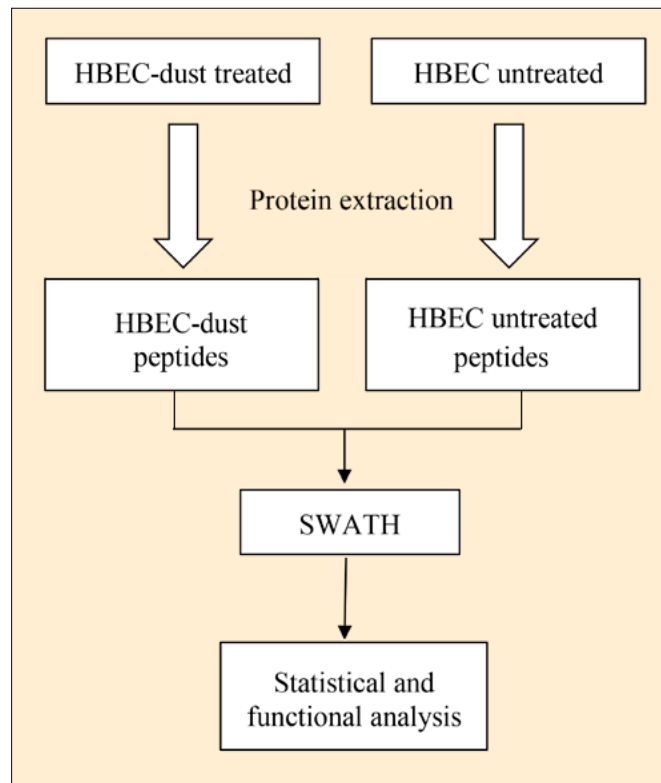


Figure 2. Quantitative proteomic workflow of HBEC both dust treated and untreated analyzed using a SWATH-MS approach.

experimental values. Next, the Δz was calculated for each protein in a pairwise manner for each sample ($z_{\text{HDE}} - z_{\text{Control}}$) and the average Δz was calculated across all samples. The z-test was then conducted for each protein using the paired sample z-test (Equation 2), where Δz_{avg} is the average Δz across all samples, D is the hypothesized mean (null hypothesis) which states that there is no significance between dust and the proteins expressed. Additionally, σ_d is the standard deviation of the pairwise differences per protein, and \sqrt{n} is the square root of the sample size (number of biological samples).

Z-score:

$$Z = (x - \mu) / \sigma$$

Equation 1

Paired sample z-test:

$$z = (\Delta z_{\text{avg}} - D) / (\sigma_d / \sqrt{n})$$

Equation 2

Western Blot to detect C3 proteins

Western Blot technique was used to identify C3 proteins extracted

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from BEAS-2B. Supernates and lysates were collected from cells both untreated and treated with HDE. Cells were washed in tissue culture flasks with phosphate buffered saline (PBS), incubated in RIPA lysis buffer for 10 minutes, then scraped and placed into microcentrifuge tubes. Tubes were centrifuged at 10,000 RPM for 7 minutes, and the supernatant fraction was collected. The protein concentration was then determined via NanoDrop. For western blotting, 50 µg of protein was loaded per well into the sample buffer, and ran on a 4-20% SDS PAGE gel at 120 V for one hour. Proteins were transferred to PVDF membranes via semi-dry transfer for 1 hour. After the transfer process, immunoblotting was performed using 5% non-fat dry milk in PBST for 1 hour. Then the primary antibody was added in 5% non-fat dry milk in PBST and incubated overnight on a shaker at 4°C. The membrane was then washed with PBST for 5 minutes and repeated three times. The secondary antibody was then added in 5% non-fat milk in PBST, and incubated for 1 hour at room temperature. Next, the membrane was washed with PBST for 5 minutes and this step was repeated three times. Then, the enhanced chemiluminescence mix was prepared utilizing the proportion of solution A and B provided by the manufacturer, and the membrane was incubated for 1–2 minutes. Finally, the results were visualized by film development in a dark room.

RESULTS

SWATH-MS Analysis

There is an estimated equal distribution of up- and down regulated proteins identified by z-scores as seen in **Figure 3**. Positive numbers indicate up-regulated proteins while negative values indicate down-regulated proteins. The p-values for the computed z-test statistic were assigned using the standard normal distribution. 346 proteins were identified to be significantly different using a p-value cutoff of <0.05. A $p < 0.05$ represents a rejection of the null hypothesis and further proves the significance between dust and protein expression. Among the 346 proteins, 214 show a $p < 0.01$ and the first two proteins (**Table 1**) illustrate the highest levels of up- and down-regulation. The statistical analysis was conducted as outlined in this paper: “Quantitative Proteomics by SWATH-MS reveals Altered Expression of Nucleic Acid Binding and Regulatory Proteins in HIV-1-Infected

Western Blot Analysis using C3 Antibody

After analyzing western blot on BEAS-2B using C3 antibodies to detect C3 proteins, we saw that when cells were exposed to dust, the complement system was activated whereas in the untreated sample, we saw the full length of C3 (**Figure 4**). Our studies show that when the complement system is activated, enzymatic cleavage of C3 occurs, the alpha and beta chains appear, and the C3 fragment is no longer in its full length. This activation leads to an

additional cascade of complement activation. When there is no dust exposure, there is no activation of proteolytic cleavage.

CONCLUSIONS AND FUTURE DIRECTIONS

SWATH-MS Analysis

The SWATH-MS proteomics data strongly elucidates our hypothesis that dust exposure activates several cell signaling pathways, specifically the complement proteins such as C3 and CD59, in an effort to reduce invading toxins in HBEC. Through SWATH-MS, we were able to identify proteins expressed during dust exposure and analyze which proteins are up- and down-regulated. For reference, we examine ICAL_HUMAN and DNMI1_HUMAN to show a correlation between dust exposure and their effects on activating specific proteins in HBEC as a response to inflammation. ICAL_HUMAN shows the highest up-regulation and encodes for the protein calpastatin. The protein is a specific endogenous inhibitor of calpain activity through four equivalent inhibitory domains. Calpains are calcium-activated neutral cysteine proteases and play an important role in inflammatory processes. Additionally, calpains are critical for inflammatory cell adhesion, chemotaxis, and inflammatory mediator processing (Zafrani). Previous research has demonstrated that calpastatin expression increases when macrophages were stimulated with TNF- α , a pro-inflammatory cytokine (Hoffmann). Our data shows that in the presence of dust, levels of calpastatin increases in response to high levels of calpains. In comparison to the upregulation of calpastatin, DNMI1_HUMAN (Dynammin-1-like protein) shows the highest down-regulation expression in the presence of dust. Commonly referred to as dynammin-related protein 1 (DRP1), recent studies on injured kidneys have shown deletion of DRP1 promotes epithelial recovery, preserves mitochondrial structure, and reduces oxidative stress (Perry). Mitochondrial dysfunction contributes to programmed cell death called apoptosis, and the study showed that decreased levels of DRP1 attenuates mitochondrial dysfunction. The preservation of mitochondrial structure is important because nearly every cell in the lungs depends on mitochondrial metabolic activities and requires constant supply of energy from oxidative phosphorylation (Cloonan). Hence, a decrease expression of CRP1 in HBEC-dust treated explains cell repair. In addition to these two proteins, the complement system is crucial for protecting the body

Table 1. Proteins Identified Using Swath-MS Proteomics

Accession	Uniprot ID	Δz
P20810	ICAL_HUMAN	14.2038493
O00429	DNM1L_HUMAN	-16.54900216
P13987	CD59_HUMAN	5.815474894

against pathogenic agents. We also identified an upregulation of CD59_HUMAN, encoding for CD59 glycoprotein. CD59 is a complement regulatory protein that inhibits MAC formation. The upregulation of this protein in the presence of dust activates the complement system by preventing complement mediated cell lysis (Budding). Previous studies have shown that cytokines secreted in inflammatory lesions can induce CD59 expression, and our data shows a high up-regulated expression of CD59 in the presence of dust exposure. The activation of the complement cascade in the presence of toxins illustrates the system's ability to distinguish between pathological and physiological challenges. In addition to triggering the system, the cascade includes several regulatory mechanisms which use membrane-bound regulators such as CD59 to prevent over-activation of the complement system (Geller). The upregulation of CD59 shows the activation of the complement cascade in dust exposed HBEC and its function to regulate the system. Our results further represent how these pathways activate a defense mechanism to remove toxins from the lungs through specific expression of proteins.

Western Analysis

Identifying complement activation through proteolytic cleavage of C3 into its alpha and beta chains is important because the production of C3b can facilitate the formation of C5 convertases, and along with the binding of C3b, fuel an amplification that leads to rapid opsonization of the target surface. Therefore, an increase in the formation of C3b initiates the lytic MAC and potent anaphylatoxin C5a, in which C3a binds to the anaphylatoxin receptor C3aR. This function mediates immune adhesion, phagocytosis, and adaptive immune stimulation (Ricklin). This experiment represents our hypothesis that HDE treatment in BEC mediates the release and activation of C3, and thus, proves our hypothesis that the complement system is activated in response to inflammation from dust.

Importance of Data and Future Directions

We were able to identify CD59 by SWATH-MS to show how it is upregulated in the presence of dust. In addition, through western blot analysis we further concluded that proteolytic cleavage of C3 in its alpha and beta chains occur in the presence of dust to activate the complement system. This reinforces the concept that in the presence of dust, the body responds through the activation of the complement system to enhance the ability of antibodies and phagocytic cells to clear toxins, promote inflammation, and finally aid in attacking the pathogen's cell membrane. Our data can be used in other lung research studies to identify up- and down-regulated proteins to understand the effects of dust exposure in HBEC and lead to potential treatments to prevent lung disease through anti-inflammatory drugs. In our future experiments, we plan to use immunofluorescence staining for C3 and CD59 on mouse bronchial epithelial cells to locate the expression of these

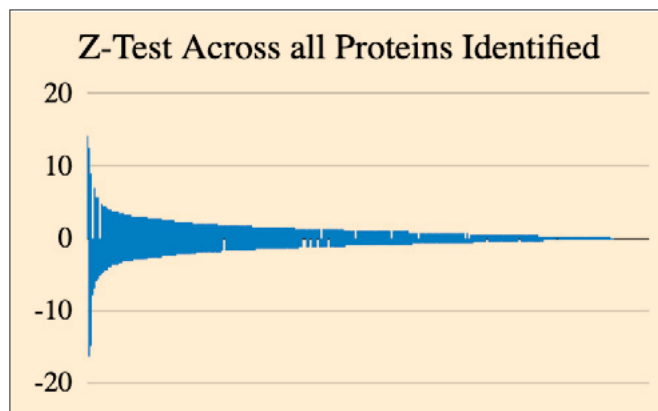


Figure 3. Y-axis illustrates an estimate of up-regulated (positive values) and down-regulated (negative values) proteins analyzed by calculating z-test from SWATH-MS.

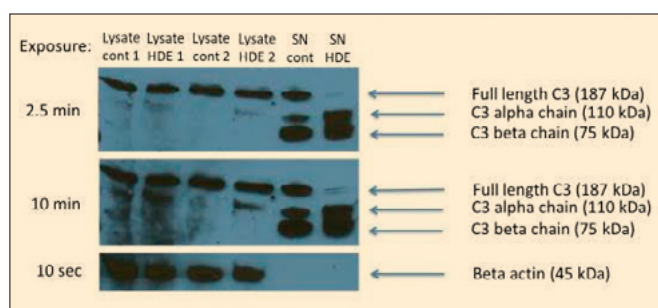


Figure 4. Western blot image using lysates and supernatant (SN) identifying C3 proteins in HBEC using 24-hour exposure of 5% HDE.

proteins in the presence of dust exposure. This will aid in detecting where the antibodies are located on the tissue and generate a better understanding of the mechanisms underlying tissue inflammation. In addition, we will further study dietary supplements such as omega-3-fatty acids, which are known to have anti-inflammatory properties, in preventing pulmonary diseases.

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