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Shambavi J. Rao April 10, 2018

Hemoglobin Induces Invasion of *Streptococcus pneumoniae* into Human Lung Cells

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Streptococcus pneumoniae (Spn) are Gram-positive bacteria known to cause many debilitating and invasive pneumococcal diseases such as pneumonia, bacteremia, and meningitis. Lethal infection is mediated by the invasion of cells primarily located in the lower respiratory tract, permitting bacteria to translocate into the bloodstream. We studied the mechanism of pneumococcal invasion by establishing an *in vitro* model to replicate invasion using cultures of human lung Calu-3 cells. Pneumococcal invasion was tested in the presence and absence of hemoglobin (Hb). This *in vitro* model was utilized to determine invasion of pneumococci through polarized human respiratory epithelial cells by counting bacteria translocated to the basolateral side of the cells. A potential invasion mechanism mediated by disruption of tight-junction (TJ) proteins was explored by measuring the trans-epithelial electrical resistance (TEER) pre-, and post-pneumococcal, infection. Through a series of experiments, I observed that hemoglobin induced pneumococcal invasion within 8 hours of incubation but a decrease in TEER within 24 hours post-infection. Whereas invasion and disruption of the TJ belt was triggered by incubation with Hb, the effectors appeared to be different given that invasive pneumococci translocated before TEER was affected. Furthermore, a mutant in a potent toxin, pneumolysin, and a mutant in the competence stimulating peptide, were unable to translocate but both mutants disrupted the TJ belt at the same extent as that observed with the wild type strain. In this thesis, we concluded that Hb triggers in pneumococci invasion of human lung cells and disruption of the TJ belt and that the effectors of this Hb-induced phenotypes may be different and required further investigation.

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I would like to sincerely thank my mentor and my honors thesis advisor, Dr. Jorge E. Vidal, for his counsel, for his guidance, and for providing me the opportunity to pursue an honors thesis in the field of microbiology and infectious disease. Dr. Vidal has significantly influenced my academic endeavors throughout the past two years. Through Dr. Vidal's constant encouragement, I learned many invaluable skills, which has helped me grow as a student and as an individual. Furthermore, I would like to genuinely thank Dr. Xueqing Wu for her invaluable discussion and advice during my research work. Dr. Wu assisted me in understanding the basics of the experiments, patiently teaching, and guiding me in the laboratory. I would also like to thank Mr. Renzo Valeriani, Dr. LaMonta Beard, Dr. Wenxin Wu, Dr. Fuminori Sakai, Mr. David Watson, and Ms. Karen Ellis for their constant support and overall guidance in the lab. Finally, I would like to thank my father, Mr. Jayakumar Rao, and mother, Dr. Anupama Rao, for their unwavering support, commitment towards life-long learning, and sharing their passion for scientific inquiry with me.

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ABSTRACT

Streptococcus pneumoniae (Spn) are Gram-positive bacteria known to cause many debilitating and invasive pneumococcal diseases such as pneumonia, bacteremia, and meningitis. Lethal infection is mediated by the invasion of cells primarily located in the lower respiratory tract, permitting bacteria to translocate into the bloodstream. We studied the mechanism of pneumococcal invasion by establishing an *in vitro* model to replicate invasion using cultures of human lung Calu-3 cells. Pneumococcal invasion was tested in the presence and absence of hemoglobin (Hb). This *in vitro* model was utilized to determine invasion of pneumococci through polarized human respiratory epithelial cells by counting bacteria translocated to the basolateral side of the cells. A potential invasion mechanism mediated by disruption of tight-junction (TJ) proteins was explored by measuring the trans-epithelial electrical resistance (TEER) pre-, and post-pneumococcal, infection. Through a series of experiments, I observed that hemoglobin induced pneumococcal invasion within 8 hours of incubation but a decrease in TEER within 24 hours post-infection. Whereas invasion and disruption of the TJ belt was triggered by incubation with Hb, the effectors appeared to be different given that invasive pneumococci translocated before TEER was affected. Furthermore, a mutant in a potent toxin, pneumolysin, and a mutant in the competence stimulating peptide, were unable to translocate but both mutants disrupted the TJ belt at the same extent as that observed with the wild type strain. In this thesis, we concluded that Hb triggers in pneumococci invasion of human lung cells and disruption of the TJ belt and that the effectors of this Hb-induced phenotypes may be different and required further investigation.

SIGNIFICANCE

The findings from this study will enhance scientific knowledge in the field of microbiology and infectious disease considering that *Streptococcus pneumoniae* is a leading cause of pneumonia globally. Developing a fundamental understanding of pneumococcal translocation from the mucosal surface of the nasopharyngeal epithelium into the bloodstream is critical for advancing clinical medicine and for discovering effective treatment plans to eliminate this disease. In my thesis, I demonstrated that the presence of hemoglobin enhances pneumococcal invasion and propagates invasive disease. Furthermore, through numerous experiments I illustrated that pneumococcal invasion, and the disruption of tight junction (TJ) proteins may occur by distinct mechanisms. Specifically, the results provide fundamental background into the mechanism of pneumococcal invasion when supplemented with hemoglobin. Thus, scientists can design experiments to target specific signal receptors present on the cell wall of pneumococcus which are recognized during an innate immune response. These studies can contribute to the development of new vaccines with increased specificity for targeting different cellular mechanisms compared to the vaccines already available for this disease (polysaccharide and conjugate vaccines). Therefore, helping to reduce the prevalence of pneumococcal disease in children and in adults.

INTRODUCTION

Streptococcus pneumoniae (Spn) are Gram-positive, facultative anaerobic bacterial pathogens. It has a capsular polysaccharide wall with peptidoglycan layers formed by glycosidic linkages of N-acetylglucosamine and N-acetylmuramic acid. Spn is transmitted through airborne respiratory droplets and acquired by direct human-human contact (11). Pneumococci propagate invasive pneumococcal disease (IPD) thereby contributing to high mortality rates and economic hardship for developing nations. Sessile pneumococci occupy a niche on the mucosal surfaces of human nasopharynx (21). However, planktonic pneumococci are located throughout the bloodstream and are responsible for fatal invasive diseases. Primarily, Spn triggers IPD such as pneumonia, bacteremia, and meningitis (6). Common symptoms of these diseases include fever, chills, and shortness of breath. Additionally, pneumococcus engages in social behaviors such as biofilm formation and quorum sensing (QS) to facilitate its growth and survival. Understanding its characteristics, its social behaviors, and its structure, provides background about the mechanism of pneumococcal virulence.

Pneumococcal pathogenesis is influenced by nasopharyngeal colonization and is highly prevalent among pediatric populations (22). Specifically, young children with comorbid conditions are immunocompromised and thus are more susceptible to pneumococcal diseases (22). The pre-existing conditions include sickle cell anemia, HIV, and diabetes mellitus. These conditions increase their risk up to 11.7 times greater than the risk faced by healthy individuals (6). Also, 11%-93% of children remain asymptomatic during Spn colonization, serving as reservoirs for pneumococcal transmission (22). Therefore, pneumococci remain the leading causative agent of childhood mortality in developing nations (21). Additionally, epidemiological studies show that Spn contributes to the death of 500,000 children annually, accounting for 5% childhood mortality

worldwide (20). However, the development of effective conjugate vaccines such as PCV7, PCV13, and PPSV 23 as well as the development of antibiotics reduces global incidence rates of Spn (22).

Pneumococcal colonization is mediated by biofilm formation in the nasopharynx. Biofilm formation is a social behavior where aggregates of bacteria secrete an extracellular matrix to protect themselves against survival threats like antibiotics (16). The characteristics of biofilms aid pneumococcal virulence by helping pneumococcal translocation from mucosal surface of nasopharyngeal cells into the bloodstream. Once, the pathogen invades cells in the lower respiratory tract and gains access to the bloodstream, invasive disease begins.

Thus, researching the importance biofilm formation in Spn provides an overview for understanding the mechanism pneumococcal invasion. Previous research with our collaborators at Georgia State University (GSU) illustrates strong evidence that hemoglobin (Hb) influences biofilm formation in Spn strains, including biofilms formed by the reference strain D39 (1). As demonstrated by the micrographs taken at successive time intervals, incubation time is directly correlated to biofilm formation, (Fig. 1). The micrographs also indicate greater biofilm biomass when pneumococci are incubated with hemoglobin in Todd-Hewitt Broth plus 0.5% Yeast Extract (THY) in comparison with the minimal biofilm formation in pure THY (absence of hemoglobin). Consequently, there is a positive correlation between the presence of hemoglobin and biofilm biomass. Therefore, we hypothesize that hemoglobin plays an important role in pneumococcal colonization.

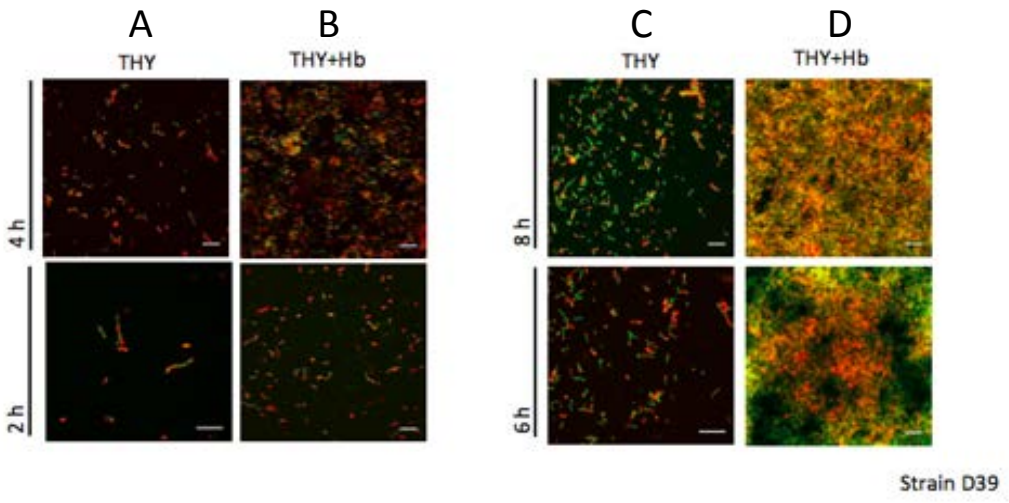

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Figure 1. Hemoglobin enhances *Streptococcus pneumoniae* strain D39 biofilm formation. Spn was incubated in THY in the absence (A, C) and in the presence (B, D) of hemoglobin. Biofilms were fixed and capsule and DNA were stained with fluorescence at each selected time point (2, 4, 6 and 8h). Green channel represents live bacterial cells, red channel shows dead bacterial cells. The stained samples were then measured by confocal microscopy and analysis by Image J.

Subsequently, the correlation between the presence of hemoglobin and biofilm formation (Fig. 1), raises questions about the exact mechanism of how hemoglobin influences this phenomenon. The structure of hemoglobin is imperative to its function and its significance in the human body and in Spn. It is likely that the quaternary structure of hemoglobin and its individual components influence biofilm formation (Fig. 1). Hemoglobin is a heterotetramer globular protein found in red blood cells and consists of two alpha and two beta subunits. By cooperative binding, the four subunits successively bind oxygen, and transports it throughout the body for aerobic respiration. In addition, hemoglobin serves as the largest iron reservoir in the human body for microorganisms (10). Iron is chelated by a porphyrin ring moiety, heme. Pneumococci process hemoglobin to release iron and amino acids which are essential for Spn survival (10). Therefore,

the structure and the components of hemoglobin help to discern hemoglobin's role in pneumococcal virulence. However, the exact mechanism of hemoglobin metabolism in Spn is currently unknown.

To cause pathogenesis of diseases like pneumonia, planktonic pneumococci obtain hemoglobin for metabolism through pulmonary hemorrhaging. Pneumonia is a common infection that occurs when Spn invades the pleural space of the lungs. Lung cells are involved in the innate immune response to minimize infections. The presence of tight junctions establishes a semipermeable barrier across lung epithelial cells to limit the passage of pneumococci through pulmonary tissue (7). Tight junctions polarize cells into apical and basolateral layers and close extracellular spaces between neighboring cells to decrease pathogenic susceptibility (7). Despite this effort, pneumococci form biofilms on the apical layer and then trigger infection.

Analyzing biofilm formation in a microenvironment similar to the environment of the thoracic cavity at the time of pneumococcal infection (i.e., presence of lung cells and hemoglobin) promotes increased understanding regarding Spn colonization and invasion. A study conducted by Dr. Xueqing Wu compares D39 Spn biofilm formation with or without lung cells. Comparing the results from Dr. Akheter's study and Dr. Wu's study illustrates hemoglobin induced biofilm formation (Hb) and produces a similar phenotype to the biofilm density seen with lung cells (Fig. 2BE) (1).

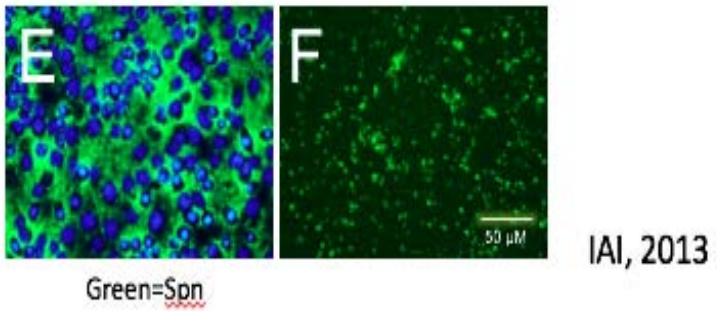

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Figure 2. Biofilm formation of Spn Strain D39 with (E) and without (F) lung cells after 8 h. Bacterial cells contains GFP (green) and human lung cell nucleus were stained with DAPI (blue).

These research studies illustrate phenotypic similarities of biofilm formation with hemoglobin and Spn growth on lung cells (Fig. 1 and Fig. 2). Thus, hemoglobin influences pneumococcal colonization on the apical layer of lung cells. Conclusively, there is strong background to understand pneumococcal colonization under conditions present in the human body, with hemoglobin and with lung cells (Fig.1 and Fig. 2). These findings led us to develop a research question to test the role of hemoglobin in IPD.

Currently, there is a gap in understanding the mechanism of pneumococcal invasion. Addressing this gap provides background for vaccine development to reduce the prevalence of pneumococcal disease. Our study begins to address this knowledge gap in pneumococcal virulence by developing an *in vitro* transwell model system which stimulates conditions similar to those present in the thoracic cavity. In this experiment, the significance of hemoglobin in pneumococcal invasion was analyzed after 8 hours and 24 hours. To account for the mechanism of pneumococcal virulence, trans epithelial resistance (TEER) was measured to determine if a disruption in the apical monolayer was correlated to the density of invasive Spn in the basolateral layer. I

hypothesized hemoglobin induces pneumococcal invasion by targeting the tight junction belt, compromising TEER.

SPECIFIC AIMS

Specific Aim 1: To develop an *in vitro* model to determine the effects of hemoglobin on pneumococcal virulence. Explore the role of hemoglobin in Spn invasion. Using an *in vitro* transwell model system that stimulated conditions similar to those in the respiratory system, pneumococcal infection was measured by TEER and invasive bacterial counts (cfu/mL) with and without hemoglobin supplementation.

Specific Aim 2: To study the mechanism of hemoglobin induced pneumococcal invasion

Aim 2.1 Explore the correlation between TEER and invasion. Using *in vitro* transwell model system, we strived to determine if the mechanism of action involved during pneumococcal infection is the same in disrupting the cellular monolayer (TEER) and in determining the density of invasive Spn (cfu/mL).

Aim 2.2 Evaluate the role of Δ*ply* and Δ*comC* genes in pneumococcal invasion. Using Spn D39Δ*ply* and D39Δ*comC* knockout mutants in D39, we examined the effects of the pneumolysin protein and competence stimulating protein on TEER, pneumococcal colonization, and invasion.

Aim 2.3 Examine which component of hemoglobin (holo form of hemoglobin, heme, iron) is responsible for invasion. I determined the component of hemoglobin influences (a) TEER decrease (b) pneumococcal colonization and (c) pneumococcal invasion.

EXPERIMENTAL METHODS

Bacterial Strains and Inoculum Preparation. *S. pneumoniae* strains utilized in this study are listed in Table 1. Strains were routinely cultured on blood agar plates (BAP) at 37°C in a 5% CO_2, humidified atmosphere, overnight before each experiment. The following day, the bacteria were harvested from plate, re-suspended in PBS, and this solution was used to prepare a 1:100 dilution. The optical density (OD) was determined at a wavelength of 600nm and this value was used to determine the volume of the suspension required to reach a final OD_{600} of 0.1M in each test group. The medium used to create the final solution varied based on the experimental conditions tested.

Table 1. Bacterial strains used in this study.

Strain	Description	Reference
D39	Avery strain, clinical isolate, capsular serotype 2	18
D39Δ*ply*	D39-derivative *ply* null mutant	18
D39Δ*comC*	D39-derivative *com*C null mutant	19

Cell Culture. Human lung Calu-3 cells (ATCC HTB-55) (Sigma Aldrich) were cultured in Eagle's minimum essential medium (EMEM; Lonza, Walkersville, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% nonessential amino acids (Sigma), 1% glutamine (Sigma), penicillin (100 U/ml), and streptomycin (100 g/ml), and the pH was buffered with HEPES (10 mM; Gibco). Cell culture medium was changed every other day.

To split cells, the cells were first detached with 0.25% trypsin (Gibco), re-suspended in the cell culture medium, and incubated at 37°C in a 5% CO2 humidified atmosphere. A week before experimentation, cells were collected, seeded into 8 transwells (TC treated, PET membrane, diameter 6.5 mm, pore size 8.0 µm) (Corning Incorporated) in a 24 transwell plate. The cells were grown until they reached 100% confluence (normally 7 days) and then were used for further tests (TEER measurements and bacterial infections).

Trans Epithelial Electrical Resistance (TEER). Prior to bacterial infection, the cells were washed three times with complete MEM without antibiotics to prevent bacterial death from the antibiotic medium previously used. Then, either 1000 µl of antibiotic free complete MEM with or without hemoglobin (20 µM) was added to bottom (basolateral side) of the respective transwell. The TEER of the untreated cells (negative control), Triton X-100 treated cells (positive control), and Spn infected cells was measured before and after the given incubation period. TEER was measured using EVOM^2 epithelial voltmeter (Ω) and STX2 electrodes (4mm wide and 1mm thick). Each electrode has an inner and outer electrode (Ag/AgCl pellet voltage sensor). The electrode was always placed for 5 minutes in ethanol to remove unwanted content. Then, it was placed in 10x phosphate- buffered saline (PBS) to neutralize of the electrode surface. The inner electrode was placed on the top (apical side) and the outer electrode (basolateral side) placed in the external medium (Fig. 3). The TEER represented the polarization of the cells and the integrity of the cellular monolayer. Moreover, this procedure was repeated to determine the TEER after a respective time frame (6, 8, or 24 hours) to determine if monolayer was compromised post Spn infection. The resistance per unit volume was calculated by multiplying the reported resistance (Ω) by the

effective membrane area (cm^2). The effective membrane area was calculated by 670 Ω x 3.14 $(d/2)^2$.

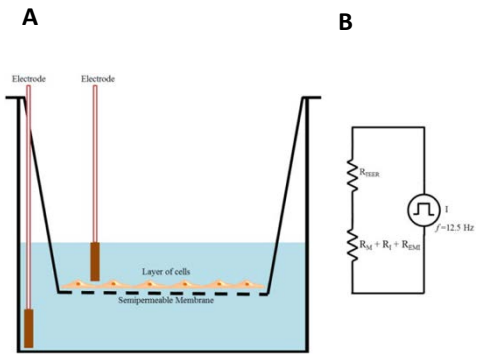

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Figure 3. (A) Diagram of the *in vitro* transwell model used to measure trans epithelial resistance (TEER) of calu-3 cells using a EVOM^2 and STX2 electrode. The companion electrodes (Ag/AgCl pellet voltage sensors) are composed of an inner and outer layer. They were placed inside the transwell with the electrode on the top of the transwell and the outer electrode in the external medium. A DC voltage of 50-80 millivolts (mV) was administered through the cellular monolayer and a resistance was given (Ω). (B) Resistance is calculated by Ohms Law. Ohms Law: V= IR where V (Volts) is the potential difference between two points, I is the current (Amperes) used, and R (Ohms) is the resistance of the system. Each individual resistance is summed (R_{apical} + $R_{basolateral}$ = R_{total}) to calculate the overall resistance of a series circuit.

Hemoglobin, Heme, and iron preparation. Hemoglobin (Sigma Aldrich) (20 µM), heme (Sigma Aldrich), and iron (Fisher scientific) were prepared by measuring the desired quantity with respect to the volume of medium required for bacterial infection. The hemoglobin solution was prepared fresh by measuring 5.8 mg of solid hemoglobin and homogenized with 4.5 mL of cell culture medium in a falcon tube to reach a final concentration of 20 µM. The solution was filtered using a syringe and filter (0.45 µm pore size). Heme was also prepared fresh prior each experiment using

Bovine Hemin in 100% dimethyl sulfoxide (DMSO). Briefly, 10 mg of hemin was dissolved in 2 mL of 100% DMSO to create a stock solution. This solution was diluted in a 1:1000 ratio (2 µl stock in 1998 µl DMSO) and sterilized using a filter (0.45 µm pore size). The absorbance of this dilution was measured at 404 nm wavelength by spectrophotometer using DMSO as the blank. Thereafter, the absorbance was using to determine the final heme concentration (10 µM) in the working solution by dividing the absorbance with the heme extinction coefficient (18800 $M^{-1}cm^{-1}$) and multiplying by the dilution factor. The iron ($FeNO_3$) (Fisher scientific) stock was prepared once and stored in 4°C to use within one month. For instance, a stock of 0.05 M $FeNO_3$ was prepared by dissolving 202 mg in 1 mL in double-distilled water (ddH_2O), and filter sterilized. Before each experiment, the iron stock solution was diluted in a 1:5 ratio in sterile ddH_2O, which was used to generate a 40 µM solution in cell culture medium.

Bacterial Infection with or without Hemoglobin. The *in vitro* transwell model was developed to measure the TEER, bacterial colonization and invasion to test the effects of pneumococcal invasion with and without hemoglobin. For each trial, 8 transwells containing confluent calu-3 cell culture were inserted into a 24 transwell plate. Among them, two wells (without hemoglobin) were used as negative controls; another two wells served as positive controls (with Triton X-100 treatment). The remaining four wells were used to test different bacterial strains (D39, D39Δ*ply*, or D39Δ*com*C) in presence or absence of hemoglobin (20 µM), heme (10 µM), or iron (40 µM). At the end of selected culture time (6, 8 or 24 h), TEER was measured as described above (Fig. 3). Subsequently, the transwells were washed with PBS and sonicated to detach the monolayer of cells with adherent bacteria. Thereafter, a serial dilution method was applied to obtain the colony counts in each sample. For example, the entire mixture from the apical and basolateral side of each

transwell were harvested and serial diluted in PBS, respectively. Then, 20 µl of the selected dilutions were plated onto blood agar plates and incubated at 37°C with 5% CO_2 overnight to obtain the number of colonies per milliliter (cfu/mL).

Statistical Analysis. Each experiment was conducted three times and the average is reported. Two tailed t-tests were performed to determine if the conditions are statistically significant ($p<0.05$) from each other. The initial TEER (pre-pneumococcal infection) was measured for each transwell in every experiment to ensure equal polarization (see supplementary materials), serving as an internal control. The limit of detection for colonized and invasive Spn was 50 cfu/mL.

RESULTS

Human lung cell *in vitro* Model stability. The stability of and integrity of the human lung cell *in vitro* model (7 days cell culture in transwell system) were tested to ensure the reliability and success of further experimentation. The untreated cells (negative controls) and Triton treated cells (positive controls) were incubated for 8 hours in hemoglobin medium without bacterial infection. The TEER was measured for each group at the end of incubation period. No TEER change was observed after cells were incubated 8 hours in hemoglobin (20 µM) medium (Fig. 4A). Triton, as a positive control, decreased the TEER significantly ($p< 0.05$) (Fig. 4AB). Furthermore, TEER (600-800 Ω/cm^2) was obtained from healthy lung cell over a 25-day period (Fig. 4C) and was comparable to the TEER (600-800 Ω/cm^2) in our model (Fig. 4A). As a result, this test ensured that Calu-3 cells (cancer cells) serve as appropriate surrogates for healthy cells and our model can be applied in this study.

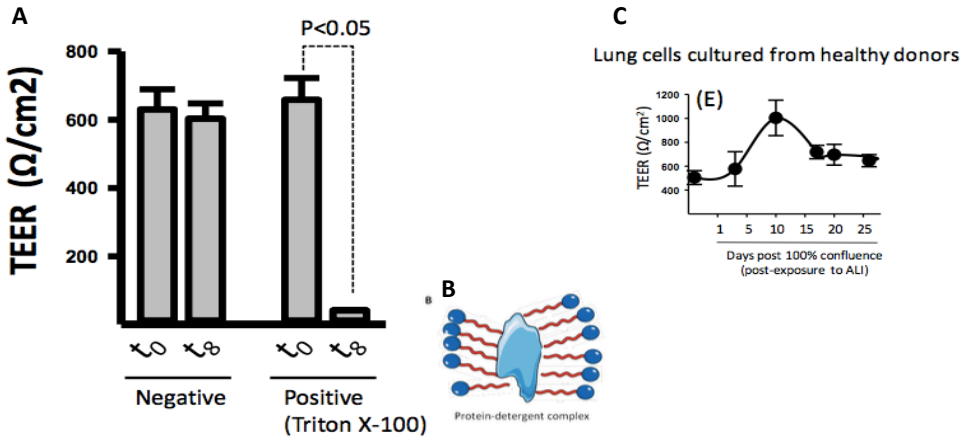

Figure 4. Polarization of Calu-3 cells and healthy lung cells are comparable. (A) The initial TEER was obtained from cultures of Calu-3 cells supplemented with 20 µM of hemoglobin (Hb+) at the beginning of the experiment (t 0h) and incubated for 8 h after which TEER (Final TEER) was again obtained. Cells were left untreated (negative control) or treated with 1% of Triton X-100 (positive control). (B) Triton X-1000 forms a protein-detergent complex with lipids and proteins in the monolayer of cells. (C) TEER measurements of lung cells of healthy donors over 25 days.

Bacterial density is not the key for invasion. To test if higher bacterial concentrations increases the chance that Spn passes through the cellular monolayer. The experiment was conducted with $2x10^6$ and $1x10^7$ cfu/mL Spn strain D39. Increased Spn colonization was observed with the higher concentration of bacteria (Fig. 5B). However, neither TEER decrease nor the presence of invasive pneumococci was observed after 6 hours of incubation with different bacterial inoculation (Fig. 5A and 5B).

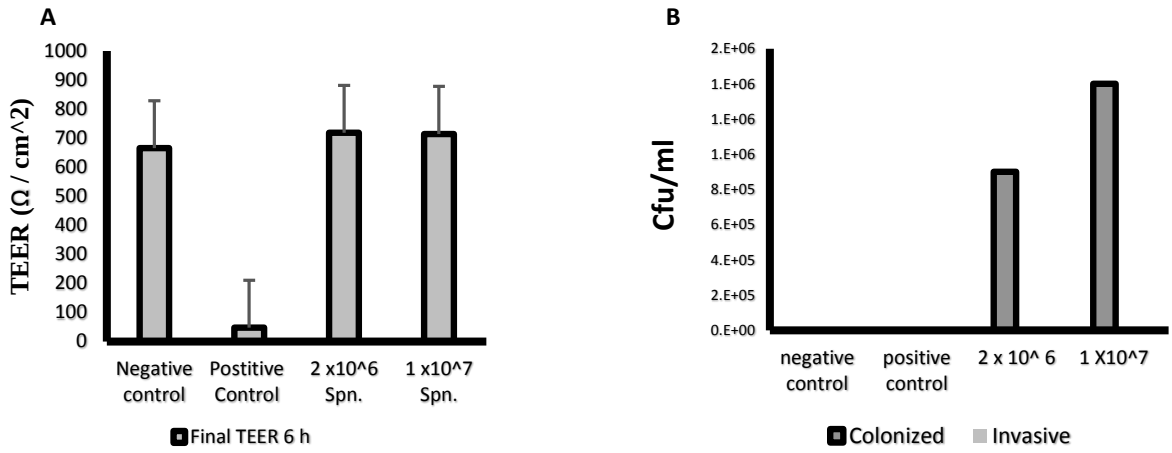

Figure 5. Spn Strain D39 Colonization is Dosage Dependent. 6 hours TEER (Ω/cm^2) (Mean+SD) measurement (A) and bacterial cell load (colonized/invasive, cfu/mL) (Mean+SD) (B) in D39 ($2x10^6$ or $1x10^7$ cfu/mL) infected transwells containing human lung Calu-3 cells. 1% of Triton X-100 treatment was applied at the end of incubation for positive control and untreated cells served as a negative control.

Hemoglobin triggered Spn bacterial virulence. To test if hemoglobin induces pneumococcal virulence at different culture times, I conducted both TEER and colonized/invasive bacterial counting experiments at 8 (Fig. 6) and 24 (Fig. 7) hours. First, in the absence of hemoglobin, TEER decrease was not observed at 8 and 24 hours (Fig. 6A and 7A). Invasive Spn was not seen at either time point (Fig. 6B, 7B, 8B), while its colonization was observed in both time points. However, in the presence of hemoglobin, at 8 hours, TEER did not significantly decrease (Fig. 6A and 8A). After 24 hours, TEER reduced significantly, ($p< 0.05$) (Fig. 7A and 8A). Interestingly, both pneumococcal colonization and invasion were seen at 8 and 24 hours (Fig. 6B and 7B). Increased Spn invasion was observed over time at 0h, 8h, and 24h (Fig. 8B).

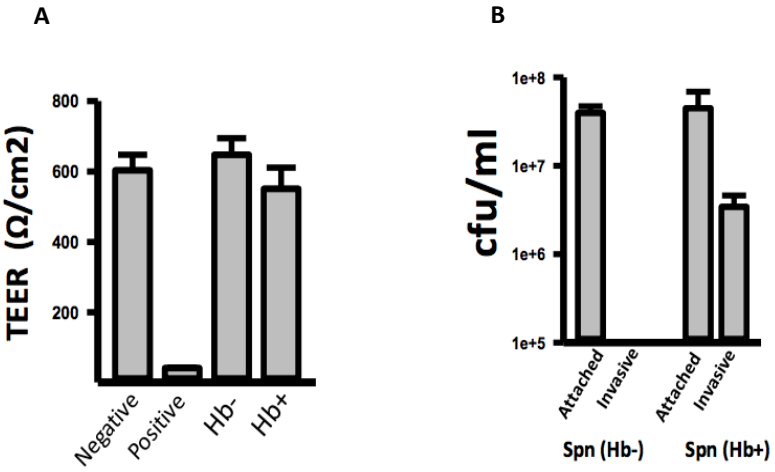

Figure 6. TEER and cellular density in the presence and absence of hemoglobin. 8 hours TEER (Ω/cm^2) (Mean+SD) measurement (A) and bacterial cell load (colonized/invasive, cfu/mL) (Mean+SD) (B) in D39 (OD_{600}=0.1) infected transwells containing human lung Calu-3 cells with or without hemoglobin (20 µM). 1% of Triton X-100 treatment was applied at the end of incubation for positive control and untreated cells served as a negative control.

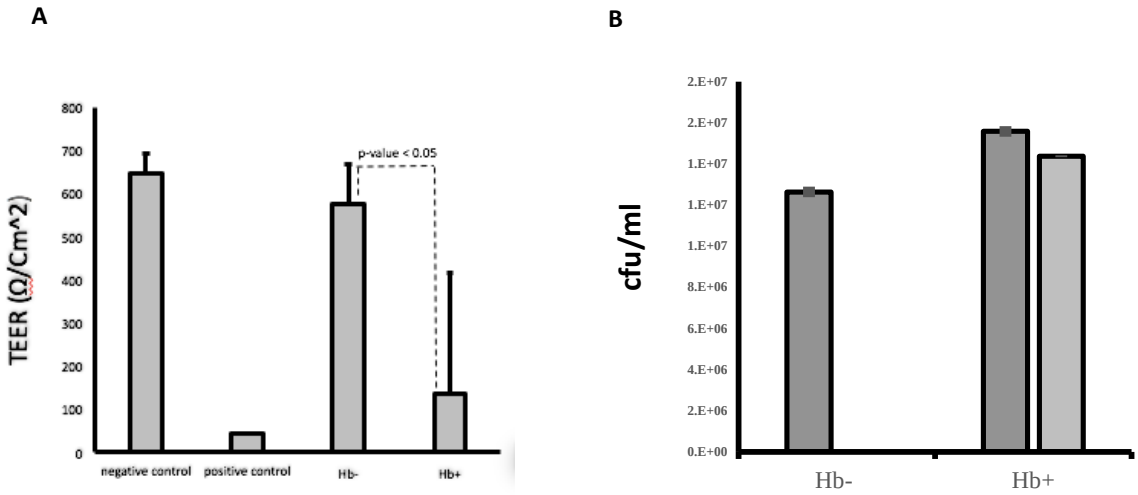

Figure 7. Hemoglobin Induces TEER Reduction and Pneumococcal Invasion. 24 hours TEER (Ω/cm^2) (Mean+SD) measurement (A) and bacterial cell load (colonized/invasive, cfu/mL) (Mean+SD) (B) in D39 (OD_{600}=0.1) infected transwells containing human lung Calu-3 cells with or without hemoglobin (20 µM). 1% of Triton X-100 treatment was applied at the end of incubation for positive control and untreated cells served as a negative control.

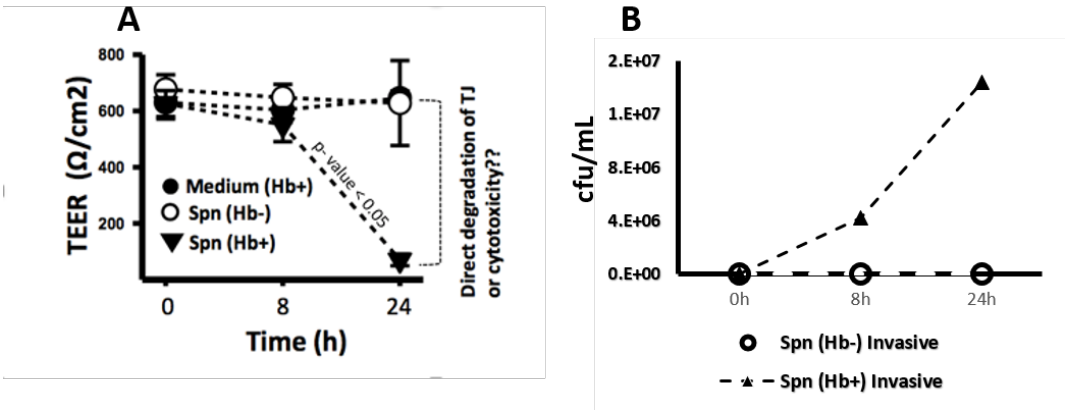

Figure 8. TEER and Spn invasion at 8 hours and 24 hours. Time course (0, 8, and 24 hours) measurement of TEER (Ω/cm^2) (A) and bacterial cell load (colonized/invasive, cfu/mL) (B) in D39 (OD_{600}=0.1) infected transwells containing human lung Calu-3 cells with or without hemoglobin.

Pneumolysin and QS system are involved in cell invasion. To test if pneumolysin or the COM system is involved in Spn cell virulence, we used two D39 mutants (D39Δ*ply* and D39Δ*comC*) in the experiment. The TEER decreased at 24 hours across the three Spn strains of D39 (Fig. 9A). Pneumococcal colonization occurred in all D39 Wild Type, Δ*ply*, Δ*comC* strains with no difference (Fig. 9B). However, prominent pneumococcal invasion was only seen in D39 WT (Fig. 9C). Spn invasion for Δ*ply* and Δ*comC* was significantly lower (p-value < 0.05) in comparison with the density of invasive bacteria of D39.

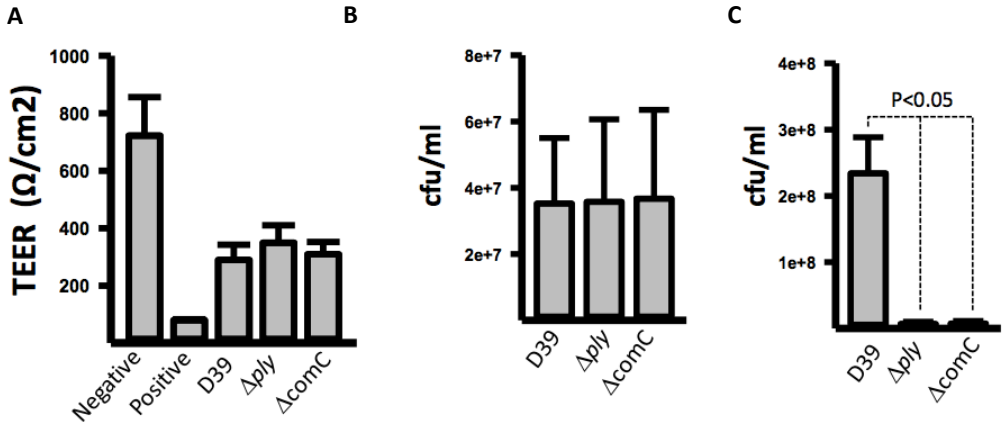

Figure 9. Δ*ply* and Δ*comC* decreases pneumococcal invasion. 24 hours TEER (Ω/cm^2) (Mean+SD) measurement (A), colonized (B) and invasive (C) bacterial cell load (cfu/mL) (Mean+SD) in D39, D39Δ*ply*, or D39Δ*com*C (OD_{600}=0.1) infected transwells containing human lung Calu-3 cells with hemoglobin (20 µM). 1% of Triton X-100 treatment was applied at the end of incubation for positive control and untreated cells served as a negative control.

The entire hemoglobin is required for TEER reduction. To further understand which part of hemoglobin is essential for Spn virulence, TEER and colonized and invasive bacterial counts (cfu/mL) were measured with holo form of hemoglobin, heme, or iron ($FeNO_3$) (Fig. 10). TEER decreased significantly ($p< 0.05$) in the hemoglobin group as expected. However, heme and iron had no effect on TEER (Fig. 10A). Further, invasive Spn cells were obtained in both hemoglobin and heme group in a similar density, while no invasive Spn cells presented in iron group (Fig. 10B).

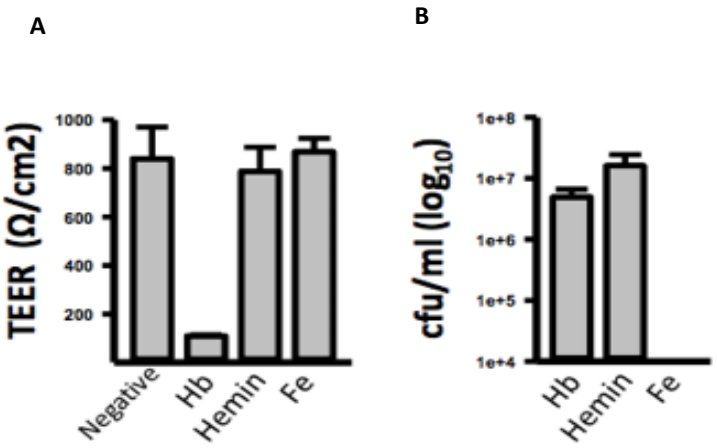

Figure 10. Hemoglobin is Required for TEER Reduction. 24 hours TEER (Ω/cm^2) (Mean+SD) measurement (A), colonized (B) and invasive (C) bacterial cell load (cfu/mL) (Mean+SD) in D39 (OD600=0.1) infected transwells containing human lung Calu-3 cells with hemoglobin (20 μM), Heme (10 μM), and iron (40 μM). Untreated cells served as a negative control.

DISCUSSION

In this study, we developed a fundamental understanding of pneumococcal translocation from the mucosal surface of the lung epithelium into the bloodstream. I first demonstrated that the presence of hemoglobin enhances pneumococcal invasion. Interestingly, such invasion and TEER decrease were regulated via different mechanisms, respectively. Moreover, the pneumolysin protein and the competence stimulating peptide did not play a role in TEER reduction but were involved in pneumococcal invasion.

The most important finding in my thesis is that hemoglobin triggers pneumococcal invasion and disruption of the TJ belt. Hemoglobin is necessary because it serves as the largest reservoir for iron in the human body (10). Free iron is not easily available throughout the human body; thus, pathogens must digest the hemoglobin molecule to acquire iron (10). Currently, the exact mechanism of hemoglobin metabolism in Spn invasion is unknown. However, Spn are

Gram-positive bacteria, therefore they may metabolize hemoglobin similarly to a mechanism discovered in other Gram-positive bacteria such as *Staphylococcus aureus*. Once hemoglobin is released from the red blood cell, free hemoglobin may bind to a G protein coupled receptor on the surface of the cell wall with the assistance of binding proteins that have strong binding affinities for hemoglobin. Previous literature states that Spn expresses two membrane proteins that binds hemoglobin and heme, respectively (14). Then the heme-iron complex is released from hemoglobin and travels from one receptor to another receptor using a transporter in the periplasm of the cell. Subsequently, this transporter transfers the heme-iron complex into the cytoplasm where heme oxygenases are present (10). These enzymes separate iron from the heme complex, and once iron enters the cytoplasm, virulence is triggered. Understanding hemoglobin metabolism in other Gram-positive bacteria provides further guidance regarding the specific mechanism that may occur during pneumococcal pathogenesis. Iron enhances micro organismal growth and serves as a chelating agent against radical oxidative species produced by the host to eliminate the bacteria (14). Moreover, hemoglobin metabolism also releases amino acids and other essential nutrients used to promote pneumococcal viability (10). These studies denote the importance of hemoglobin for Gram-positive bacteria. Thus, pneumococci may also behave in a similar manner to acquire necessary factors from hemoglobin to achieve Spn invasion.

We hypothesized in the beginning, that pneumococcal invasion occurs through the tight junction belt. Therefore, implying when Spn invades the epithelial cell affects the tight junctions, compromising the cellular monolayer (TEER decrease). Our findings depict that Spn invasion first occurred at 8 hours, but TEER decrease was only induced at a later culture time (24 hours). This observation suggests that Spn enters the cell without disruption of the monolayer or tight junction belt. There are numerous mechanisms which molecules and pathogens use to enter a cell. Small

non-polar molecules passively diffuse through the membrane. Pneumococci may enter the cell by capitalizing upon natural membrane transport mechanisms (23). Some bacteria secrete a protein which allows them to reside in a vacuole, enter the cell via phagocytosis, and once the pathogen enters the cell it lysis the vacuole (23). Many invasive bacteria have shown to use the clathrin endocytosis process where the pathogen binds to a receptor on the plasma membrane and then triggers a signal leading to internalization (23). Specifically, glycosaminoglycans have shown to be involved in bacterial adherence to lung cells (12). According to those previous findings, Spn may be using multiple ways to achieve invasion without disrupting the cell monolayer at an early culture time. Further studies are needed to address this specific mechanism.

Even at 24 hours, the decrease of TEER only occurs with hemoglobin supplementation. Hemoglobin provides the pathogen with the nutrients to survive and to replicate as time progressed within the *in vitro* model. Our results indicate that TEER is slowly decreasing, that may result from pneumococci degrading the cellular monolayer overtime. The TEER decrease may be related to tight junction protein function failure, for example, claudin, which plays a prominent role in maintaining alveolar epithelial cell function (9). The first extracellular loop of claudin forms an electrostatic selectivity filter and the second loop of this protein is responsible for cellular adhesion (2). Specifically, a previous study illustrated the downregulation of claudin 7 and claudin 10 *in vivo* mice models when infected with Spn (4). Claudin 7 can cause a decrease in TEER by increasing cation and anion permeability (2). Hemoglobin metabolism releases copious amounts of iron (cation) therefore potentially increasing the permeability of the electrostatic filter, leading to a downregulation of claudin proteins and ultimately a decrease in TEER. Furthermore, unlike in human body, where blood cells are abundant, in our *in vitro* model, when human lung cells cultured with bacteria without hemoglobin, we did not observe any TEER decrease. This may

result from a lack of necessary resources from hemoglobin or blood cells for Spn to degrade the cellular monolayer or to defect tight junction proteins.

To explore the virulence factors of Spn that may involve pneumococcal invasion and TEER decrease, pneumolysin and competence stimulating protein were evaluated in this study using two respective mutants. The pneumolysin protein encodes for a potent toxin, pneumolysin (Ply), which promotes hemolysis thereby releasing hemoglobin from red blood cells and triggering an upregulation of cytokine expression (3). After pneumolysin releases hemoglobin, the protein is processed, iron is released, and pathogenesis occurs (18). Also, the presence of hemoglobin and the Ply gene plays a role in biofilm formation which helps to mediate pneumococcal colonization (1) (15). Once pneumococci are colonized, they can subsequently invade into the basolateral surface. Another Spn biofilm regulator is the COM quorum sensing (two-component regulatory system) which controls early Spn biofilm formation on human respiratory epithelial cells in a different model (19). In Spn biofilm, competent cells intend to kill non-competent bacteria (fratricide), then release pneumolysin (5). The COM system influences the genetic competence affecting pneumococcal virulence in the nasopharynx (8). Further studies test the effects of the mutation (Δ*comC*) of this gene on biofilm formation and density. These studies indicate the necessity of the *comC* regulatory system in producing early biofilms on human cells (19). The competence stimulating peptide (CSP) is encoded by *comC* and promotes infection through a peptide pheromone signaling pathway, leading to the expression of genes involved in a positive feedback loop (8). A previous study showed that the inability to secrete CSP produced a dosage dependent reduction in bacterial colonization (8). Also, in another model, the D39Δ*comC* mutant showed decreased biofilm formation in the bioreactor (19). However, prominent colonization by D39Δ*comC* was observed in our experiment. The different observations may be attributed to the

fact that our experiment was conducted in a closed system rather using the continuous-flow system of the bioreactor.

In this current study, it is evident that neither the pneumolysin protein nor the competence stimulating peptide (CSP) are responsible for disrupting the cellular monolayer (TEER) in early culture time (8 hours). Nevertheless, we found that Ply and CSP may play a role in pneumococcal invasion because a scarcity of invasive Spn was observed in the presence of prominent TEER diminution. Previous studies suggest that pneumolysin-deficient mutants trigger lower levels of virulence as observed *in vivo* (13). Further, when Ply was administered directly to lung cells pneumonia like symptoms were observed (13). Therefore, these studies support our finding which illustrated decreased invasive Spn in the absence of Ply. Based on previous studies of the function pneumolysin and competent stimulating protein, we demonstrated Ply and *comC* are involved in Spn invasion by using hemoglobin as a mediating factor.

TEER reduction occurred in the presence of the holo form of hemoglobin but TEER did not change in the presence of either the heme or the iron molecule. Thus, the holo form of hemoglobin may be crucial to triggering virulent activity. Hemoglobin contains four heme groups, iron, and a protein subunit. Heme contains one heme group and lacks the protein complex. A previous study which mainly analyzed the effects of hemoglobin in etiologic agents like Spn showed releases essential biomolecules (i.e., amino acids) required for pneumococcal metabolism (10). The whole hemoglobin molecule has an abundance and variety of amino acids bound together while the heme group contains fewer and mainly is composed of histidine. Hence, metabolism of hemoglobin or heme may be a necessary process for Spn to release toxin. Additionally, bacterial invasion was observed with the supplementation of hemoglobin molecule and heme but not with

iron (20 μM). Iron is an element and cannot be metabolized further, therefore the absence of pneumococcal invasion can be attributed to the deficiency of essential nutrients in iron (10).

Overall from this research study, the role of hemoglobin in triggering pneumococcal pathogenesis was exemplified through: early pneumococcal invasion without monolayer disruption (TEER reduction). Analyzing the significance of hemoglobin during Spn virulence addresses current knowledge gaps in understanding the mechanism of pneumococcal disease. Consequently, these findings can lead to long term implications in global health in the future.

FUTURE DIRECTIONS

This study fundamentally describes a mechanism of pneumococcal invasion. However, this data has great potential to further discern the definitive action of Spn translocation. The next steps include conducting a time trial experiment to determine the exact time point during the incubation period in which pneumococcal invasion and TEER decrease occurs which illustrates the moment of bacterial virulence. Furthermore, the RNA of the cellular monolayer has been extracted. Conducting gene expression studies from these experiments will help to determine the mechanism of TEER decrease. By understanding the upregulation and downregulation of specific mRNA genes will help to determine whether TEER decrease is due to the compromise of tight junction proteins or cytotoxicity. Moreover, additional auxiliary experiments can be conducted to discern whether pneumococcal invasion is regulated by internal signaling or hemoglobin metabolism. An experiment to test whether Spn secretes a substance to promote hemoglobin metabolism can help to answer this question. Furthermore, an experiment to test whether the pneumolysin protein (ply gene) is responsible for hemolysis of red blood cells can be conducted *in vitro* using this model. Also, we can test the role of hemoglobin variants seen in hematologic diseases such as anemia and

sickle cell disease to determine if these conditions influence IPD. Also, it is important we develop a more specific positive control to test the integrity of the cellular monolayer. For example, using a compound which specifically targets the ZO protein in the tight junction complex can provide certainty that TEER decrease results from TJ insult.

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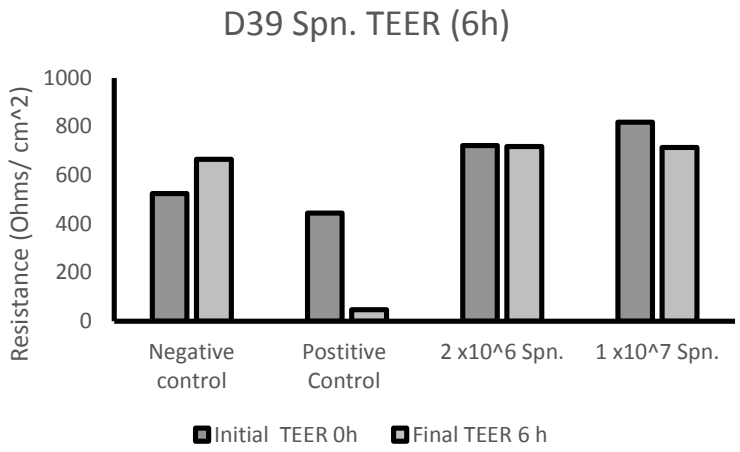
Appendix

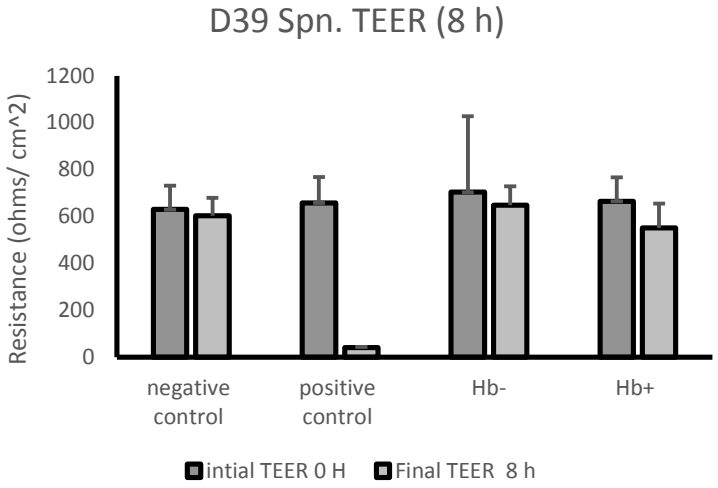
The author of this thesis provided an appendix to include further supporting data obtained for her thesis work.

Appendix author: Shambavi J. Rao

Appendix Results

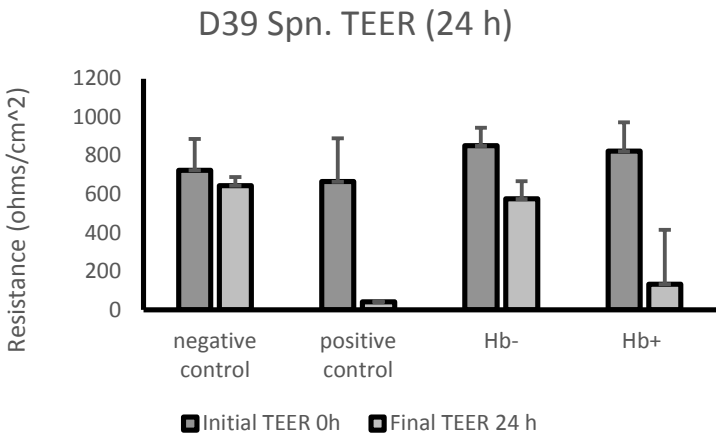


Supplementary Figure 1. Transepithelial resistance (TEER) of human lung Calu-3 cells is unaffected upon disruption of the monolayer. TEER was obtained from cultures of Calu-3 cells at the beginning of experiments (t_0, dark grey bars). Cells were left untreated (negative control) or treated with 1% of Triton X-100 (positive control) and incubated 6 h (t_1, light grey bars) after which TEER was obtained. Cells were also infected with either 2 $x10^6$ D39 WT Spn or 1 x 10^7 D39 WT Spn.



Supplementary Figure 2. Transepithelial resistance (TEER) of human lung Calu-3 cells is decreases upon disruption of the monolayer. TEER was obtained from cultures of Calu-3 cells at the beginning of experiments (t_0,

dark grey bars). Cells were left untreated (negative control) or treated with 1% of Triton X-100 (positive control) and incubated 8 h (t_1, light grey bars) after which TEER was obtained. Cells were also infected with D39 WT Spn with or without the supplementation of hemoglobin.



Supplementary Figure 3. Transepithelial resistance (TEER) of human lung Calu-3 cells is decreases upon disruption of the monolayer. TEER was obtained from cultures of Calu-3 cells at the beginning of experiments (t_0, dark grey bars). Cells were left untreated (negative control) or treated with 1% of Triton X-100 (positive control) and incubated 24 h (t_1, light grey bars) after which TEER was again obtained. Cells were also infected with D39 WT Spn with or without hemoglobin supplementation.