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Determining the molecular mechanisms to activate cathelicidin for increased resistance against *Streptococcus pneumoniae*

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2020

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

Determining the molecular mechanisms to activate cathelicidin for increased resistance against *Streptococcus pneumoniae*

By Anika Sinha

Understanding the interactions between pathogenic organisms and the host immune system is imperative to developing innovative treatment methods. In the case of *Streptococcus pneumoniae* (SPN), identification of naturally-occurring host antibiotics that can be upregulated is of the utmost importance. This is due to its increasing resistance to antibiotics and the continued rank as the leading cause of childhood morbidity and mortality worldwide. Previous research suggests that cathelicidin, which is a host-produced cationic antimicrobial peptide (CAMP), may be a factor that can be induced to prevent and combat infection, as SPN remains susceptible to attack by such factors. The production of cathelicidin is transcriptionally regulated by the Vitamin D (VD) receptor, so it can be induced by VD or 4-phenylbutyrate (PBA), which acts in conjunction with this receptor. I hypothesized that macrophages treated with PBA and VD would be able to increase killing of SPN. To investigate these interactions, differentiated human *THP-1* macrophages were treated with varying concentrations of PBA, VD, or both. These were then incubated with either TIGR4 or D39 strains of SPN and bacterial killing was indicated by detecting a decrease in colony-forming units (CFU) in the treatment group compared to the negative control. There was a decrease in CFU of TIGR4 when the macrophages were treated with PBA and VD. However, this reduction was not significant. There was no significant decrease in CFU of D39 when the macrophages were treated with PBA and VD. This provides limited support of the PBA and VD induction hypothesis. Future experiments will test increased doses of PBA and VD, as well as increased incubation times with treatment before infection, in order to investigate the synergistic hypothesis further. The effects of treatment on cathelicidin mRNA and protein concentrations will also be measured in order to elucidate these mechanisms.

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## Introduction

Understanding pathogen-host interactions is imperative for developing treatments to enhance host resistance to infection. *Streptococcus pneumoniae* (SPN) is one of the most prominent Gram-positive opportunistic pathogens. This harmful bacterium causes infections such as pneumonia, meningitis, otitis media, acute sinusitis, and sepsis. The most common way to treat bacterial infection has been through the use of antibiotics. Researchers identified antibiotic-resistant SPN soon after the introduction of the first antibiotic, penicillin, as a treatment to the general population (Tomasz, 1997). Rates of resistance to other traditionally-used antibiotics, such as cephalosporin, carbapenem, erythromycin, azithromycin, clarithromycin, and clindamycin, have rapidly evolved since, due to efficient uptake and integration of DNA encoding antibiotic resistance factors from other bacterial pathogens (Weiser et al., 2018). This has greatly incentivized researchers to examine preventative strategies for infection and transmission (Weiser et al., 2018). Currently, there are different serotype-specific vaccines available for prevention of SPN infection, but these are not always effective as they may not protect against all serotypes (Wang et al., 2019). Researchers are developing protein based vaccines, but these are not available to the public yet (Pichichero et al., 2016). This is why it is crucial to investigate other methods of treatment and prevention of SPN infections.

An alternative method of approaching the problem of SPN infection is through the use of naturally-occurring factors of the innate immune system that have prophylactic potential. One such factor is cathelicidin, a cationic antimicrobial peptide (CAMP) that is produced by circulating immune cells, such as macrophages and neutrophils, as well as epithelial cells resident in the surfaces that SPN infects (LaRock and Nizet, 2015). The process of killing SPN by CAMPs is depicted below (Figure 1). Normally, cathelicidin is activated when extracellular proteases, such

as neutrophil proteinase 3, cleave a prodomain of the protein, which produces the active peptide LL-37 (Sørensen et al., 2001). However, there are many other cells that synthesize cathelicidin and there may be a diversity of other proteases that can activate it (Murakami et al., 2004; Sorensen et al., 2003; Yamasaki et al., 2007).

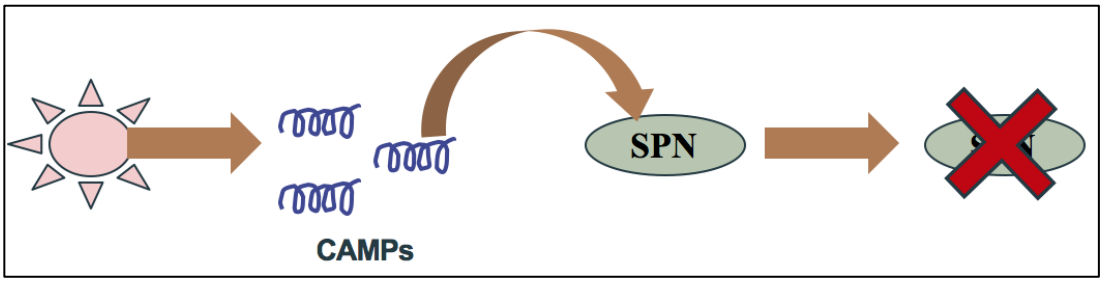


**Figure 1: Process of killing SPN by CAMPs.** A variety of host cells, such as macrophages, epithelial cells and polymorphonuclear leukocytes, can produce CAMPs. CAMPs are generally attracted to the negative membranes of SPN and can cause membrane disruption, which ultimately results in bacterial cell death.

Other bacterial pathogens utilize several strategies to evade killing by CAMPs, such as increasing the net charge of their cell surface in order to mimic host cells, reducing CAMP binding to the bacterial surface, synthesizing surface proteins that inhibit immune function, the possible use of multidrug efflux pumps, direct destruction of CAMPs, and utilizing environmental response regulators that react to CAMP by increasing virulence activity; SPN may utilize none or some of these methods (LaRock and Nizet, 2015). Although SPN has developed a great level of resistance towards conventional antibiotics, previous studies have suggested that SPN remains susceptible to physiological levels of CAMPs, which may vary depending on the site and type of CAMP (LaRock and Nizet, 2015). The bacteria may not be able to develop a strong enough resistance to them (LaRock and Nizet, 2015). Thus, inducing and increasing expression of CAMPs, such as cathelicidin, could be protective against SPN infection.

In humans, cathelicidin is transcriptionally-regulated directly via the Vitamin D (VD) receptor (Gombart et al., 2005; Wang et al., 2004). Epidemiological evidence suggests that low VD levels correlate with increased susceptibility to infections such as community-acquired

pneumonia (Jovanovich et al., 2014; Kim et al., 2015). Additionally, it has been found that 4-phenylbutyrate (PBA) also induces cathelicidin expression, and the VD receptor serves as a crucial transcription factor required for this up-regulation (Steinmann et al., 2009; Kulkarni et al., 2014). A model of this regulation is shown below (Figure 2). Clinical trials treating *Mycobacterium tuberculosis*-infected individuals with VD and PBA showed that there is a substantial increase in activated cathelicidin concentration in the treatment groups as compared to the placebo groups, showing therapeutic benefit (Mily et al., 2015). However, randomized control trials have shown conflicting results in the protective effects of VD alone (Bergman et al., 2013). Hence, hyper-activation effects of VD and PBA may be the key to designing a therapeutic strategy to combat SPN infection.

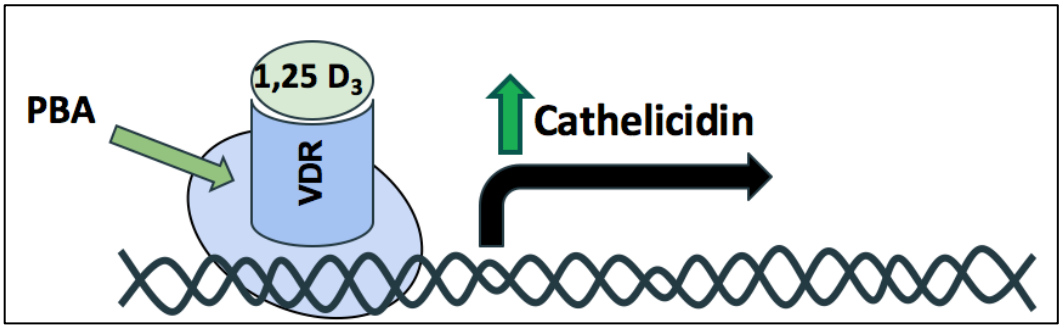


**Figure 2: Transcription of the gene that encodes cathelicidin can be induced with VD and PBA.** The VD receptor (VDR) is a crucial transcription factor for this upregulation.

This research project focused on evaluating whether synergistic treatment with PBA and VD at varying concentrations could activate or upregulate cathelicidin in macrophages in order to increase resistance against SPN and kill SPN. We propose a mechanism through which SPN synthesizes one or more extracellular proteases that cleaves cathelicidin at a position that produces an active form of the antimicrobial peptide. We hypothesize that the limiting step in the generation of mature cathelicidin is often proteolysis, and that VD and PBA induced hyper-activation of cathelicidin can increase resistance against SPN because this pathogen encodes a protease that activates cathelicidin. A model of the mechanism tested in this study is shown below (Figure 3).

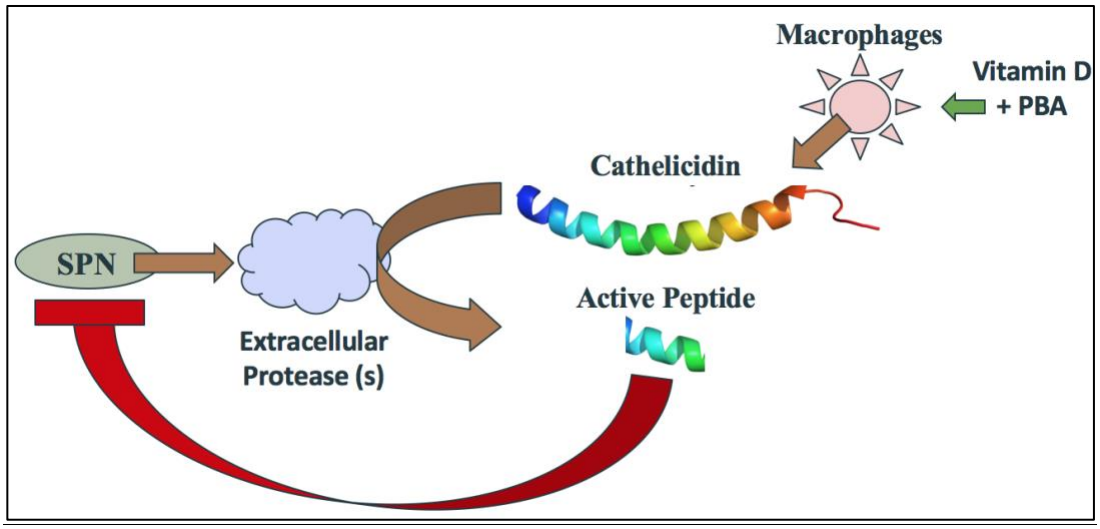


**Figure 3: Proposed mechanism of activation of cathelicidin to increase resistance against SPN.** We hypothesize that SPN secretes a protease that cleaves cathelicidin, which can be produced by a variety of host cells, in a way that generates an active form of the peptide. The host cell used in this study were *THP-1* macrophages. Since proteolysis of cathelicidin is proposed to be a limiting factor in the production of the active peptide, it was hypothesized that hyper-activation of cathelicidin production via treatment with PBA and VD would help overcome this limitation and increase killing of SPN.

This model implies that both VD and PBA potentially could have beneficial impacts on the host innate immune system's ability to fight infection. Individuals of African descent have lower levels of VD and lower cathelicidin expression (Liu et al., 2006), as well as a higher rate of pneumococcal infection (Wortham et al., 2014). Therefore, since low VD is considered to be a risk factor, VD supplementation and PBA treatments may be protective for such at-risk populations. Comprehending the specific molecular mechanisms that impact these effects will further aid in developing feasible preventative treatments to combat SPN infection.

## Methods

*Stocks of SPN and Concentration Calculation*

First, two widely-used laboratory wildtype SPN strains were grown on sheep blood agar overnight in a 37°C 5% $CO_2$ incubator (Table 1). After incubation, inoculation loops were used to collect bacteria from the plates and the different bacteria were placed in 50 mLs of Thioglycolate broth (THY) media and allowed to grow in a 37°C and 5% (v/v) $CO_2$ incubator for five to six hours. The optical density at 600 nanometers was measured periodically. The optimum amount of genomic processes and protein synthesis occurs in the early to mid logarithmic phase. Therefore, once the optical density at 600 nanometers reached about 0.3, which indicated that the bacteria were in the early logarithmic growth phase, the bacteria were spun down at 1000 x g for 10 minutes, and then the THY media was aspirated.

| Bacteria | Description | Plasmid | Growth/Selection |
|---|---|---|---|
| *S. pneumoniae* | TIGR4 wildtype (virulent serotype 4) | pMV158GFP | TetR |
| *S. pneumoniae* | D39 wildtype (virulent serotype 2) | pMV158GFP | TetR |

**Table 1: Description of *S. pneumoniae* strains used in this study.**

Next, the bacteria were resuspended in 25 mLs of Dulbecco's phosphate-buffered saline (DPBS) solution, which was made to be 10% glycerol. After mixing thoroughly, 0.5 mL aliquots of each stock were made by flash freezing with dry ice and ethanol. Stocks of each strain were stored at -80°C.

To calculate the concentration of the stocks, a tenfold dilution series was performed in a 96 well plate for each individual stock. 5 µL of each dilution was then plated on sheep blood agar

plates. The SPN were then grown overnight in a 37°C and 5% $CO_2$ incubator. The number of colonies at the lowest dilutions was counted and average CFU/mL was calculated by multiplying the CFU by the dilution factor.

*Killing of SPN with induced THP-1 cells*

First, human *THP-1* monocytes were cultured at 37°C and 5% $CO_2$. The cells were centrifuged at 160 rcf for 5 minutes, and then the supernatant was aspirated. The cell pellet was resuspended in 1 mL of RPMI 1640, 10% heat-inactivated fetal bovine serum (hiFBS). The live cell concentration was measured by trypan blue exclusion using the Olympus Cell Counter Model R1. Based on the live cell concentration, dilutions were calculated in order to achieve the desired cell concentration of 2e5 cells/mL in RPMI +10% hiFBS. Phorbol 12-myristate 13-acetate (PMA; Sigma) was added to the THP-1 cells, in a 1:1000 ratio, in order to induce differentiation into macrophages. 100 μL of cells were plated into each sample well of a 96 well plate. The cells were incubated at 37°C in a 5% $CO_2$ environment for 24 hours.

After growth in PMA, it was confirmed that the macrophages were alive and adherent to the wells by visual inspection on an inverted light microscope. Macrophages were then treated with varying concentrations of PBA (Sigma) alone, VD (Sigma) alone, or both. Sample concentrations included 2 mM of PBA, 4 mM of PBA, and 100 nM of VD. For the negative control, some macrophages, that would be later incubated with SPN, received no treatment. Four technical replicates were performed. The cells were incubated with these treatments overnight at 37°C in a 5% $CO_2$ environment.

Next, the bacterial stocks made earlier were diluted with Dulbecco's phosphate-buffered saline (DPBS) in order to achieve the desired infection concentration of 2e5 CFU/mL. Separate

macrophages were each incubated with this concentration of either TIGR4 or D39. Using a multichannel pipette, 10 μL of bacteria was added to each of the sample wells to reach this infection concentration, excluding the samples that only contained the macrophages as a control for contamination. The macrophages were incubated with the SPN for 3 hours in a 5% $CO_2$ environment at 37°C.

After 3 hours of incubation, the macrophages were lysed in 0.05% Triton X-100 to release any intracellular bacteria. Next, a 10-fold dilution series was performed for each sample. These dilutions ranged 1e-1 to 1e-4. 5 μL of each sample at each dilution was plated on sheep blood agar plates. The SPN were then grown overnight in a 37°C and 5% $CO_2$ incubator. The number of colonies at the lowest dilution was counted and average CFU/mL, across four technical replicates, with standard deviation was calculated. Average CFU/mL for each treatment, across three biological replicates, with standard deviation was then calculated. Statistical comparisons between the different treatments for each wildtype strain of SPN were performed using a paired Student's t-test using the software package Prism 8 (Graphpad).

## Results

*Killing of SPN with induced THP-1 cells*

In this assay, THP-1 differentiated macrophages were induced via incubation with either 2 mM PBA, 4 mM PBA, 100 nM VD, 2 mM PBA with 100 nM VD, or 4 mM PBA with 100 nM VD for 24 hours. After this acclimation period, the macrophages were infected with either TIGR4 or D39 for 3 hours. The macrophages were then lysed with Triton X-100, dilutions were performed for each sample, and the sample dilutions were plated on sheep blood agar. Average growth, as measured in CFU/mL, of TIGR4 across 3 biological replicates is shown below (Figure 4). The

highest amount of average TIGR4 growth was regularly seen in the negative control, in which the macrophages were only incubated with SPN and received no cathelicidin-inducing agents (VD or PBA). However, this did not approach statistical significance by a paired Student's t-test (Figure 4). The lowest amount of average TIGR4 growth was seen in the 2 mM PBA + 100 nM VD treatment, but this was not statistically different from the other treatments (Figure 4). Generally, the amount of SPN recovered was lower in the samples treated with higher concentrations of PBA in addition with VD (Figure 4).

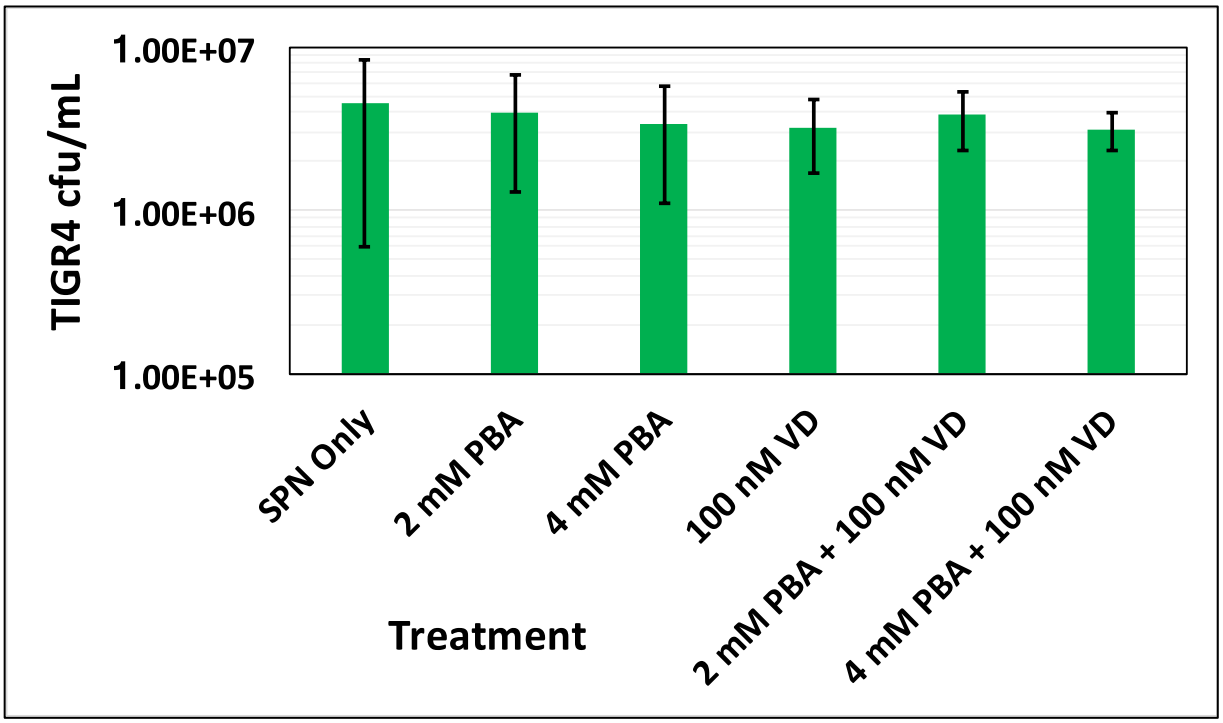


**Figure 4: Effect of PBA and VD on macrophage killing of SPN TIGR4.** *THP-1* differentiated macrophages were incubated with PBA only, VD only, or both for 24 hours. Next, the macrophages were infected with TIGR4 for 3 hours and then lysed with 0.05% Triton X. Dilutions were performed and each sample was plated on sheep blood agar. Average CFU/mL across 3 biological replicates was calculated. Standard deviations were calculated for treatments tested across the biological replicates, and statistical significance was determined through a paired Student's t test.

Average growth, as measured in CFU/mL, of D39 across 3 biological replicates is shown below (Figure 5). The highest amount of average D39 growth was seen in the 2 mM PBA + 100 nM VD treatment, but this was not statistically different from the other treatments (Figure 5). The lowest amount of average D39 growth was seen in the 100 nM VD treatment, but this was not

statistically different from the other treatments (Figure 5). SPN strain D39 was not more susceptible to macrophages treated with PBA or VD (Figure 5).

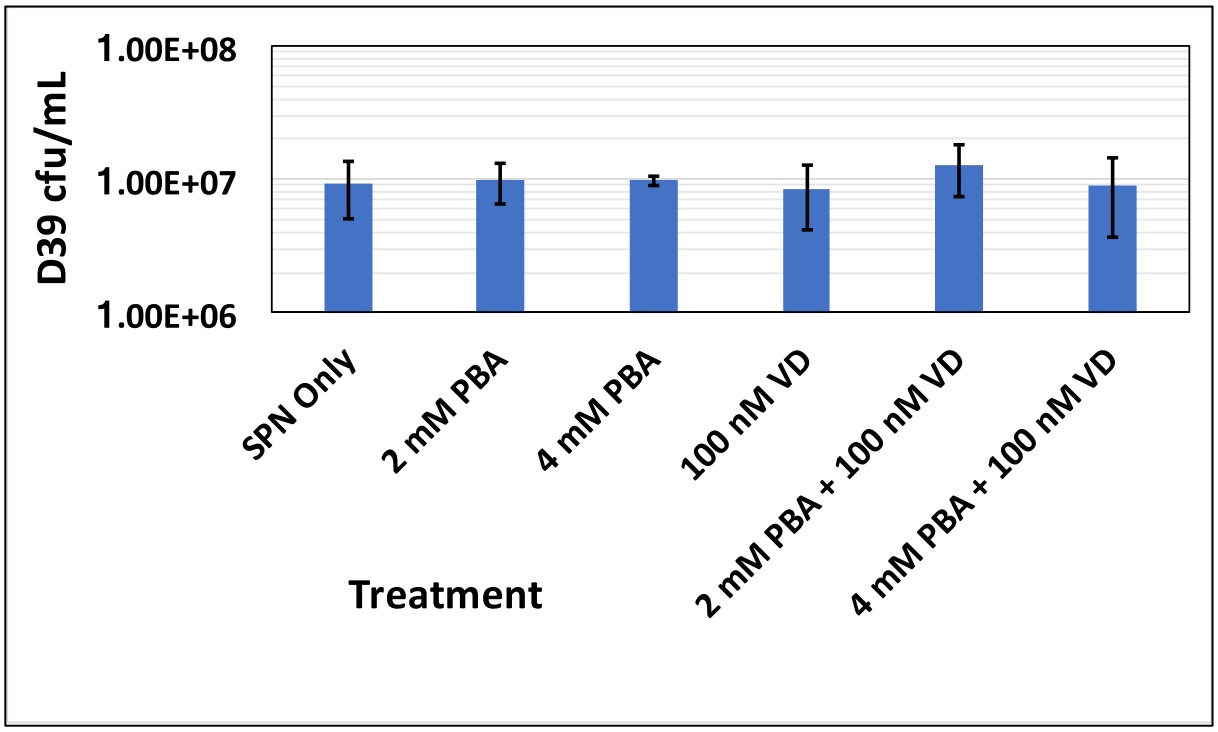


**Figure 5: Effect of PBA and VD on macrophage killing of SPN D39.** *THP-1* differentiated macrophages were incubated with PBA only, VD only, or both for 24 hours. Next, the macrophages were infected with D39 for 3 hours and then lysed with 0.05% Triton X. Dilutions were performed and each sample was plated on sheep blood agar. Average CFU/mL across 3 biological replicates was calculated. Standard deviations were calculated for treatments tested across the biological replicates, and statistical significance was determined through a paired Student's t test.

## Discussion

This research project examined whether PBA and VD induction of cathelicidin in macrophages could increase killing of SPN. In human bronchial epithelial cells, it has been shown that cathelicidin mRNA and inactive protein levels increased with the PBA and VD doses used in this current study (Kulkarni et al., 2014). In addition, epidemiological studies found that low VD levels correlate with increased susceptibility to SPN infection (Jovanovich et al., 2014; Kim et al., 2015). Therefore, since SPN remains susceptible to physiological levels of cathelicidin (LaRock and Nizet, 2015), it was hypothesized that cathelicidin induction via PBA and VD treatment of differentiated macrophages would increase killing of SPN.

Two different wildtype strains of SPN were tested. The first strain tested was TIGR4, the virulent serotype 4 strain that has been widely studied (Tettelin et al., 2001). There was a slight dose response of induction of the macrophages, as decreased growth of TIGR4 was seen in the 4 mM PBA treatment group compared to the 2 mM PBA group (Figure 4). There was slightly decreased growth of TIGR4 in the 4 mM PBA + 100 nM VD condition as compared to other treatment conditions, suggesting that there was limited synergy between these inducers of cathelicidin (Figure 4). However, statistical analysis from three biological replicates indicated that this decrease did not reach significance. The second strain tested was D39, the virulent serotype 2 strain that has also been widely studied (Lanie et al., 2006). There was no significant dose response of induction to kill D39 seen at the concentrations of PBA and Vitamin D tested in this study (Figure 5). Across three biological replicates, individually performed with four technical replicates, CFU/mL for each treatment was moderately consistent. This suggests evidence against the synergistic induction hypothesis.

One possibility for the slightly different trends between TIGR4 and D39 is that different serotypes and strain backgrounds of SPN may have different susceptibility to CAMPSs (Habets et al., 2012). However, the difference in reduction of growth of SPN was not significantly different between the two strains, but this idea is important to keep in mind for future research testing clinical isolates of SPN. Therefore, it is possible that VD and PBA treatment will be more effective against strains that are less resistant to CAMPs. It remains crucial to understand and characterize these mechanisms of action in well-studied strains such as TIGR4 and D39 so that scientists may better prepare to evaluate efficacy of synergistic treatment when it comes to clinical isolates.

The results found in this current study conflict with the evidence found previously, which overwhelmingly supports the synergistic induction hypothesis. This may have been due to

regulation that led to decreased cathelicidin concentrations at the post-translational stage of production in macrophages. It was previously shown that 2 mM or 4 mM PBA and 100 nM VD, which were the doses used in this current study, increased mRNA and inactive protein levels of cathelicidin in human bronchial epithelial cell lines (Kulkarni et al., 2014). To further investigate whether post-translational regulation is occurring in the *THP-1* macrophages used in this study, quantitative real-time PCR could be conducted in order to detect changes in cathelicidin mRNA levels in response to PBA and VD treatment. Western blot analysis could then be used to examine the levels of inactive cathelicidin protein. GAPDH mRNA and protein could be measured as a control. Comparing the levels of cathelicidin mRNA and protein will allow for the detection of post-translational processes that may have led to poor SPN killing.

Another explanation for these conflicting results could be that the doses used in this current study did in fact increase cathelicidin mRNA and inactive protein concentrations, but this increase was not sufficient enough to have a significant impact on cathelicidin activation and killing of SPN. For *Shigella flexneri*, 97-99% of killing was observed when the level of cathelicidin, with and without the presence of 40 mM PBA, was at 0.90 μM and 0.68 μM respectively (Raqib et al., 2006). In order to investigate this in the context of the current study, the concentration at which the active form of cathelicidin inhibits SPN growth could be determined by performing a minimum inhibitory concentration (MIC) assay. A two-fold dilution series of active cathelicidin in THY media could be performed with TIGR4 and D39. The MIC value could then be compared to the active cathelicidin protein levels, measured by Western Blot, produced during the *Killing of SPN with induced THP-1 cells* experiment detailed previously. Depending on the results of future experiments, doses of PBA and VD could also be increased in order to determine whether this

treatment has a significant effect on activation of cathelicidin and killing of SPN. This will also allow for the evaluation of any synergistic effects that may be occurring.

Another possibility is that the incubation periods with the treatment, and later on with the SPN, were not long enough for the macrophages to acclimate or kill the SPN. Due to time constraints, incubation with PBA and VD only lasted 24 hours, which is the lowest amount of time in the range of the incubation period (24 - 48 hours) used in other studies (Kulkarni et al., 2014). Therefore, going forward, the incubation period with PBA or VD or both will be elongated to 48 hours. Incubation of macrophages with *Mycobacterium tuberculosis* lasted only 2 hours in the study that showed that treatment with PBA sped up the decline of viable *Mycobacterium tuberculosis* (Mily et al., 2015), so a longer infection period may not solve the problem of not being able to detect a significant decrease in growth of the bacteria. However, it is important to be thorough and test every possibility, so future experiments may include longer infection periods as well.

In addition to the future experiments mentioned previously, other cell types that produce cathelicidin, such as epithelial cells, could be tested in the same method used in this study in order to elucidate whether synergistic induction can boost killing of SPN with other specific cell types. Macrophages were chosen in this study as they do not produce any proteases that can cleave cathelicidin and produce an active form, which is why it was hypothesized that it is a bacterial protease that cleaves it (LaRock and Nizet, 2015). The same is true for epithelial cells, so a logical next step would be to test such cells in order to observe any changes in synergistic induction effects, if there are any differences at all.

In conclusion, this study provided limited support for the hypothesis that PBA and/or VD could increase the killing of SPN, as decreased growth of TIGR4 was seen with treatment. This

research will deepen the understanding of the efficacy of treatment with PBA and VD in boosting the innate immune system cells to increase killing of SPN. Low VD has been shown to be a risk factor for SPN infection (Jovanovich et al., 2014; Kim et al., 2015), and it has also been shown that SPN remains susceptible to cathelicidin (LaRock and Nizet, 2015), so the synergistic induction method with PBA has immense potential for effective treatment. Determining the efficacy of this synergistic treatment could allow for the further development of novel therapeutic strategies that will combat dangerous diseases mainly caused by SPN, such as pneumonia, meningitis, and sepsis. SPN is the leading cause of pneumonia mortality globally (Troeger et al., 2018), and increasing antibiotic resistance in this species has galvanized researchers to search for other solutions to this massive problem. In the future, this research on increasing activation of naturally-occurring factors of the immune system to provide prophylaxis will help to decrease the threat of infection toward at-risk populations worldwide.

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