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Tissue factor expression, regulation, and signaling in human airway cells

Michael D. Davis
Virginia Commonwealth University, mdavis35@vcu.edu

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Tissue factor expression, regulation, and signaling in human airway cells

A dissertation submitted as a partial fulfillment of the degree requirements for the Doctor of Philosophy of Physiology and Biophysics at Virginia Commonwealth University

By

Michael D. Davis, RRT
Bachelor of Science in Biomedical Science
Lynchburg College, 2008

Advisor: Bruce K. Rubin, M.Engr., M.D., M.B.A., FRCPC
Jessie Ball DuPont Distinguished Professor and Chair, Department of Pediatrics

Virginia Commonwealth University
Richmond, Virginia
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Abbreviations

ALI: Air-Liquid Interface
ANOVA: Analysis of Variance
AP-1: Activator Protein 1
cDNA: Complementary Deoxyribonucleic Acid
CD14: Cluster of Differentiation 14
CXCL8: Chemokine (C-X-C Motif) Ligand 8
DNA: Deoxyribonucleic Acid
ELISA: Enzyme-Linked Immunosorbent Assay
FV: Factor V
FVII: Factor FVII
FVIIa: Activated Factor FVII
FX: Factor X
FXa: Activated Factor X
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase
IkB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor
IKK: IkB Kinases
IL-1: Interleukin 1
IRF3: Interferon Regulatory Factor 3
LBP: LPS Binding Protein
LDH: Lactate Dehydrogenase
LPS: Lipopolysaccharide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MD2</td>
<td>Lymphocyte Antigen 96</td>
</tr>
<tr>
<td>MEK/ERK</td>
<td>Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinases</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal Human Bronchial Epithelium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerize Chain Reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>TF</td>
<td>Tissue Factor</td>
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<td>TFPI</td>
<td>Tissue Factor Pathway Inhibitor</td>
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<tr>
<td>TGF-beta</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-Like Receptor 4</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor Necrosis Factor Alpha</td>
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Abstract

TISSUE FACTOR EXPRESSION, REGULATION, AND SIGNALING IN HUMAN AIRWAY CELLS

By Michael Denning Davis, RRT, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Physiology and Biophysics at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Advisor: Bruce K. Rubin, M.Engr., M.D., M.B.A., FRCPC

Jessie Ball DuPont Distinguished Professor and Chair, Department of Pediatrics

Rationale: Tissue Factor (TF) is a transmembrane glycoprotein that canonically functions as the initiator of the coagulation cascade. Increased levels of TF have been associated with inflammatory airway diseases. Since lipopolysaccharide (LPS) is known to elicit and inflammatory response in airway epithelium, we hypothesized that airway epithelial cells release TF when exposed to LPS. Since TF aids in local wound healing, we also hypothesized that inhibition of TF would decrease NHBE growth. The specific aim of this work was to evaluate the effects of LPS exposure on TF production and release from airway epithelia and determine the signaling pathways involved. A secondary aim was to evaluate the effects of TF inhibition on NHBE growth.

Methods: Normal human bronchial epithelial cells were grown in submerged cell culture and exposed to LPS as well as several intracellular signaling pathway agonist and inhibitors.
**Measurements:** Tissue Factor mRNA and protein were measured in culture media and cell lysate by reverse-transcriptase polymerize chain reaction and enzyme-linked immunosorbent assay, respectively. Signaling pathways were evaluated using selective agonists and inhibitors.

**Main results:** TF protein levels increased nearly two-fold in cell media after exposure to LPS (p < 0.01). This did not occur in the presence of an MEK/ERK inhibitor (PD98059) or a SMAD inhibitor (SB431542). TF protein levels also increased nearly ten-fold in the presence of TGF-beta (p < 0.05). mRNA of TF and TGF-beta was not altered by LPS or TGF-beta exposure. NHBE grown in the presence of Tissue Factor Pathway Inhibitor grew significantly slower than those grown in standard media (P < 0.05).

**Conclusions:** NHBE release TF when exposed to LPS. This phenomenon is post-translational and may be mediated by an autocrine mechanism involving MEK/ERK signaling that increases TGF-beta which then leads to the release of TF. Our data suggest that this airway epithelium release of TF serves as a local repair function.
Chapter 1: Introduction

1.1 Background

Tissue Factor (TF) is a 47 kDa transmembrane glycoprotein that structurally resembles a cytokine receptor and canonically functions as an initiator of the coagulation cascade\textsuperscript{1-5}. Vascular endothelium, fibroblasts, smooth muscle cells, and epithelial cells constitutively express TF\textsuperscript{1-5}. When exposed to Factor VII (FVII), which is present in circulating blood, TF activates FVII (FVIIa) binds with FVIIa and forms a complex that triggers the Factor X (FX) pathway of coagulation\textsuperscript{1-5}. This leads to self-amplified activation of FVII, thrombin production, and eventually clot formation and tissue growth (Image 1). Lipopolysaccharide (LPS) can also induce expression of TF in monocytes and vascular endothelium via AP-1 and NF-kB which also lead to thrombin/fibrin clot formation in blood\textsuperscript{4}. Both of these indicate that TF activates in response to blood presence or local inflammation\textsuperscript{1-5}. An endogenous inhibitor of TF called Tissue Factor Pathway Inhibitor (TFPI) balances TF activity \textit{in vivo} by interfering with TF/FVIIa complexes as well as FX\textsuperscript{6,7}. 
1.2 TF and Disease

Most translational research involving TF has focused on its role in coagulopathies\textsuperscript{1,2,4,5}. However, in the past three decades TF has been identified in blood and other fluids. Serum TF expression increases in physiologic disturbances including disseminated intravascular coagulation\textsuperscript{8}, hemorrhage\textsuperscript{5}, and disorders that increase capillary permeability such as sepsis and systemic inflammatory disorders\textsuperscript{9,10}. TF has also been shown to promote cell proliferation and migration\textsuperscript{3}.

Increased levels of systemic and local tissue TF have also been associated with pulmonary diseases including pulmonary fibrosis\textsuperscript{11-13} and acute lung injury\textsuperscript{10,14}. Since all systemic blood flows through the vasculature in the areas affected by these disorders and these disorders are associated with capillary membrane leakage, local increases in TF caused by these disorders could stem from increased circulating TF levels leaking into the airways.

1.2.1 TF and Inflammatory Airway Disease

In 2009, Brims et al reported that subjects with severe asthma had higher levels of TF in their sputum than subjects with moderate asthma or control subjects, even while undergoing treatment\textsuperscript{15}. Increased levels of TF have been reported in airway fluids from subjects with inflammatory airway diseases including pneumonia\textsuperscript{16,17}, bronchopulmonary dysplasia\textsuperscript{18}, and asthma\textsuperscript{15,19}. This could be related to tissue remodeling and wound healing commonly associated with these disorders. These findings were initially attributed to vascular leakage into
the airway lumen, however Park et al reported in 2012 that cultured normal human bronchial epithelium (NHBE) produced and released TF bound to exosomes in response to cyclic stress\textsuperscript{20}. Since cyclic stress stretches and can rupture cell membranes, and TF is known to aid in wound healing and hemostasis\textsuperscript{5,21,22}, it is probable that the release of TF in this setting would function to aid in tissue repair from potential stretch damage.
Image 1: Cartoon of TF Activity.

1. Upon exposure to serum, extravascular TF forms a complex with activated Factor VII (FVIIa).
2. Activated Factor X (FXa) interacts with Factor V (FV) to activate Factor VII (FVII) to Factor VIIa (FVIIa). 3. FXa forms a complex with activated FV, generating thrombin. 4. This thrombin perpetuates activation of FV and FVII, leading to 5. Platelet activation and continued coagulation. (modified from Eilertsen 2004)
1.3 Tissue Factor Pathway Inhibitor

TFPI is an endogenous compound that canonically inhibits the activity of TF\(^6,7\). It binds with activated FX and reversibly inhibits its ability to promote thrombin/fibrin formation\(^6,7\). Subsequently, TFPI binds with TF/FVII complexes and prevents their ability to further activate the FX pathway (Image 2). Vascular endothelium synthesizes most of this ~40 kDa modular protein\(^6\). Over the past two decades, TFPI has been shown to be synthesized in small amounts by most human cell types. The exceptions to this are hepatocytes, erythrocytes, neutrophils, and lymphocytes\(^6,7\).

Although the specific mechanism of TFPI activation remains unknown, several factors are known to increase TFPI expression. Cells that constitutively express TFPI increase synthesis in response to plasma exposure\(^6,7\), shear stress\(^6,23\), and thrombin presence\(^24\). Inflammatory stimulation, such as that by LPS, TNF-\(\alpha\), and IL-1, has been shown to decrease TFPI expression\(^25\). Decreased levels of TFPI have been associated with acute lung injury\(^26,27\), pulmonary fibrosis\(^11\), and acute respiratory distress syndrome\(^14\).

Although cellular expression of TFPI increases with TF expression, TFPI does not increase above the basal levels of TF which makes it incapable of blocking TF in the setting of acute inflammation\(^14,28,29\). This suggests that TF and TFPI normally exist in a balanced state but that TFPI cannot balance abnormally increased amounts of TF. This observation has led to interest in therapeutic applications of exogenous TFPI\(^17,27,30-32\).
Image 2: Cartoon of TFPI Activity.

TFPI inhibits both FXa and TF/FVIIA complexes. Modified from Bajaj 2001.
1.4 Airway Cell Models

Airway cell culture can be used as models of healthy and diseased airway. Human airway cells are commercially available and a variety of models have been developed. Two types of these models were used for the work reported here – differentiated and undifferentiated cell culture. Each of these models has specific benefits. Below is a brief background on these models.

1.4.1 Undifferentiated Airway Cell Culture

When grown under submerged conditions, NHBE cells stay in an undifferentiated, basal-type form similar to that seen during growth and wound repair which makes this model helpful when studying these states. This model of cell culture is one of the simplest; the cells are grown on a surface such as a flask or culture well and media is layered on top of them (Image 3a). Submerged cell culture techniques have been used for decades and are well established in airways cells. Being submerged in cell media also protects these cells from the environmental contamination. Due to the robust nature of this culture model and the widely available established techniques, it is convenient to use.
1.4.2 Differentiated Airway Cell Culture

The development of semi-permeable membranes conducive to cell culture has enabled the culture of polarized and differentiated cells at an air-liquid interface (ALI) in which the media is able to feed cells from below a membrane on which they are grown (Image 3b-c). This is beneficial to airway researchers since NHBE in vivo are exposed to air apically. These ALI conditions allow cells to fully differentiate from their submerged precursor form to polar pseudostratified ciliated columnar epithelium. The final product is a functioning piece of airway epithelium.

These models allow investigators to evaluate the response to stimuli applied to the apical or basolateral sides of airway cells which are representative of the airway lumen and parenchyma, respectively. Because these cells are exposed directly to the atmosphere, this model is more vulnerable to contamination. Also, the techniques required to maintain cell growth are precise and less forgiving. Finally, this model takes approximately a month to reach differentiation. These features contribute to the complex nature of this differentiated airway cell culture\textsuperscript{34, 35}. 

- 8 -
(A) In the classic model of submerged cell culture, cells are grown on the bottom surface of a cell culture flask and remain submerged under cell media. When growing cells in an air-liquid interface, cells are grown atop a semipermeable membrane that allows nutrients from media to pass through and feed the cells. (B) Initially, the cells maintain submerged underneath media but eventually the apical media is removed and (C) cells are able to fully differentiate. Modified from Stem Cell Technologies (www.stemcell.com).
1.5 LPS-induced Airway Inflammation Model

LPS is a well-described initiator of inflammatory responses throughout the body\textsuperscript{35-37}. Although classically used as a model for bacterial infection since Gram-negative bacteria express LPS on their cell membrane, LPS is now used as an airway inflammation model due to its consistent activation of toll-like receptor 4 (TLR-4)\textsuperscript{35-37}. LPS binds to TLR-4 and activates intracellular signaling pathways, including mitogen-activated protein kinase/extracellular signal-regulated kinases (MEK/ERK) and IkB kinases (IKK) which lead to AP-1 and NFkB activation, respectively (Image 4)\textsuperscript{35-37}. 
Image 4: Cartoon depiction of signaling pathways affected by LPS activation of TLR-4.

Pathways evaluated in these experiments, MEK/ERK and IKK, are circled in red. Modified from public domain.
1.6 Specific Aims

Since many of the diseases associated with TF in airway fluid have been inflammatory airway diseases, I hypothesized that an inflammatory insult may induce NHBE release of TF similar to that seen after cyclic stress of NHBE\textsuperscript{20}. Specifically, I hypothesized that LPS exposure would induce TF release. The specific aims of this work were to identify whether NHBE produce and release TF in response to LPS stimulation and to elucidate the mechanism(s) by which that occurs. A secondary aim was to evaluate functions of airway epithelial TF; specifically, although I initially hypothesized that TF was pro-inflammatory and would promote the production of inflammatory mediators, following the studies to address Specific Aim 1 it appeared that TF was more likely involved in cell growth and repair, and so I evaluated the effects of TF inhibition on the rate of cell growth.
Chapter 2: Methods

2.1 Submerged Airway Epithelial Cell Culture

Primary NHBE cells used for all experiments described were healthy, non-smoker donor cells purchased from Lonza (Basel, Switzerland). Cell media used was Bronchial Epithelial Growth Media with the SingleQuot Supplement Kit (Lonza) that included: hydrocortisone, gentamicin, retinoic acid, bovine pituitary extract, recombinant human insulin, transferrin, triiodothyronine, epinephrine, and human epidermal growth factor. Cells were grown under submerged conditions until confluent in 75 cm$^2$ cell culture flasks (Image 3a). After confluence, which takes 5-6 days, the cells were exposed removed from the flask by trypsin exposure for five minutes after which trypsin neutralizing solution (TNS) was added. These cells were then counted by a digital cell counter (Bio-Rad Laboratories Inc., Hercules, CA, USA) with viability determined by trypan blue staining and then passaged into 15.6 mm cell culture wells (Corning, New York, USA) at 20,000 cells per well under 500 uL of media per well which lead to approximately 10% confluence. The cells were then grown again until confluent (5-7 days). Cell media was changed every 48 hours.
2.1.1 Evaluation of Confluence/Cell Growth

Immediately after each media change, cells were viewed via light microscopy and confluence was noted as percentage of the culture well covered by cells. For all experiments, confluence was considered to be the point at which >80% of culture well was covered by cells. Only culture wells that grew at the same pace were included in comparative experiments. When comparing different levels of confluence, percentage estimates were verified using ImageJ to count cells in photographs taken during light microscopy (https://imagej.nih.gov/ij).

2.2 Air-Liquid Interface Airway Epithelial Cell Culture

Primary NHBE cells were grown in ALI culture until fully differentiated as pseudostratified ciliated columnar epithelial cells. Cells were grown under submerged conditions as described above until confluent in a 75 cm² cell culture flask and then passaged into 6.5 mm transwell culture wells (Corning) at 200,000 cells per well under 100 uL of media and above 500 uL of media per well (Image 3b). Cell media was homemade and contained the same supplements described above (Section 2.1) but in 10-fold higher quantities to sustain the higher number of cells grown. 36 hours after confluence, apical media was removed. Ciliary beat was observed under light microscopy after 10 days to verify differentiation (Image 3c).
2.3 Biomarker Analysis

2.3.1 Enzyme-Linked Immunosorbent Assay

TF and TGF-beta protein levels were quantified using commercially available enzyme-linked immunosorbent assays (ELISA) from R&D Systems (R&D Systems, Inc., Minneapolis, MN, USA). Assays were run according to the manufacturer’s specifications. TF protein was measured in undiluted cell media. Standard curves were initially run in parallel using the manufacturer-recommended reagent diluent and cell media as a diluent to rule out artifact or a matrix effect caused by the presence of cell media in samples; no difference was noted and subsequent standard curves were run using the manufacturer-recommended reagent diluent. TGF-beta protein was measured in cell lysate after lysis with RIPA buffer. During initial assay optimization the manufacturer-recommended reagent diluent was compared to RIPA buffer to rule out potential artifact in samples. No difference was noted between diluents and manufacturer-recommended reagent diluent was used for subsequent standard curves.
2.3.2  *Polymerase Chain Reaction*

After LPS exposure, cells were washed three times with PBS and total RNA was extracted using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories Inc.). Total RNA was used to synthesize the first-strand cDNA (Script TM cDNA synthesis kit; Quanta BioSciences, Inc., Gaithersburg, MD, USA). Reverse Transcription Polymerase Chain Reaction (PCR) was performed on the C1000TM thermal cycler equipped with CFX96TM real-time PCR system (Bio-Rad Laboratories Inc.). Total RNA was determined in samples via use of a Nanodrop 200c spectrophotometer (Thermo Fisher Scientific, Waltham, MA) after which samples were normalized to RNA concentration. For the relative quantification of TF mRNA expression, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping control. Perfecta SYBR Green (Quanta BioSciences, Inc.) was used as a DNA intercalator dye to monitor amplified DNA quantification and real-time quantitative PCR curves were analyzed by CFX Manager software (Bio-Rad Laboratories Inc.) to obtain threshold cycle values for each sample. TF primers used were forward sequence 5’-ACAGAGTGTGACCTCACCGACGA-3’ and reverse sequence 5’-CCTGCAGGGTAGGAGAAGACCC-3’.

2.3.3  *Colorimetric Assay*

Lactate dehydrogenase (LDH) was measured using the Pierce LDH Cytotoxicity Assay which is a commercially available colorimetric assay (Pierce Biotechnology, Rockford, Illinois, USA).
Assays were run according to manufacturer’s specifications. LDH was measured in undiluted cell media.
2.4 Evaluation of Signaling Pathways

Cells were exposed to 10 μg/mL LPS in the basolateral media for several different time courses; the time of exposure at which the largest protein signal was observed was 24 hours (which has been previously reported), so, we subsequently used this time point for all LPS exposures. We have previously reported that 10 μg/mL LPS will activate MEK/ERK without causing cell death and used this model for our reported experiments.36

Specific pathways by which LPS induces TF release were evaluated via selective antagonist exposures in separate experiments. For these experiments, the selective antagonist was added to cell media along with LPS or vehicle for the duration of the exposure. Excipients for each antagonist were added to control groups to prevent artifact. PD98059 was used to evaluate MEK/ERK signaling, PS1145 was used to evaluate IKK signaling, and SB431542 was used to evaluate SMAD signaling (R&D Systems, Inc.).

2.5 TF Inhibition

TF has an endogenous specific inhibitor called TFPI (Image 2). This commercially available compound functions in vivo to balance TF.6, 7, 18, 28, 29 To evaluate the function of TF in cell growth, we added TFPI (R&D Systems, Inc) to cell media during each media change. Since NHBE grown in submerged cell culture are precursors to differentiated NHBE that canonically function
during tissue development and wound healing, we used this culture model (Image 3a) for these experiments.

2.6 Statistical Analysis

Sample sizes for each group were determined to be appropriate to achieve a level of significance of 0.05 and a power of 0.80 (G*Power Software, Universitat Kiel, Germany). Statistical analyses were performed using SigmaStat (Systat Software, Inc., San Jose, CA). Student’s unpaired, two-tailed t-test was used to evaluate protein and mRNA levels comparing control groups and exposure groups. Wilcoxon rank-sum test was used for non-Gaussian distributed data as determined by the Shapiro-Wilk test. For multiple group exposures, analysis of variance (ANOVA) or ANOVA on ranks was used. A Bonferroni correction was used to account for multiplicity. A p-value of less than 0.05 was considered statistically significant.
Chapter 3: Release of TF from NHBE after LPS Exposure

3.1 Rationale and Hypothesis

We sought to determine whether NHBE produce and release TF in response to LPS stimulation and to elucidate the mechanism by which that occurs. We used airway cell culture models for all experiments since these allow for evaluation of local airway epithelial biomarker production. We hypothesized that cultured airway epithelial cells produce and release TF and that this production and release is increased by LPS exposure. Specifically, I hypothesized that these increases were mediated by MEK/ERK and SMAD signaling mediated by TGF-beta (Image 5).
Image 5: Schematic diagram of experiments.

NHBE Grown in Submerged Cell Culture

TF release in response to inflammation:
24-hour 10 µg/mL LPS or vehicle exposure

TGF-beta increase in response to inflammation:
24-hour 10 µg/mL LPS or vehicle exposure

MEK/ERK signaling pathway evaluation
24-hour PD98059 + 10 µg/mL LPS or
24-hour PD98059 + vehicle exposure

TF release in response to TGF-beta:
24-hour 1000 pg/mL TGF-beta or vehicle exposure

TGF-beta receptor/SMAD signaling pathway evaluation:
24-hour SB431542 + 10 µg/mL LPS or
24-hour SB431542 + vehicle exposure
3.2 Results

3.2.1 Differentiated NHBE does not release TF after LPS exposure

Primary NHBE cells from three separate donors were grown in ALI culture conditions until fully differentiated as pseudostratified ciliated columnar epithelial cells. TF protein levels were measured via ELISA in apical and basolateral media. TF protein was not statistically different between control or LPS groups at any time point (Figure 1). Since LPS is known to induce an increase in CXCL8 release from NHBE, we evaluated CXCL8 levels in media as a positive control for LPS function. CXCL8 was increased in the LPS groups compared to the control groups (p < 0.01 Figure 1). These results indicate that differentiated pseudostratified ciliated columnar epithelial cells do not release TF in response to LPS exposure.
Figure 1: Differentiated NHBE does not release TF when exposed to LPS.

Cells grown in ALI were exposed to 10 ug/mL LPS in the basolateral media for 24 hours. TF was measured via ELISA in (A) apical and (B) basolateral media. No statistically significant change was noted between control and LPS groups. Results were normalized to reflect TF change vs. control. (C) LPS exposure significantly increased released CXCL8 protein (p < 0.05 indicated by asterisk). All results are reported as means plus and minus standard error of the mean.

A.

\[
\begin{array}{c|c|c}
    & N = 9 & P < 0.67 \\
\hline
    \text{Control} & \text{LPS} \\
\end{array}
\]
B.

- Graph showing control and LPS conditions with N = 6 and P < 0.45.

C.

- Graph showing CXCL8 pg/mL with Control and 10 μg/mL LPS conditions with N = 4 and P < 0.05.
3.2.2 TF protein release from undifferentiated NHBE increases after LPS exposure

Since differentiated pseudostratified ciliated columnar epithelium did not respond to LPS exposure by releasing TF, we evaluated TF release from a submerged cell culture model. The undifferentiated precursor airway cells that develop in submerged conditions are representative of the basal cell phenotype found in the growing and healing airway. We repeated a time course evaluation for both TF protein and TF mRNA levels to determine the optimal time of exposure to LPS and found TF protein levels to be the highest after 24 hours of LPS exposure and TF mRNA signal to be the highest after 3 hours of exposure. NHBE cells were exposed to 10 ug/mL E. coli LPS added to 500 uL of cell media for either 3 hours or 24 hours.

NHBE exposed to LPS demonstrated a nearly 2-fold increase (p < 0.01) in released TF protein compared to the control groups (Figure 2a). TF mRNA levels were not affected by LPS exposure (Figure 2b).
Figure 2: Undifferentiated NHBE releases TF protein when exposed to LPS.

(A) NHBE exposed to 10 ug/mL LPS for 24 hours released > 50% more TF protein than those exposed to vehicle (p < 0.001). Three different cell lines were used to verify this observation and data were normalized to reflect change vs. control. (B) TF mRNA did not increase after exposure to 10 ug/mL LPS for 3 hours. Results normalized to reflect change vs. control. Results are reported as means with error bars indicating standard error.

A.

B.
3.2.3  *LPS-induced TF release from NHBE is mediated by MEK/ERK signaling*

LPS has been reported to induce TF release from monocytes via MEK/ERK signaling and AP-1 activation\(^1\text{-}^4\). It also activates IKK signaling pathways which increase NFKB\(^3\text{7}\). To evaluate the signaling pathways involved in LPS-induced TF release from NHBE, we exposed cells to a MEK/ERK-selective inhibitor (PD98059, 10 + 20\(\mu\)M) and an IKK-selective inhibitor (PS1145, 15 + 30\(\mu\)M) in the presence of 10 \(\mu\)g/mL LPS or vehicle. Inhibitor concentrations were selected based on previously reported experiments\(^3\text{6,}^3\text{8}\). TF protein levels in cell media were measured by ELISA after 24 hours of exposure.

NHBE treated with PD98059 did not release more TF when exposed to LPS than the control group (Figure 3a). Treatment with PS1145 did not prevent LPS-induced increased TF release from NHBE (Figure 3b). These results suggest that TLR-4 activation by LPS leads to TF release via MEK/ERK signaling and not IKK signaling.
Figure 3: LPS-induced TF release from NHBE is mediated by MEK/ERK signaling.

(A) NHBE exposed to 10 ug/mL LPS for 24 hours released more TF than control group (p < 0.05, indicated by asterisks); NHBE exposed to 10 ug/mL LPS for 24 hours in the presence of 20 uM PD98059, a MEK/ERK inhibitor, did not release more TF than control. (B) The addition of 15 or 30 uM PS1145 did not alter the release of TF after exposure to 24 hours of ug/mL LPS compared to control group (p < 0.05, indicated by asterisks). Results normalized to reflect change vs. control and reported as means with error bars indicating standard error.
3.2.4 **NHBE TGF-beta levels increase after LPS exposure**

TGF-beta contributes to wound healing and tissue remodeling in airway disease\(^{39-42}\). To evaluate the effects of LPS on TGF-beta in NHBE, we exposed cells to 10 µg/mL LPS for time points as described above; the largest levels of TGF-beta were found at 24 hours of exposure and this duration was used for subsequent experiments. TGF-beta protein levels were then evaluated in cell media and lysate by ELISA.

NHBE exposed to LPS demonstrated a 10-fold increase in TGF-beta protein in cell lysate compared to the control group (\(p < 0.05\), Figure 4A). There was no change in TGF-beta protein in cell media (Figure 4B). These results indicate that TLR-4 activation increases intracellular TGF-beta protein as well as released TF protein levels.

3.2.5 **TF protein is released from NHBE after exposure to TGF-beta**

We then investigated the effect of TGF-beta on TF. We exposed NHBE to several concentrations of TGF-beta for 24 hours after which we quantified TF protein levels in cell media by ELISA. The cells used in these experiments were grown in submerged conditions.

NHBE exposed to TGF-beta demonstrated a dose-dependent increase in released TF protein compared to the control groups (\(p < 0.01\), Figure 5A). TF mRNA levels were not affected by TGF-beta exposure (Figure 5B). These results, paired with the demonstration that LPS leads to increased intracellular but not released TGF-beta, suggest that LPS-induced TF protein release may be mediated by autocrine upregulation of and signaling by TGF-beta.
Figure 4: NHBE TGF-beta protein levels increase after exposure to LPS.

(A) Levels of TGF-beta protein did not increase in cell media after 24 hour exposure to LPS (results normalized to reflect change vs. control). (B) Levels of TGF-beta protein in NHBE cell lysate increased after 24 hour exposure to 10 ug/mL LPS (p < 0.05, indicated by asterisk). Results are reported as means with error bars indicating standard error.

A.

**TGF-beta levels in cell media**

<table>
<thead>
<tr>
<th>Control</th>
<th>10 ug/mL LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 12</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>
B.

![Graph showing TGF-b pg/mL with N=8, P<0.05](image)

Control  
LPS

N = 8  
P < 0.05
Figure 5: NHBE exposed to TGF-beta for 24 hours release TF in a concentration-dependent manner.

(A) NHBE exposed to 1000 pg/mL, 500 pg/mL, or 250 pg/mL TGF-beta for 24 hours released more TF than control group (p < 0.01 indicated by **, p < 0.05 indicated by *). NHBE exposed to 125 pg/mL TGF-beta did not release more TF than control (A). Results are reported as means with error bars indicating standard error. (B) No change in TF mRNA was found after exposure to 1000 pg/mL TGF-beta (results normalized to reflect change vs. control).
3.2.6  *NHBE TF release after TGF-beta exposure is mediated by SMAD signaling*

TGF-beta activates the TGF-beta receptor complex and initiates SMAD and SMAD-independent signaling pathways\(^{39,40}\). To evaluate signaling pathways involved in TGF-beta-induced TF release from NHBE, we exposed cells to different concentrations of SMAD-selective inhibitor (SB431542) in the presence of 10 \(\mu\)g/mL LPS or vehicle as described above; inhibitor doses were selected based on previously reported experiments (Figure 6)\(^{39}\). TF protein levels in cell media were measured by ELISA after 24 hours of exposure.

NHBE treated with 20 \(\mu\)M SB431542 did not release more TF than the control group when exposed to LPS (Figure 6). These results suggest that the release of TF after exposure to LPS is mediated by SMAD signaling through the TGF-beta receptor complex. This also supports the hypothesis that LPS-induced TF protein release is mediated by autocrine TGF-beta signaling.
Figure 6: LPS-induced Increased TF release from NHBE is mediated by SMAD signaling.

NHBE exposed to 10 ug/mL LPS or 1000 pg/mL TGF-beta for 24 hours released approximately 50% more TF compared to control (p < 0.01). NHBE exposed to 10 ug/mL LPS + 100 uM SB431542, a SMAD inhibitor, did not release more TF compared to control and released less TF than those treated with LPS or TGF-beta (p < 0.01). Lower doses of SB431542 had no effect on TF release. Results normalized to reflect change vs. control and reported as means with error bars indicating standard error; statistical significance indicated by asterisks.
Chapter 4: Effects of TF Inhibition on NHBE Growth

4.1 Rationale and Hypothesis:

Having previously demonstrated that NHBE grown under submerged conditions constitutively release TF, we decided to evaluate the effect of inhibiting TF in these cells on cell growth. Since our results showed that NHBE release TF after exposure to LPS by a mechanism mediated by TGF-beta superfamily signaling, we hypothesized that this was part of a local wound healing/repair mechanism. Specifically, we hypothesized that selective inhibition of TF via exposure to TFPI would increase the time required for NHBE to reach confluence under submerged conditions.

4.2 Results – TFPI Decreases NHBE Growth

To evaluate the effects of TFPI on cell growth, we added TFPI to cell media throughout the course of submerged cell culture. Several doses of TFPI were used in different groups and compared to a vehicle group used as a control. The cells used in these experiments were grown in submerged conditions described above (Image 2a) with media changed every 48 hours. Confluence was evaluated via light microscopy prior to each media change. The control group reached confluence after six days at which point confluence was measured in each group and
recorded. After the final media change and confluence evaluation, LDH levels in each group were measured to rule out cytotoxic effects of TFPI.

NHBE treated with TFPI grew significantly slower than the control group, reaching less than 50% confluence by the time the control group was fully confluent (Figure 7a, P < 0.001). This phenomenon was dose-dependent. LDH was not different between any of the groups (Figure 7b, P < 0.001), indicating that the lower numbers of cells was not caused by cytotoxicity. These results suggest that TF aids cell growth which is a function closely related to wound repair\textsuperscript{28, 41, 42}. These results also indicate that inhibition of TF can occur without local cytotoxicity.
Figure 7: TFPI slows NHBE growth in a dose-dependent manner.

(A) NHBE exposed to 1000 pg/mL or 500 pg/mL TFPI grew significantly slower that those exposed to 250 pg/mL TFPI and the control group (p < 0.05). Images of each exposure group are below their respective column in the bar graph. Results are reported as means with error bars indicating standard error. (B) LDH was not significantly different between the control or treatment groups (p > 0.05).
B.

N = 12
P > 0.05
Chapter 5: Discussion

The canonical functions of TF as initiator of coagulation and subsequent clot formation is integral to wound healing\textsuperscript{5, 21, 22}. The increased levels of TF found within the inflamed airway (including during asthma\textsuperscript{15, 19}, pneumonia\textsuperscript{16, 17}, bronchopulmonary dysplasia\textsuperscript{18}, and acute respiratory distress syndrome\textsuperscript{14, 27}) suggest that TF might have other roles as well. The airway source of TF, its mechanism for production and release, and the function of TF within airways are largely unknown. Non-canonical functions of TF have been reported that further support the role of TF in cell growth. Specifically, TF has been shown to reduce the activation of apoptosis pathways and promote metastasis\textsuperscript{3}.

\textit{In vitro} dysregulation of TF via selective inhibitors or knock-down models leads to systemic hemorrhage; fatal pulmonary hemorrhage occurs in TF knockout mice shortly after birth\textsuperscript{5, 28, 30}. TF levels have been shown to be profoundly increased at sites of active wound healing and inhibition of TF by administration of TFPI is associated with increased healing/clotting time\textsuperscript{21, 22}. This knowledge supports the theory that TF supports wound healing/repair and that TFPI inhibits these processes.

LPS is a well-described initiator of inflammatory responses\textsuperscript{35-37}. LPS binds to TLR-4 and activates intracellular signaling pathways, including MEK/ERK\textsuperscript{37}. We have previously reported that 10 ug/mL LPS will activate MEK/ERK without causing cell death and used this model for our reported experiments\textsuperscript{36, 38}. These experiments resulted in similar TF release from NHBE as that reported after cyclic stress\textsuperscript{20}.
In 2009, Brims et al reported that subjects with severe asthma had higher levels of TF in their sputum than subjects with moderate asthma or control subjects, even while undergoing treatment\textsuperscript{15}. Park et al reported a significant increase in released TF protein from NHBE after exposure to cyclic stress levels similar to those reported in the asthma airway\textsuperscript{20}. Since cyclic stress can cause epithelial injury, the TF function to create a “patch on a hole” when vascular endothelium ruptures could serve similarly within the airways. Also, TGF-beta regulates many of the processes surrounding wound healing and is increased in many cell types after exposure to LPS\textsuperscript{39-42}. Local increases of TGF-beta in the airways are associated with airway remodeling in severe asthma, which is thought to involve a dysregulated and excessive wound healing processes\textsuperscript{39,40,42}. It is also possible that increases in local TF could contribute to airway remodeling; this possibility is supported by our finding that inhibition of TF slows the growth of airway cells.

When interpreted as a whole, our experiments indicate a complex sequential mechanism leading to TF release from NHBE after exposure to LPS. Our initial experiment which demonstrated the release of TF from NHBE after LPS exposure is only novel due to the cell types evaluated. This phenomenon has been observed in cell types typically involved in hemostasis including monocytes and vascular endothelium\textsuperscript{1-4}. Groups have also reported findings similar to ours regarding MEK/ERK signal mediation of LPS-induced TF and TGF-beta release\textsuperscript{1-4,43,44}. The data presented here is novel due to the cell types evaluated, but more importantly because it ties together several processes previously thought to be independent of one another. Through selective inhibitors and agonists applied in a stepwise manner, we have demonstrated that LPS-induced TLR-4 activation (1) leads to MEK/ERK signaling (2) and
subsequent increases in TGF-beta levels (3), SMAD-signaling (4), and TF protein release (5) from undifferentiated NHBE cells (Image 6). Since TGF-beta levels were not increased in cell media, it is likely that this is an autocrine mechanism and not paracrine. It is possible that this mechanism is responsible for LPS-induced TF release from other cells types, although this has yet to be investigated.
Image 6: Cartoon illustration of proposed autocrine mechanism.

Exposure of NHBE to LPS leads to TLR-4 activated, MEK/ERK-mediated increase in TGF-beta. This leads to TGF-beta receptor activation and SMAD-mediated TF release. The curved arc represents the cell membrane.
TFPI is a strong inhibitor of TF in vitro and in vivo. It prevents TF from forming clots or promoting tissue growth by destabilizing activated TF/FVII complexes or FX and therefore preventing the coagulation cascade. Airway epithelia can produce FVII constitutively that can bind with TF. Along with maintaining hemostasis, these complexes promote cell migration and organized tissue formation as seen during cell growth and wound healing. Our experiments evaluating the effects of TF inhibition by TFPI on NHBE cell growth suggest a local repair/wound healing function of TF within the airways.

Unlike the ALI model used by Park et al., the submerged cell culture conditions we used prevent cells from fully differentiating to pseudostratified ciliated columnar epithelia in vivo and instead stay in a form that resembles basal cell morphology. We initially exposed differentiated cells grown in ALI to LPS and saw no increase in TF (Figure 1). Since submerged, undifferentiated cells are actively growing and dividing, and these processes are similar to wound healing (a process that begins with basal cells in the epithelium), this could explain why we did not observe the release of TF from differentiated NHBE when we exposed them to LPS. Rather, differentiated cells may release TF in response to damage. Of note, the bottom layer of cells grown in air-liquid interface differentiate as basal cells; in discussions with Dr. Park, I learned that the TF release she reported did not come from pseudostratified ciliated columnar epithelium but rather from basal cells in their culture model (J-A Park; April 2017, personal communication). Since cyclic stress is more likely to disrupt the layers of cells than LPS exposure, this could further explain why we did not see TF protein released from differentiated cells.
A confounder in our results is the lack of an observed increase in TF mRNA after exposure to LPS. Park et al noted an increase in both TF mRNA and released TF protein after exposing airway cells to cyclic stress. We exposed several different cell lines to LPS at various time courses and never found a difference in TF mRNA between control and treatment groups. Park et al reported that the TF mRNA increase and protein release was mediated by PKC signaling whereas we report that LPS induced TF release is mediated by MEK/ERK signaling. It is possible that MEK/ERK signaling leads to increased release of TF protein via exosomes (as reported by Park et al) but not an increase in TF mRNA signal.

These studies had several limitations. Most notably, we did not evaluate the effects of TF on wound repair in differentiated airway epithelium nor the mechanism by which TF acts. TF/FVII complexes have been shown to activate intracellular signaling of mitogen-activated protein kinases, protease-activated receptors, and apoptotic pathways. We intend to evaluate these remaining questions in the future to better characterize the role of TF within the airways and as potential therapeutic targets.

Signaling pathways were evaluated by selective inhibitors as opposed to RNA interference or knockout cells. We intend to evaluate phosphorylation of ERK to further support the role of this signaling pathway in TF release and decrease any likelihood that our results are related to non-specific effects of the MEK/ERK inhibitor.

We did not design experiments to evaluate specific mechanisms of TFPI delaying NHBE growth; rather, these data are descriptive. Further studies to evaluate the effects of TFPI on intracellular signaling pathways associated with TF/FVII complex formation and the effect of
TFPI on the cell cycle are planned. It also would be worthwhile to evaluate the effects of other reported inhibitors of TF such as AP-1\(^{47}\).

Our data from diverse cell lines using stepwise inhibition and stimulation demonstrate that NHBE release TF in response to LPS exposure. This release is mediated by TGF-beta signaling and regulated by MEK/ERK and SMAD signaling pathways which may function as an autocrine mechanism. Inhibition of TF by TFPI slows airway cell growth, similar to the slowed clotting and healing that occurs in other settings when TF is inhibited. Based upon the results we report, inhibitors of TF and the pathways leading to its release during inflammation could be therapeutic targets for severe asthma.
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Vita

Michael D. Davis, RRT

Michael.D.Davis@vcuhealth.org

**Education**

Associate of Applied Science, Cardiopulmonary Science, Central Virginia Community College 2005

Bachelor of Science, Biomedical Science, Lynchburg College 2008

Doctor of Philosophy, Physiology and Biophysics, Virginia Commonwealth University 2017

**Previous Research Experience**

Ph.D. Candidate (December 2014 – Present)

Virginia Commonwealth University

Department of Physiology and Biophysics

Advisor: Bruce K. Rubin, M.Engr., M.D., M.B.A., FRCPC

Research Respiratory Care Practitioner (March 2015 – Present)

Virginia Commonwealth University Medical Center

Department of Respiratory Care Services

Respiratory Research Specialist (January 2010 – Present)

Airbase Therapeutics

Advisor: John F. Hunt, M.D.
Senior Respiratory Therapy Research Scientist (March 2008 – Present)
University of Virginia School of Medicine
Department of Pediatrics, Division of Respiratory Medicine and
Department of Anesthesiology, Division of Neuroanesthesiology
Principal Investigators: John F. Hunt, M.D. and Thomas N. Pajewski, M.D., Ph.D.

Project Director – Exhaled Breath Research (February 2012 – January 2015)
Virginia Commonwealth University
Department of Adult Health and Nursing Services
Principal Investigator: Alison J. Montpetit, RN, Ph.D.

Ph.D. Student (August 2013 – December 2014)
Virginia Commonwealth University
Department of Physiology and Biophysics
Advisor: Bruce K. Rubin, M.Engr., M.D., M.B.A., FRCPC

Clinical Research Assistant (June 2007 – February 2008)
University of Virginia School of Medicine
Department of Pediatrics, Division of Respiratory Medicine
Principal Investigators: Brian K. Walsh, RRT, Ph.D. and John F. Hunt, M.D.
**Certifications/Licenses**

Virginia Department of Health Professions
Board of Medicine
Respiratory Care Practitioner (RCP): License Number 0117005205
06/06/2005-04/30/2020

National Board for Respiratory Care (NBRC)
Registered Respiratory Therapist (RRT): Registry number 89486
06/24/2005-06/30/2020

American Academy of Pediatrics (AAP)
Helping Babies Breathe (HBB) Master Trainer
Completed on 06/2011

**Professional Societies**

American Association for Respiratory Care (AARC)
Member in Good Standing
2007-Present
Reviewer – Clinical Practice Guidelines
2011-Present
International Association for Breath Research

Member in Good Standing

2012-Present

Member of Global Task Force for Breath Standardization

2013-Present

**AWARDS/ACCOLADES**

Robert W. Ramsey Award: Most Outstanding Doctoral Student

Virginia Commonwealth University School of Medicine

Department of Physiology and Biophysics

05/2014

Denny Hamlin Foundation Scholar

Virginia Commonwealth University School of Medicine

Department of Pediatrics

12/2014 – Present

**PEER REVIEWING**

*Journal of Respiratory Care*

2008-Present

*Journal of Breath Research*

2012-Present
Heart & Lung: The Journal of Acute and Critical Care
2012-Present

Analytica Chimica Acta
2013-Present

British Journal of Clinical Pharmacology
2016-Present

Biomedical Physics and Engineering Express
2016-Present

Publications (* indicates MDD as first or corresponding author)

Administration of inhaled pulmonary vasodilators to the mechanically ventilated neonatal patient.

Davis MD*, Donn SM, Ward RM
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**Invasive Blood Gas Analysis and Monitoring.**

Davis MD*

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**Exhaled breath condensate pH assays.**

Davis MD*, Hunt J.

**Exhaled breath condensate: an overview.**

Davis MD*, Montpetit A, Hunt J.


**High titers of IgE antibody to dust mite allergen and risk for wheezing among asthmatic children infected with rhinovirus.**


**Select Scientific Meeting Abstracts and Presentations**


*Lecture/Presentation:* “Plastic Bronchitis and Pulmonary Lymphatic Abnormalities: New Frontiers in Pulmonary Medicine” – Invited Guest Lecturer, University of Manchester Division of Respiratory Medicine, Manchester, England, 04/2016


Lecture/Presentation: “Assessment and Treatment of the Respiratory Patient” – National Grand Rounds, JFK Hospital, Monrovia, Liberia, 10/2015

Lecture/Presentation: “Exhaled Breath Condensate Biomarkers in Critically Ill, Mechanically Ventilated Adults” – International Association of Breath Research 10th Anniversary Summit, Vienna, Austria, 09/2015

Lecture/Presentation: “Advances in Respiratory Monitoring Have Improved Outcomes – a Pro/Con session” – 58th International Respiratory Conference of the AARC, Las Vegas, NV, 11/2014

Poster Presentation: “Airway Acidity in Critically Ill, Mechanically Ventilated Adults” - 8th International Conference on Breath Research & Cancer Diagnosis, Torun, Poland, 07/2014
**Keynote Lecture:** “Relationships between exhaled nitric oxide, volumetric capnography and pulmonary status in patients with rib fractures” - 8th International Conference on Breath Research & Cancer Diagnosis, Torun, Poland, 07/2014

**Poster/Presentation:** “Methods for Evaluating the Pulmonary Effects of Swimming in Chlorinated Water” - 58th International Respiratory Conference of the AARC/Journal of Respiratory Care, 11/2012

**Grants/Funding**

Mallinckrodt Pharmaceuticals, Investigator Initiated Study

Comparing the inflammatory effects of nitric oxide and two formulations of epoprostenol on human bronchial epithelium.

Role: Principal Investigator

Amount: $100,000

Award Date: 07/2016

American Respiratory Care Foundation Frances B. Parker Grant Fund

The Role of Tissue Factor in Inflammatory Airway Disease.

Role: Principal Investigator

Amount: $20,000

Award Date: 01/2016