Engineered Cationic Antimicrobial Peptides Differ in Their Ability to Limit *in vitro* Growth and Viability of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*

by

Alyssa Marie Jespersen

BS in Biology, The Catholic University of America, 2016

Submitted to the Graduate Faculty of the

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2021

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Alyssa M. Jespersen

It was defended on

April 16, 2021

and approved by

Moses T. Bility, PhD, Assistant Professor, Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Berthony Deslouches, MD, PhD, Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Thesis Advisor: Joshua T. Mattila, PhD, Assistant Professor, Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Engineered Cationic Antimicrobial Peptides Differ in Their Ability to Limit *in vitro* Growth and Viability of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*

Alyssa M. Jespersen, MS

University of Pittsburgh, 2021

Abstract

Mycobacterium tuberculosis is a major global health concern, causing almost 2 million deaths annually, and the emergence of multidrug resistant strains is a problem in the treatment of infections. Identification of alternatives for traditional antimicrobial treatments for *M. tuberculosis* infection is vital to combat multidrug resistant strains. Engineered cationic antimicrobial peptides with antibacterial activities may be an alternative to treating bacterial infections like tuberculosis. Here we assessed the bactericidal abilities of three antimicrobial peptides, A4S7, D8, and WLBU2, on two *Mycobacterium* species and *Escherichia coli*, a gram-negative bacterium. Bacterial cultures were incubated with varying concentrations of antimicrobial peptides and were assessed through OD measurement at varying times. *E. coli* was used as a positive control to confirm the peptides were active against a bacterium with a typical gram-negative cell wall, whereas we used *M. bovis* BCG as a surrogate for *M. tuberculosis* where we could test the peptides' activity against the mycobacterial cell wall, which has a more complex structure than other bacteria, under BSL2 conditions. Bactericidal ability and permeability by the peptides were assessed through staining with viability dyes and flow cytometry. We investigated the effect of these peptides on intracellular infection with *M. tuberculosis* by treating the cells with peptides before and after bacterial infection. All peptides affected the growth of all species, with α4S7 having great bactericidal effects of *E. coli* growth at the lowest inhibitory concentration. *M. tuberculosis* and *M. bovis* were affected by all peptides and experienced permeabilization in the higher concentrations of peptide.

Monocyte-derived macrophages from nonhuman primates treated with a4S7 and D8 prior to exposure to *M. tuberculosis* had fewer bacteria than cells treated after. *M. tuberculosis*, *M. bovis* BCG, and *E. coli* experienced inhibited growth when treated with 10 μM or greater of antimicrobial peptides, but little difference was seen during intracellular infection. Our results suggest that engineered cationic antimicrobial peptides are active against *M. tuberculosis* and our data warrants further investigation into these agents as potential tools for treating the challenging infections caused by mycobacteria.

Table of Contents

1.0 Introduction 1

1.1 *Mycobacterium tuberculosis* and Tuberculosis 1

1.1.1 TB Transmission, Disease, and Treatment 1

1.1.2 Multidrug Resistant and Extensively Drug Resistant TB 4

1.2 Antimicrobial Peptides 4

1.2.1 Antimicrobial Peptides and Mtb 5

1.2.2 Limitations of Antimicrobial Peptides 6

1.3 Engineered Cationic Antimicrobial Peptides 6

2.0 Hypothesis and Specific Aims 9

2.1 Aim I: Determine if eCAPs have antimycobacterial activity against *M. bovis* BCG and Mtb 9

2.2 Aim II: Determine if eCAPs can inhibit intracellular mycobacterial survival and replication 10

3.0 Materials and Methods 11

3.1 Bacterial Culture 11

3.2 Cell Culture and Differentiation of Monocyte-Derived Macrophages 11

3.3 Antimicrobial and Cytolytic Activity Assays 12

3.4 Bacteria and Cell Culture Microscopy 13

3.5 Antibiotic Sensitivity Assays 14

3.6 Live/Dead Intracellular Infection Experiments 15

3.7 ImageJ Analysis of Live/Dead Experiments 16

4.0 Results 17

4.1 Aim I: Determine Whether eCAPs are Capable of Killing Mtb 17

4.1.1 eCAP Effect on Bacterial Growth Rates and Killing.....17

4.1.2 Permeabilization of Bacteria by eCAPs 20

4.1.3 eCAPs Effect on Bacterial Antibiotic Sensitivity 24

4.1.4 Aim I Conclusions 26

4.2 Aim II: Determine Whether eCAPs are Capable of Treating Intracellular Mtb Infection in Macrophages 26

4.2.1 eCAP Tolerance by Human Cell Lines and Monocyte-Derived Macrophages 27

4.2.2 Effect of eCAPs on Intracellular Infection Using Live/Dead Reporter Mtb 30

4.2.3 Aim II Conclusions.....33

5.0 Discussion..... 34

5.1 eCAPs Do Not Inhibit Replication or Induce Bacterial Killing on Mtb and *M. bovis* BCG 34

5.2 eCAPs Do Not Permeabilize Mtb and *M. bovis* BCG As They Permeabilize *E. coli* 35

5.3 eCAPs Synergize with Penicillin/Streptomycin to Improve Their Activity Against *M. bovis* BCG 36

5.4 Human Cell Lines and Monocyte-Derived Macrophages are Degraded by High Concentrations of eCAPs..... 37

5.5 eCAPs do not Prevent Intracellular Mtb Infection 38

5.6 Public Health Significance 38

5.7 Future Directions 39

Bibliography 40

List of Figures

Figure 1: Illustration of the mycobacterial cell wall. 2

Figure 2: Growth curves of *E. coli, M. bovis* BCG, and Mtb after incubation with various concentrations of eCAPs. 18

Figure 3: CFU/mL of *E. coli, M. bovis* BCG, and Mtb after eCAP treatment. 19

Figure 4: Illustration of membrane impermeable dye mechanism. 20

Figure 5: Permeabilization of bacteria by eCAPs. 22

Figure 6: Morphological changes to *E. coli* and *M. bovis* BCG after eCAP incubation. 23

Figure 7: Diagram of proposed effect of permeabilized bacteria on antibiotic entry to cells. 24

Figure 8: eCAPs enhance antibiotic sensitivity in *E. coli* and *M. bovis* BCG. 25

Figure 9: Permeabilization of human cell lines by eCAPs. 27

Figure 10: THP-1 degredation by eCAPs. 28

Figure 11: Permeabilization of monocyte-derived macrophages by eCAPs. 29

Figure 12: Schematic of live/dead Mtb reporter strain. 30

Figure 13: Proportion of Mtb infected monocyte derived macrophages after pre-treatment or post-treatment with eCAPs. 32

Figure 14: Proportion of monocyte-derived macrophages containing viable Mtb after pre-treatment or post-treatment with eCAPs. 32

1.0 Introduction

1.1 *Mycobacterium tuberculosis* and Tuberculosis

Mycobacterium tuberculosis (Mtb) is a facultative intracellular pathogen that causes infection in humans and is the causative agent of tuberculosis (TB) (1). TB is a global health concern and is the leading cause of death due to an infectious agent, infecting approximately 10 million and killing 1.6 million people in 2018 (2). TB is a historic disease and is responsible for mortality across millennia. Evidence of TB has been observed in Egyptian mummies dating back to 2400 BC and was referred to as consumption as early as the 17th century (3). While historically significant, TB is a current concern and is also the leading cause of death for people living with HIV (4, 5). Most cases of TB and Mtb infection occur in Sub-Saharan Africa and South East Asia, which are also geographic regions that represent the epicenter of the HIV epidemic (2, 6).

1.1.1 TB Transmission, Disease, and Treatment

Mtb is transmitted between individuals through transfer of aerosolized particles (7). An uninfected individual can be exposed to Mtb after interacting with an infected individual when that person coughs, speaks, or sneezes, releasing Mtb containing particles into the air, which can be inhaled by the uninfected individual (7, 8). Droplets containing the bacteria can remain in the air for hours prior to inhalation. Once inhaled, Mtb can be engulfed by alveolar macrophages in the lungs, where the bacteria are either destroyed by the cell or persists within it (9). The mycobacterial cell wall is a complex structure that aids in the transmission of Mtb. Like gram-negative and gram-

positive bacteria, peptidoglycan is a major component of the mycobacterial cell wall (Figure 1) (10). In Mtb, peptidoglycan is recognized by the host immune response, recruiting immune cells to the site of infection, allowing for cells to become infected and for transmission in the lung (11). Unlike other bacteria, the cell wall also contains glycolipids, arabinogalactan, mycolic acids (Figure 1). These complex structures make it difficult for common detergents to desiccate Mtb and are targets for antibiotics against Mtb (10).

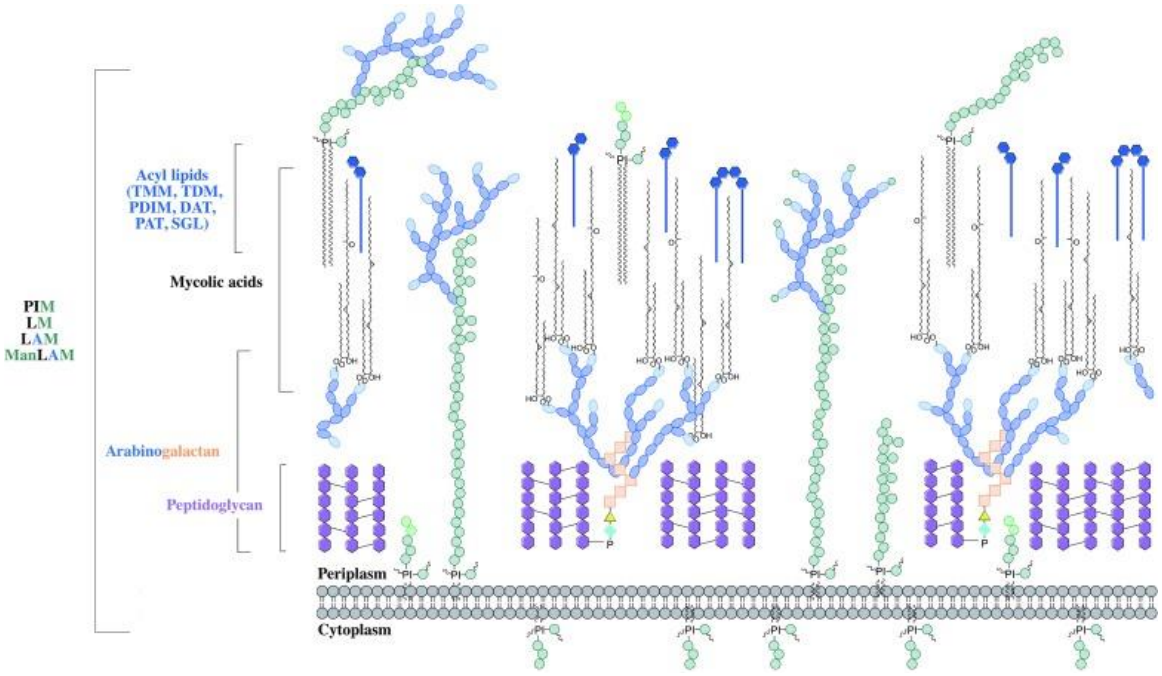

Figure 1: Illustration of the mycobacterial cell wall.

After exposure to the pathogen, individuals can be described as either having latent or active TB. Only a small fraction of individuals exposed to Mtb develop primary TB disease, the majority develop latent infection. Latent TB is defined as infection with Mtb that results in a persistent immune response to infection without clinical symptoms of disease and nearly one third of the world's population is believed to be latently infected, with a higher proportion in areas endemic to TB (12, 13). Individuals with latent infection cannot transmit Mtb, but can undergo reactivation, where latent infections transition to active disease (14). Individuals with active

disease experience symptoms of cough with sputum and possibly blood, fever, chest pain, night sweats, and weight loss (15). Infection can lead to the formation of granulomas, typically found in the lungs, but can also form in other tissues, like lymph nodes. Granulomas are considered the hallmark of TB and are structures of bacteria, infected cells, immune cells, and necrosis, that can act as immune barriers, preventing further dissemination of bacteria, and as bacteria refuge within the tissue (16).

Mtb infection can be diagnosed through several methods. The tuberculin skin test involves injecting a solution containing Mtb proteins under the top layer of skin and measuring swelling at the site to determine if an immune response occurs (17). This method will determine if an individual has been infected with a *Mycobacteria* species, but does not differentiate between Mtb and *M. bovis* BCG, the bacteria used in the Mtb vaccine. Additionally, the interferon-γ release assay (IGRA) is a blood test that measures the development of an interferon-γ response in the blood after exposure to Mtb specific proteins, like ESAT-6 (17, 18). This test does not cross react with the BCG vaccine, so it is the preferred test for people who have been vaccinated (17). Positive results of either test can be confirmed by a positive sputum culture for Mtb or a chest x-ray for the presence of granulomas (17, 18).

Treatment of TB is dependent upon early diagnosis and several antibiotic drugs. The first phase of treatment uses first line drugs isoniazid, rifampicin, pyrazinamide, and ethambutol for two months (19). The second phase of treatment uses a combination of isoniazid plus rifampicin for four months. If these are not effective, intravenous drugs like amikacin, kanamycin, or capreomycin can be used (19). The long course of treatment and side effects caused by the drugs can lead to lack of compliance, leading to ongoing disease, potential transmission, and the development of drug resistant bacteria (2, 20).

1.1.2 Multidrug Resistant and Extensively Drug Resistant TB

A challenge of combating TB is the rise of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of Mtb. MDR TB is resistant to both isoniazid and rifampicin, two of the first line drugs used when treating TB, and XDR TB is resistant to rifampicin, isoniazid, any fluoroquinolone, and one of three injectable drugs, capreomycin, kanamycin, and amikacin (21). Incidence of MDR and XDR TB has increased and 3.1% of new cases and 18% of previously treated cases globally are due to MDR TB (2). Lack of compliance to current treatments has led to the increase in resistant strains, which are extremely difficult to treat and require a longer course of treatment with first, second, and third line drugs (22).

1.2 Antimicrobial Peptides

Antimicrobial peptides are a group of peptides characterized by their potent activity against bacteria, viruses and fungi and are common components of the immune response (23, 24). These peptides are commonly in the form of a β-sheet or α-helix, with the latter being the most common and most studied class of antimicrobial peptide (23). Their antimicrobial activity is achieved by targeting the cell membrane to create pores, inducing rapid killing of their target (25-27). Most antibacterial peptides are cationic, which target bacterial cell membranes and destroy the membrane's lipid bilayer structure (23, 24). Additionally, many of these peptides are also amphipathic, containing both hydrophilic and hydrophobic regions, allowing them to interact with lipid and phospholipid groups within the membrane (23, 28). The amphipathic nature allows the peptides to interact with the target membrane and destroy it through different mechanisms,

including aligning perpendicularly to the membrane and directly forming pore, and several peptides coating a small region of the membrane before penetrating the lipid bilayer, forming pores (29, 30).

1.2.1 Antimicrobial Peptides and Mtb

Antimicrobial peptides are an integral component of the immune response and are also involved in the anti-Mtb response. Cathelicidins, including LL-37, are a family of antimicrobial peptides found in the lysosomes of macrophages and polymorphonuclear leukocytes in humans and are involved in the anti-Mtb immune response. LL-37 plays a large role during mycobacterial infection, including contributing to the recruitment of T cells to the site of infection and modulate the expression of inflammatory and anti-inflammatory cytokines (31, 32). Production of LL-37 in alveolar macrophages, neutrophils, and monocyte-derived macrophages has been observed to be induced after infection with Mtb through stimulation of toll-like receptors (TLRs) by Mtb DNA (33). TLR-2 and TLR-4 are over expressed during Mtb infection and recognize Mtb lipoprotein, lipomannan, and phosphatidyl-myo-inositol, and induce signaling that activate macrophages, dendritic cells and polymorphonuclear cells at the site of infection and initiate the adaptive immune response (34). Patients with active TB infection have also been shown to have higher levels of LL-37 in their serum compared to patients infected with other diseases, suggesting that LL-37 is a major component of the innate response to Mtb (35).

Defensins, another family of antimicrobial peptides, are also involved in the immune response to mycobacterial infection. Gene analysis of the peripheral blood mononuclear cells of TB patients have shown that they overexpress defensins compared to healthy individuals (36). These peptides have been observed to bind Mtb within the macrophage phagosome, and both α-

defensin and β-defensin expression can be induced by the mycobacterial cell wall in epithelial cells and eosinophils (37-39).

1.2.2 Limitations of Antimicrobial Peptides

While antimicrobial peptides are a potential alternative to traditional antibiotics, there are limitations to their use. As host produced proteins, they are unstable, unable to provide long lasting antimicrobial activity on their own (24). Additionally, these peptides are susceptible to protease degradation, contributing to their short lifespan during the immune response (24, 40, 41). The effect of antimicrobial peptides on the host cells is another concern regarding their use as therapy. Several have been observed to have hemolytic activity, limiting their clinical uses (42, 43). Cationic antimicrobial peptides, though they have greater antimicrobial activity, are more toxic to human cells at therapeutic concentrations, limiting their use in drugs (42).

1.3 Engineered Cationic Antimicrobial Peptides

To overcome the limitations of naturally occurring antimicrobial peptides, engineered cationic antimicrobial peptides (eCAPs) have been developed. Naturally occurring antimicrobial peptides are diverse in their structures and amino acid sequences, causing them to have a range of antimicrobial abilities across the different families of peptides (23). Prior to developing the eCAPs, the lentivirus lytic peptide 1 (LLP1), a peptide derived from the human immunodeficiency virus 1 (HIV-1) transmembrane protein, was observed to have an α-helical structure and proposed to be involved in HIV-1 pathogenicity (44). Studies with LLP1 showed that this peptide was more potent

than other host-derived antimicrobial peptides, and lead researchers to substitutions and additions to the amino acid sequence could increase selectivity and potency of the peptides for pathogens and decrease the toxicity against host cells (45-47).

After observing the effects of LLP1, eCAPs were designed to have an α-helical structure to increase their potency against pathogens. These were designed to be amphipathic, with cationic and hydrophobic regions, allowing the eCAPs to be able to interact with both regions of the bacterial cell membrane (48). To increase potency and selectivity, the eCAPs were designed with only three amino acids in their sequence: arginine, to comprise the cationic and hydrophilic region of the α-helix; valine, to increase hydrophobicity; and tryptophan, to increase the peptide length (48, 49). Additionally, some eCAPs use the D-enantiomer of valine, which increased bacterial killing and decreased hemolysis of host cells, increasing the peptide's safety for mammalian cells (50).

The antimicrobial activity of eCAPs has been investigated in gram-positive bacteria, gram-negative bacteria, and in the formation of biofilms. eCAP WLBU2 was more effective at killing *Pseudomonas aeruginosa* in culture with human serum, human monocytes, and skin fibroblasts with less adverse effects on the host cells than naturally occurring antimicrobial peptide LL-37, which requires a higher concentration to be effective against the bacteria (49). WLBU2 was also more effective at clearing *P. aeruginosa* infection from the airways during intratracheal infection in a murine model compared to LL-37, both in culture and when delivered intratracheally (51). Both WLBU2 and D8, an eCAP that incorporates the D-enantiomer of valine, proved more effective against multidrug resistant strains of *P. aeruginosa* and *Acinetobacter baumannii* than LL-37, and were less cytotoxic to mammalian cells than LL-37 (52). The effects of eCAPs against gram-positive and gram-negative bacteria led us to question if they could also be effective against

Mtb and other *Mycobacteria* species. The complexity of the mycobacterial cell wall and the rise of multidrug resistant strains presents the need for alternative treatments for infection, and eCAPs may be a potential alternative.

2.0 Hypothesis and Specific Aims

I propose to determine how three eCAPs, α4S7, D8, and WLBU2, interact with Mtb and *Mycobacterium bovis* BCG, the bacteria used in the Mtb vaccine. I hypothesize that eCAPs kill Mtb and can be used to prevent and treat intracellular infection in macrophages at concentrations that are tolerated by the host cells. I will test this hypothesis with the following specific aims.

2.1 Aim I: Determine if eCAPs have antimycobacterial activity against *M. bovis* BCG and Mtb

Aim Ia: Determine how eCAP treatment affects Mtb growth in culture, I will incubate *M. bovis* BCG and Mtb with various concentrations of α4S7, D8, and WLBU2. OD will be measured across several timepoints to determine the growth curve of the bacteria and cultures will be plated after 6 days to measure total CFU after eCAP treatment.

Aim Ib: Determine whether eCAPs permeabilize Mtb like other bacteria, *M. bovis* BCG and Mtb will be stained with a membrane impermeable dye after incubation with eCAPs and analyzed using flow cytometry to determine the proportion of cells that have been permeabilized.

2.2 Aim II: Determine if eCAPs can inhibit intracellular mycobacterial survival and replication

Aim IIa: Determine which concentrations of eCAPs are tolerated by host cells, human cell lines will be incubated with various concentrations of eCAPs prior to being stained with a membrane impermeable dye. Cells will then be mounted on slides and the proportion of permeabilized cells will be measured though fluorescent microscopy.

Aim IIb: Determine if eCAPs can prevent and limit intracellular Mtb infection, macrophages will be incubated with eCAPs either before or after infection with a live/dead reporter strain of Mtb that expresses a fluorescent marker constitutively and another when transcriptionally active. The proportion of infected cells and the proportion of live cells will be determined though microscopy.

3.0 Materials and Methods

3.1 Bacterial Culture

For bacterial culture experiments, the Erdman strain of Mtb was used, as well as *Mycobacterium bovis* BCG and *Escherichia coli* as controls. *M. bovis* BCG was used as a model mycobacterium that can be safely used outside of BLS3 containment and *E. coli* was used as a positive control to demonstrate eCAP activity against a bacterium with a 'typical' gram negative cell wall. Mtb and *M. bovis* BCG were cultured in 7H9 media with oleic acid, dextrose, and catalase at 37°C with 5% CO_2. *E. coli* strain DH5α was culture in LB broth at 37°C with 5% CO_2. Experiments with *E. coli* and *M. bovis* BCG were performed under BSL-2+ conditions and experiments with Mtb were performed under BSL-3 conditions in the University of Pittsburgh Regional Biosafety Laboratory. Prior to use in all assays, the optical density (OD) of all bacterial cultures was measured by spectroscopy at 600 nm to confirm the bacteria were in mid-log phase.

3.2 Cell Culture and Differentiation of Monocyte-Derived Macrophages

Human cell lines U937, a monocyte cell line, THP-1, a monocyte cell line, and A549, a lung epithelial cell line, were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Cells were inspected via microscopy prior to use in experiments to confirm that they were morphologically normal and free of contamination.

Monocyte-derived macrophages were differentiated from cynomolgus macaque monocytes from Percoll gradient isolated PBMCs. $1x10^7$ PBMCs were used for $CD14^+$ cell isolation using human anti-CD14 magnetic beads. $CD14^+$ monocytes were differentiated in RPMI supplemented with 20% FBS, GM-CSF, and M-CSF for 7 days at 37°C with 5% CO_2. Macrophages were lifted off plates using trypsin, washed, and counted before being seeded into new culture plates the day prior to use in experiments.

3.3 Antimicrobial and Cytolytic Activity Assays

Mtb, *M. bovis* BCG, and *E. coli* were incubated with eCAPs α4S7, D8, and WLBU2 to assess antimicrobial and cytolytic activity. eCAPs were diluted to concentrations 0.1 μM, 1 μM, 10 μM, 20 μM, and 40 μM in 7H9 for Mtb, serial twofold dilutions from 40 μM to 0.625 μM in 7H9 for *M. bovis* BCG, and serial twofold dilutions from 20 μM to 0.156 μM in LB for *E. coli*. The bacteria were seeded into wells of a 96-well plate at a starting culture OD 600nm of 0.05 with their respective dilutions of eCAPs and incubated at 37°C with 5% CO_2. The bacteria were also incubated in additional wells with untreated medium and penicillin/streptomycin treated medium as positive and negative growth controls. Measurements of OD 600nm were taken using a spectrophotometer at timepoints 0, 3, and 6 days for Mtb and *M. bovis* BCG, and at timepoints 0, 0.25, 0.5, 1, 4, and 6 hours for *E. coli*. The OD was used to calculate the colony forming units (CFU) per mL based on a standard curve based on bacteria plated. At the final timepoint, samples of each treatment condition were plated on 7H11 plates (Mtb and *M. bovis* BCG) or LB plates (*E. coli*) at dilutions 10^{-4}, 10^{-6}, and 10^{-8}. Colony forming units (CFU) per mL were determined using

the bacteria counted on the plates to determine bacterial killing. Additionally, to assess membrane permeabilization, at the final timepoint, samples were stained with a 1:2,500 dilution of Sytox Orange, a membrane impermeable dye, and fixed in 2% paraformaldehyde (PFA). After staining, samples were analyzed using flow cytometry on the BD LSRFortessa flow cytometer maintained by the Department of Infectious Diseases and Microbiology. Analysis was completed using FlowJo.

Human cell lines and monocyte-derived macrophages were incubated with eCAPs α2S7, D8, and WLBU2 to assess their effects on mammalian host cells. Cells were incubated with eCAPs diluted in R10 in twofold serial dilutions from 40μM to 0.625μM and incubated at 37°C with 5% CO_2. After incubation for 24 hours, the cells were stained with a 1:2,500 dilution of Sytox Orange and fixed with 2% PFA. To determine the proportion of permeabilized cells, samples were analyzed using flow cytometry on the BD LSRFortessa flow cytometer and analysis was completed using FlowJo.

3.4 Bacteria and Cell Culture Microscopy

To assess the effect of eCAPs on bacteria morphology, *M. bovis* BCG and *E. coli* were incubated with 0.1 μM, 1 μM, 10 μM, and 20 μM of α2S7, D8, and WLBU2 for 18 hours. After incubation, the bacteria were stained with a 1:2,500 dilution of Sytox Orange and fixed with 2% PFA. Cells were then transferred to slides using cytospins at 1,600rpm for 5 minutes. Coverslips were affixed to the slides using Prolong Gold with DAPI. Fluorescent microscopy was performed using a Nikon e1000 epifluorescent microscope operated by the Nikon NIS Elements software. Image lookup tables (LUTs) were adjusted according to an untreated control to minimize

autofluorescence and background for each fluorophore and the same settings were applied to each image. The length of individual bacterium was measured using the measurement tool in the NIS Elements Analysis software and were plotted using GraphPad Prism.

To assess the effect of eCAPs on mammalian cells, THP-1 cells were similarly incubated with 0.1 μM, 1 μM, 10 μM, and 20 μM of α2S7, D8, and WLBU2 in R10 for 18 hours. After incubation, the cells were stained with a 1:2,500 of Sytox Orange before being transferred to two slides via cytospins at 1,600rpm for 2 minutes. One slide was additionally stained with hematoxylin and eosin. Coverslips were affixed to all slides using Prolong Gold with DAPI. Fluorescent and light microscopy was performed using a Nikon e1000 epifluorescent microscope operated by the Nikon NIS Elements software with similar adjustments made as referenced above.

3.5 Antibiotic Sensitivity Assays

To assess the effect of eCAPs on bacterial antibiotic sensitivity, *E. coli* and *M. bovis* BCG were incubated with 10 μM of α4S7, D8, and WLBU2 and serial 10-fold dilutions from 0.0001 u/mL to 100 U/mL of penicillin/streptomycin, including an untreated control. Bacteria without eCAPs were also incubated with the dilutions of penicillin/streptomycin to determine a baseline of antibiotic sensitivity. OD 600nm was taken using a spectrophotometer to at time 0 to determine the starting density of the culture. After 6 hours for *E. coli* or 6 days for *M. bovis* BCG, the OD 600nm was taken for all samples to determine the bacterial growth under eCAP and antibiotic treatment. The ODs were plotted using GraphPad Prism.

3.6 Live/Dead Intracellular Infection Experiments

A live/dead reporter strain of Mtb was used to assess the ability of eCAPs to target bacteria in an intracellular environment. This strain constitutively expresses mCherry fluorescence and will express GFP if transcriptionally active after induction by tetracycline. Cells that are transcriptionally active and express both mCherry and GFP were considered live and cells that only expressed mCherry were considered dead. These experiments were partially performed in the University of Pittsburgh Regional Biosafety Laboratory at BSL-3 conditions.

Monocyte-derived macrophages from cynomolgus macaques, isolated and differentiated according to the procedures previously stated, from four animals were seeded into the wells of two 12-well chamber slides at 50,000 cells per well for each animal and incubated at 37°C with 5% CO_2 overnight prior to use in downstream assays. Prior to infection 10 μM of α2S7, D8, and WLBU2 were added to two wells of one slide per animal. The live/dead Mtb was added at a MOI of 5 to each well of both slides and incubated for four hours at 37°C with 5% CO_2. After incubation, all wells were washed with PBS. New media was added to the eCAP treated slide and eCAPs were added to the untreated slide. Tetracycline was then added to the wells of all slides to induce the live Mtb to express GFP and were incubated overnight. After incubation, the slides were fixed with 2% PFA and coverslips were affixed using Prolong Gold with DAPI. Slides were analyzed using fluorescent microscopy as described above and images were taken across several regions of each well.

3.7 ImageJ Analysis of Live/Dead Experiments

Images obtained of the live/dead Mtb infected monocyte-derived macrophages were analyzed using ImageJ. TIFs containing the DAPI (nuclei), FITC (GFP), and TRITC (mCherry) channels of each image were opened in ImageJ. Channels were separated into individual windows using the Split Channels function. Using the Image Calculator function, a composite image containing pixels that only expressed both FITC and TRITC was created using the FITC and TRITC images. Using the Images to Stack function, the DAPI, new composite image, and TRITC channels were combined into a stacked image. Individual infected cells were then determined by eye. Using the region of interest tool, a box was made around each infected cell and the pixel expression for the composite and TRITC channels were measured using the Measure function. To determine the proportion of pixels that express both FITC and TRITC, or live, the fluorescent measurement of the composite channel was divided by the fluorescent measurement of the TRITC channel. The proportion of live Mtb and proportion of infected cells for each treatment group were plotted using GraphPad Prism.

4.0 Results

4.1 Aim I: Determine Whether eCAPs are Capable of Killing Mtb

The bactericidal activity of AMPs is accomplished by forming pores and degrading the cell walls of the target bacteria (25-27). eCAPs α2S7, D8, and WLBU2 function similarly, but it is not known if they are similarly functional against *Mycobacteria* due to the complexity of their cell wall (10, 53). The purpose of this aim is to assess the ability of eCAPs to kill Mtb, *M. bovis* BCG, and *E. coli* and determine if the eCAPs are permeabilizing the bacteria.

4.1.1 eCAP Effect on Bacterial Growth Rates and Killing

E. coli, M. bovis BCG, and Mtb were incubated with various concentrations of α2S7, D8, and WLBU2 and the OD was measured across several timepoints. The OD was used to calculate the CFU/mL of each treatment group, which were plotted to create a growth curve. *E. coli* was more susceptible to the eCAPs, particularly α2S7, at high concentrations, resulting in inhibited replication, similar to that of a penicillin/streptomycin treated negative control (Figure 2). *M. bovis* BCG and Mtb were more resistant to α2S7 and WLBU2, but showed concentration-wise susceptibility to D8, with less replication at the higher concentrations across the day 3 and day 6 timepoints, and growth similar to the no peptide control in the low concentrations (Figure 2). Several concentrations for eCAPs across the later timepoints were determined to have significantly reduced replication when compared to a no peptide treated control (Two-way ANOVA).

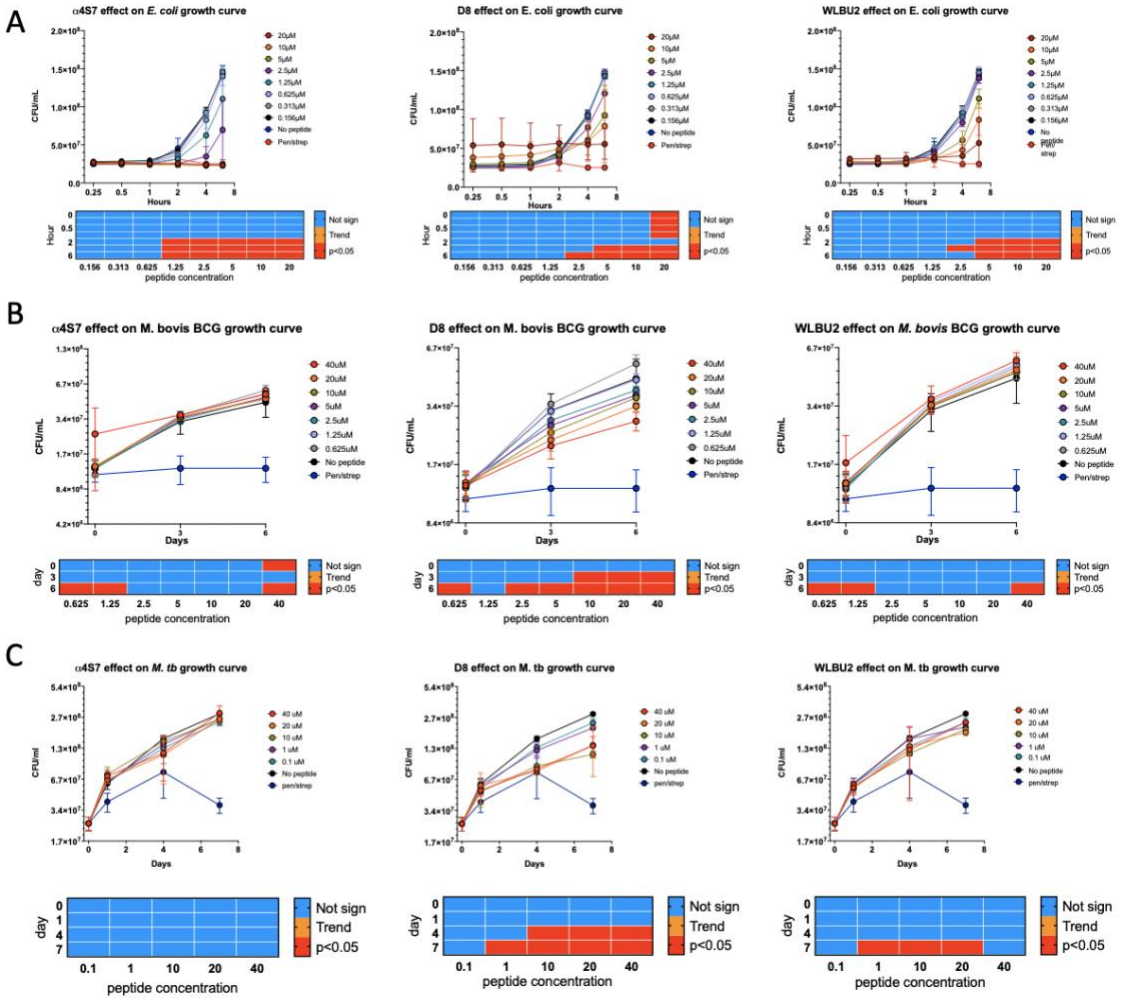

Figure 2: Growth curves of *E. coli, M. bovis* BCG, and Mtb after incubation with various concentrations of eCAPs.

Growth curves of *E. coli* (A), *M. bovis* BCG (B), and Mtb (C), after incuabtion with α4S7, D8, and WLBU2 across several timepoints. Heatmaps below the graphs represent the results of a Two-way ANOVA comparing each condition to the no peptide control. P<0.05.

To understand whether the reduced growth rate was due to bacterial killing by eCAPs, *E. coli*, *M. bovis* BCG, and Mtb were plated after incubation with the various concentrations of α2S7, D8, and WLBU2. Similar to the growth curves, *E. coli* was most susceptible to α2S7 and showed no growth at 2.5μM of α2S7 and above. There was an increase in *E. coli* killing as the

concentration of D8 and WLBU2 increased, with no bacterial growth at 20μM of D8 (Figure 3). *M. bovis* BCG and Mtb were more resistant to eCAP treatment than *E. coli.* There is a slight reduction in *M. bovis* BCG growth as the concentration of α2S7 and WLBU2 increases, and no change after D8 treatment (Figure 3). Mtb was most resistant to α2S7 and there was a reduction in growth after D8 and WLBU2 treatment as the concentration of the eCAPs increases (Figure 3). The difference in bacterial growth was not significantly different for *E. coli*, *M. bovis* BCG, and Mtb after eCAP treatment compared to a no peptide treated control (Friedman Test).

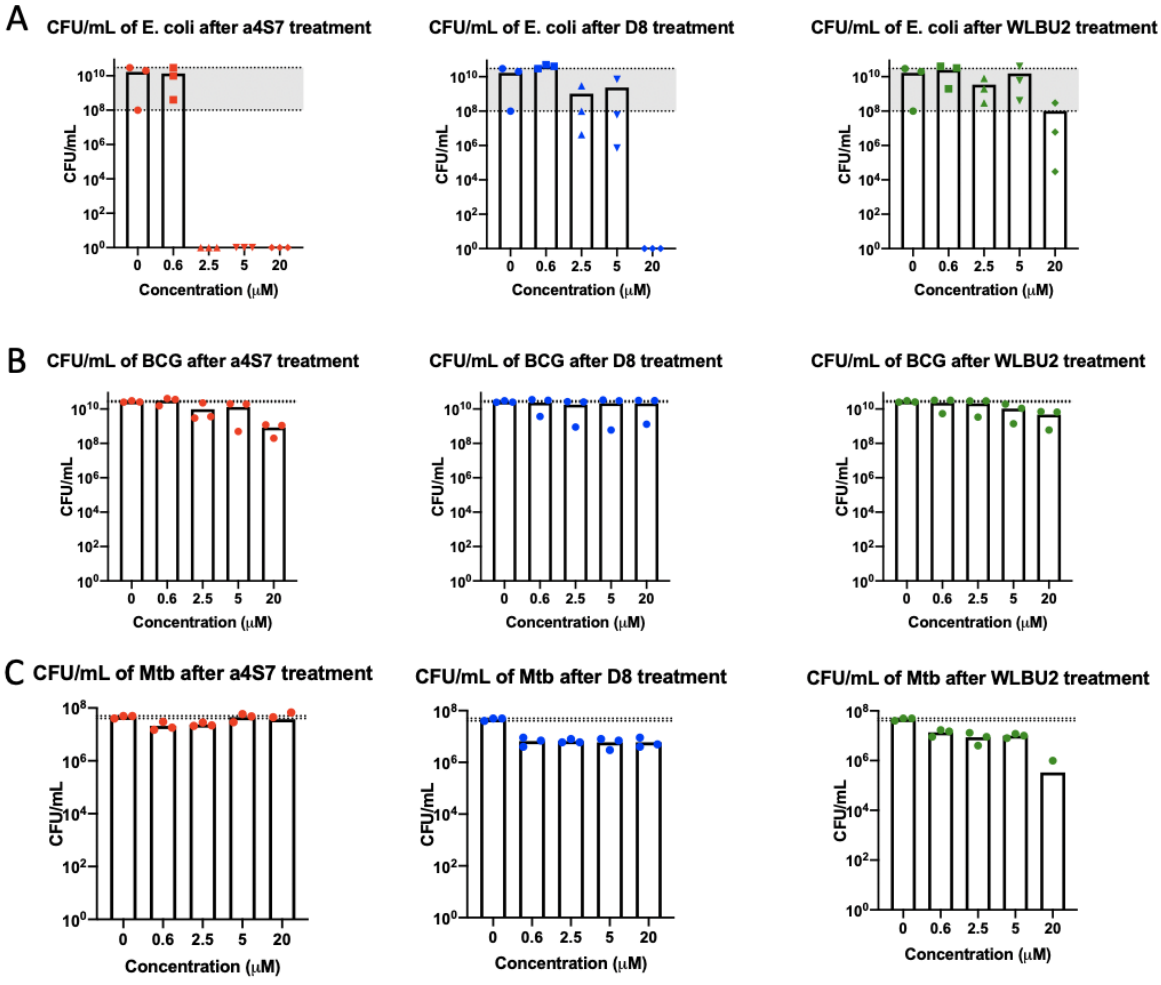

Figure 3: CFU/mL of *E. coli, M. bovis* BCG, and Mtb after eCAP treatment.

CFU/mL of *E. coli* (A), *M. bovis* BCG (B), and Mtb (C)) were determined by plating after samples were incubated with various concentrations of α4S7, D8, and WLBU2. *E. coli* plates were incubated overnight and *M. bovis* BCG and Mtb plates were incubated for 3 weeks prior to counting colonies. No peptide control is represented by grey bar. Non-parametric Friedman Test was used to determine significance between no peptide control and each condition. $P<0.05$.

4.1.2 Permeabilization of Bacteria by eCAPs

To assess whether the reduction in bacterial replication and growth was due to permeabilization by eCAPs, a membrane impermeable dye was used. Sytox Orange is a membrane impermeable dye that will stain the DNA of a cell with a red fluorescent marker if the cell's membrane or cell wall has been permeabilized. If the membrane remains intact, the dye is unable to cross the membrane and the cell will remain unstained (Figure 4).

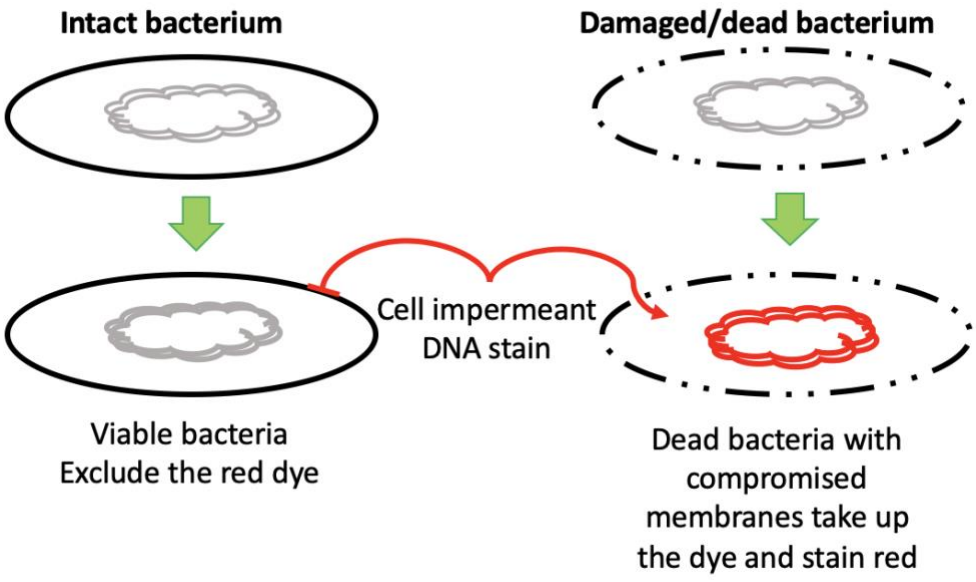

Figure 4: Illustration of membrane impermeable dye mechanism.

E. coli, *M. bovis* BCG, and Mtb incubated with various concentrations of eCAPs as described above were stained with the membrane impermeable dye Sytox Orange and the proportion of permeabilized cells were analyzed using flow cytometry. *E. coli* was permeabilized by α2S7 starting at 1.25μM and was completely degraded and unable to be analyzed using flow cytometry at 5μM and above (Figure 5). *M. bovis* BCG and Mtb appear to be permeabilized by the eCAPs based on the proportion of cells stained by the dye, but cells treated with a no peptide control show a range of permeabilization. Though permeabilization for *M. bovis* BCG starting at 20 μM appears to be significant compared to the no peptide control (Two-way ANOVA), it cannot be confirmed due to the range of permeabilization in the untreated control (Figure 5).

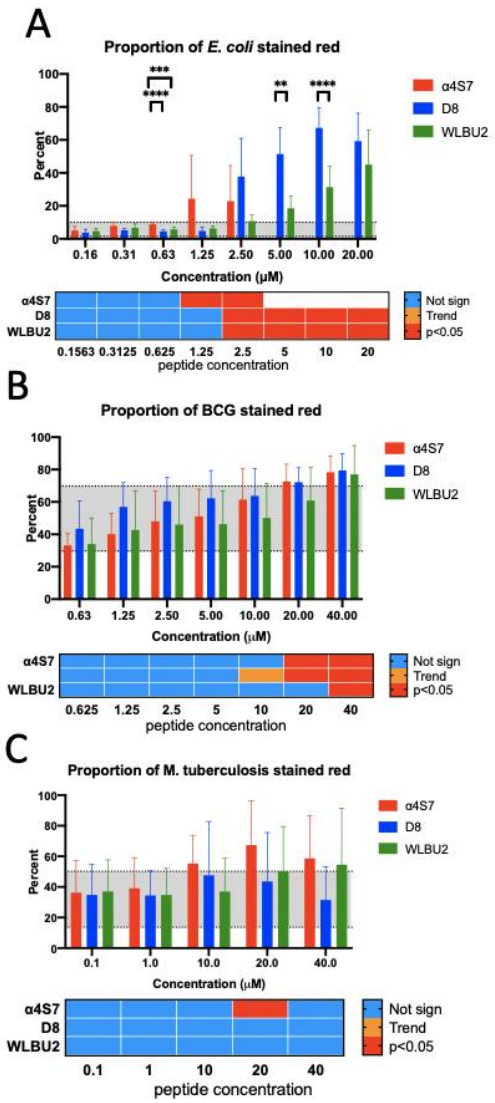

Figure 5: Permeabilization of bacteria by eCAPs.

Proportion of *E. coli* (A), *M. bovis* BCG (B), and Mtb (C) stained by Sytox Orange representing permeabilization by several concentrations of α4S7, D8, and WLBU2. The grey shaded bars represent the range of untreated conrol permeabilization. Heatmaps below the graphs represent the results of a Two-way ANOVA comparing each condition to the no peptide control. P<0.05

After determining that eCAPs permeabilize *E. coli* but not *M. bovis* BCG or Mtb, we wanted to determine if they were inducing morphological changes to the individual cells. *E. coli* and *M. bovis* BCG were analyzed using fluorescent microscopy after incubation with eCAPs. After treatment with 1μM of WLBU2 and at 20 μM for all eCAPs, *E. coli* cells increased in length and became longer as the concentration of eCAP increased (Figure 6). Similarly, *M. bovis* BCG also showed an increase in length that corresponded to the increase in eCAP concentration, but the change did not significantly differ from untreated cells, unlike *E. coli* (Figure 6).

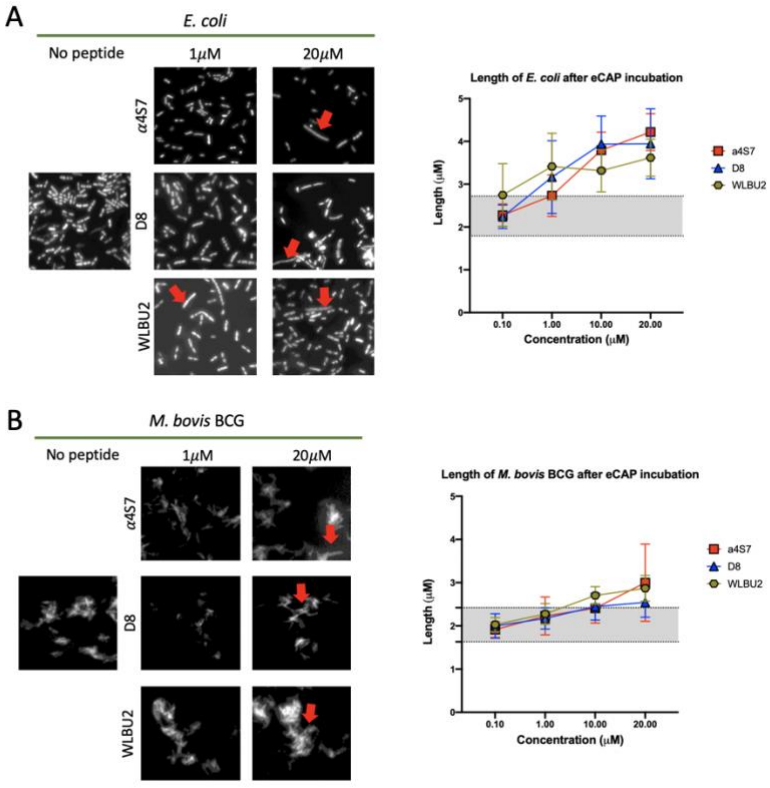

Figure 6: Morphological changes to *E. coli* and *M. bovis* BCG after eCAP incubation.

Images of *E. coli* (A) and *M. bovis* BCG (B) after incubation with α4S7, D8, and WLBU2. Red arrows indicate elongated cells. Graphs represent change in length at different concentrations of eCAP. Grey shaded bars represent range of untreated control.

4.1.3 eCAPs Effect on Bacterial Antibiotic Sensitivity

We observed that eCAPs permeabilize *E. coli* and that there is a range of permeabilization in *M. bovis* BCG and Mtb, we wanted to determine if eCAP treatment can affect bacterial antibiotic sensitivity. Antimicrobial peptides and eCAPs function by forming pores in the cell wall and cell membrane of bacteria. We hypothesized that the pores formed by eCAPs would allow antibiotics to enter the bacteria faster and require a lower concentration to kill the cells, ultimately lowering the minimum inhibitory concentration (Figure 7).

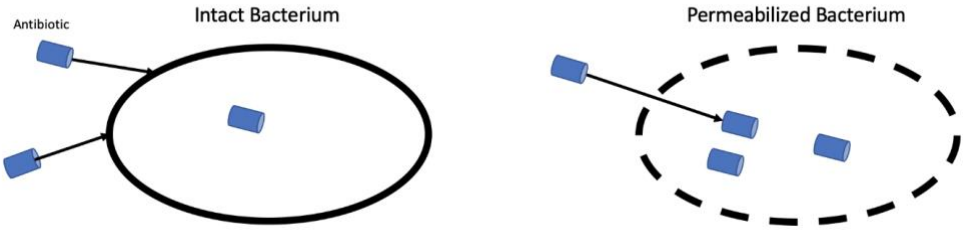

Figure 7: Diagram of proposed effect of permeabilized bacteria on antibiotic entry to cells.

E. coli and *M. bovis* BCG were incubated with both 10 μM of α2S7, D8, and WLBU2 tenfold dilutions of penicillin/streptomycin. After incubation, the OD was measured and plotted to determine the change in minimum inhibitory concentration to the antibiotics in response to eCAP treatment. For *E.* coli, incubation with only penicillin/streptomycin resulted in a lack of bacterial growth, with the OD equal to that of the baseline starting OD, and bacterial sensitivity to the antibiotics starting at 10 U/mL. When incubated with α4S7 and WLBU2, the bacteria became more sensitive to the antibiotics and had an OD equal to that of the baseline at 0.001 U/mL, while incubation with D8 did not increase antibiotic sensitivity at the same rate (Figure 8). For *M. bovis* BCG, incubation with only penicillin/streptomycin resulted in a lack of growth, with the OD equal

to that of the baseline starting OD, and bacterial sensitivity to the antibiotics starting at 0.1 U/mL (Figure 8). When incubated with eCAPs in addition to the antibiotics, samples incubated with α4S7 showed greater sensitivity and reached baseline at 0.01 U/mL, while incubation with D8 and WLBU2 did not differ from the no peptide control (Figure 8).

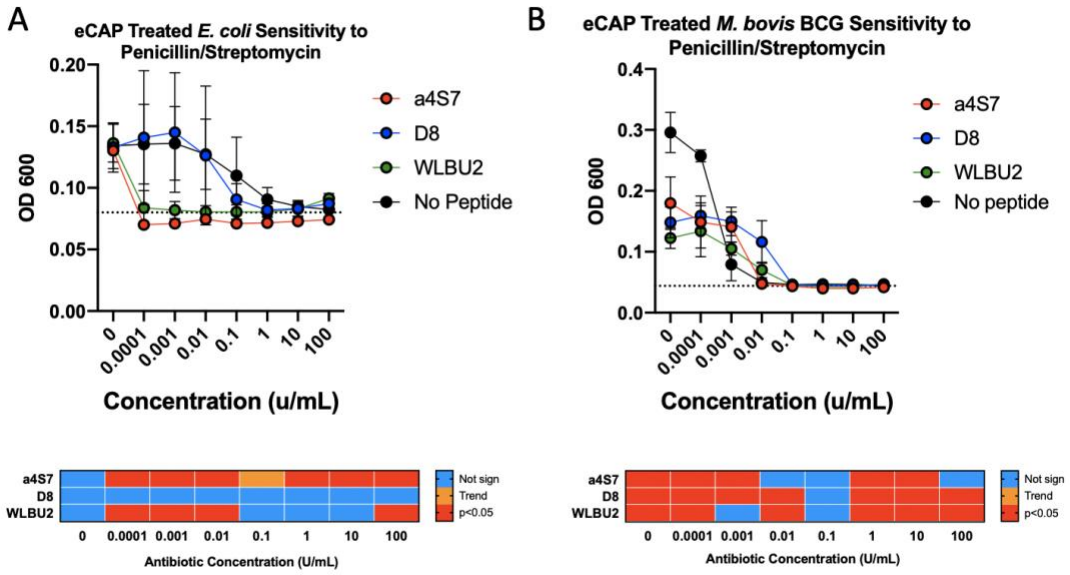

Figure 8: eCAPs enhance antibiotic sensitivity in *E. coli* and *M. bovis* BCG.

OD 600nm of *E. coli* (A) and *M. bovis* BCG (B) after treatment with serial dilutions of penicillin/streptomycin with or without α4S7, D8, and WLBU2. *E. coli* was incubated for 6 hours and *M. bovis* BCG was incubated for 6 days prior to taking the OD. The dotted lines represent the baseline OD of the culture determined at time 0. Heatmaps below the graphs represent the results of a Two-way ANOVA comparing each condition to the antibiotic-only no peptide control. $P<0.05$

4.1.4 Aim I Conclusions

In this aim, we observed that eCAPs reduce bacterial replication, specifically at 1.25 μM and above of α4S7 for *E. coli*, 2.5 μM and above of D8 for *M. bovis* BCG, and 1 μM and above of D8 for Mtb. Though this reduction was observed, *M. bovis* BCG and Mtb were not permeabilized by the eCAPs at any concentration and *E. coli* was permeabilized by α4S7. Additionally, incubation with eCAPs induced morphological changes to *E. coli* and *M. bovis* BCG, elongating individual cells. Incubation with eCAPs and penicillin/streptomycin resulted in an increase in antibiotic sensitivity for *M. bovis* BCG when incubated with α4S7, decreasing the concentration of antibiotic needed to prevent bacterial growth.

4.2 Aim II: Determine Whether eCAPs are Capable of Treating Intracellular Mtb Infection in Macrophages

After observing how α4S7, D8, and WLBU2 interact with the bacteria themselves, we wanted to determine how the eCAPs interact with host cells. For eCAPs to be considered as a therapeutic for Mtb and other pathogens, their effects on host cells and their ability to combat intracellular infection needs to be understood. The purpose of this aim is to understand how eCAPs interact with host cells and how they interact with Mtb during infection.

4.2.1 eCAP Tolerance by Human Cell Lines and Monocyte-Derived Macrophages

To determine whether eCAPs have adverse effects against mammalian cells, human cell lines U937, THP-1, and A549 were incubated with various concentrations of α4S7, D8, and WLBU2. After incubation, the cells were stained with membrane impermeable dye Sytox Orange, and the proportion of permeabilized cells was analyzed using flow cytometry. U937 cells showed resistance to α4S7 and D8 at concentrations below 20 μM and 40 μM, respectively, but was permeabilized by WLBU2 starting at 1.25 μM (Figure 9). THP-1 cells were permeabilized by all eCAPs at 5 μM and above. Though it appears the proportion of permeabilized cells for THP-1s treated with D8 decreases starting at 1 0μM, the cells were being degraded and were unable to be analyzed using flow cytometry (Figure 6). A549 cells showed an increase in permeabilization to α4S7 and WLBU2 starting at 10 μM, and were permeabilized by D8 at 40 μM (Figure 9). Significant changes in permeabilization are shown in the heatmaps below the graphs and were determined by using a Two-way ANOVA to compare each sample to the no peptide treated control.

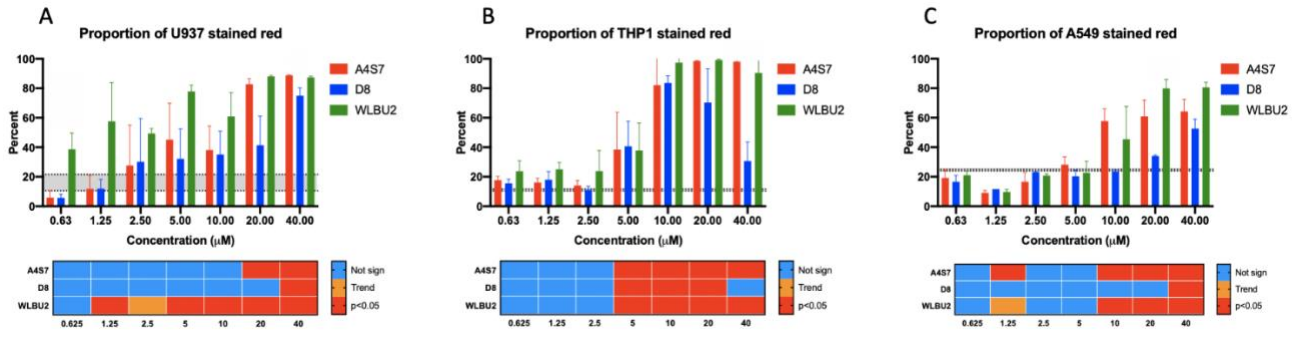

Figure 9: Permeabilization of human cell lines by eCAPs.

Proportion of permeabilized U937 (A), THP-1 (B), and A549 (C) human cell lines by various concentrations of α4S7, D8, and WLBU2. The grey shaded bars represent the range of untreated conrol permeabilization. Heatmaps below the graphs represent the results of a Two-way ANOVA comparing each condition to the no peptide control. P<0.05

After observing how eCAPs permeabilize human cell lines, we wanted to visualize morphological changes to the cells after incubation with eCAPs. THP-1 cells were incubated with various concentrations of α4S7, D8, and WLBU. After, the cells were adhered to slides and stained with hematoxylin and eosin and were analyzed using microscopy. Cells treated with 0.1 μM and 1 μM of all eCAPs were morphologically similar to cells that were not treated with eCAPs and appeared to be normal. At 10 μM of α4S7 and D8, the cells began to degrade and ceased to appear as normal, healthy cells. At 20 μM of all eCAPs, the cells were completely degraded and permeabilized (Figure 10), suggesting that THP-1 cells cannot tolerate eCAPs above 10 μM.

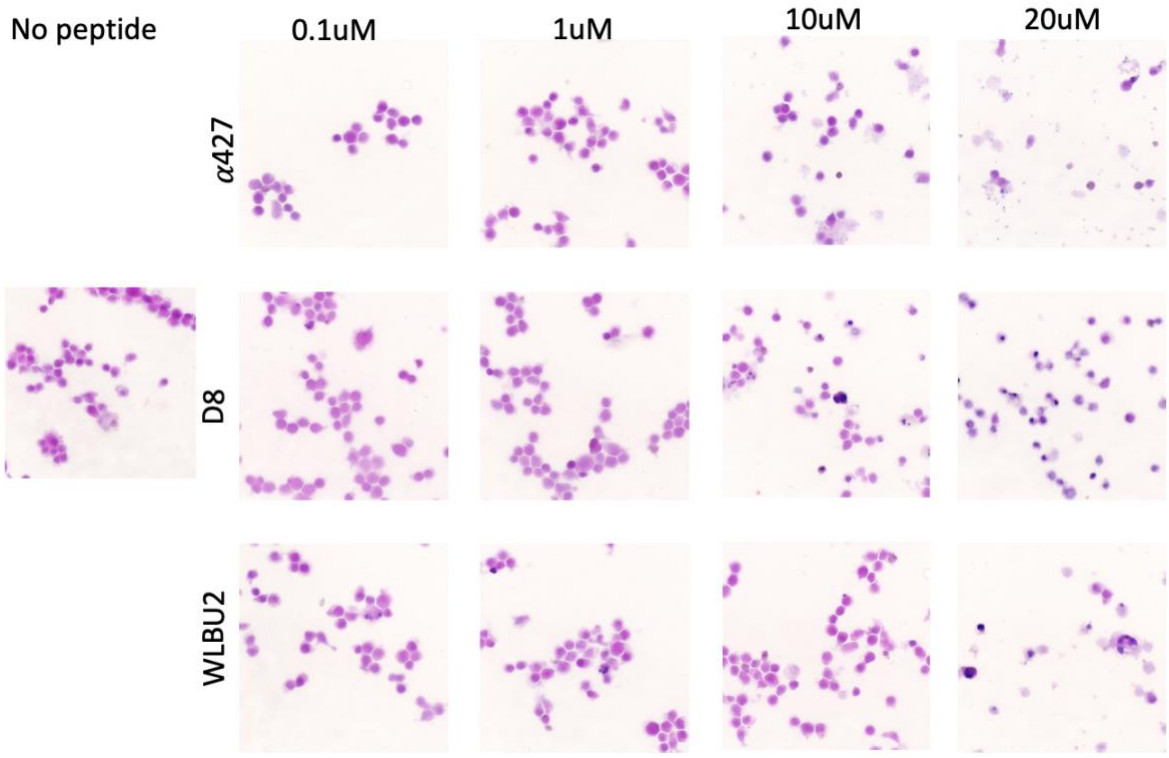

Figure 10: THP-1 degredation by eCAPs.

THP-1 cells were incubated with several concentrations of α4S7, D8, and WLBU2 prior to staining with hematoxylin and eosin. Cells were analyzed using microscopy.

While human cell lines are useful, they are derived from cancer cells and may have membrane compositions that differ from non-transformed primary cells. To determine if eCAPs had similar effects of primary cells as they had on cell lines, these experiments were repeated with monocyte-derived macrophages isolated from cynomolgus macaques. Monocyte-derived macrophages from five animals were incubated with various concentrations of α4S7, D8, and WLBU prior to staining with Sytox Orange. The proportion of permeabilized cells was determined using flow cytometry. For α4S7 and WLBU2, there was an increase in permeabilization for all animals starting at 5 μM. D8 was better tolerated by the macrophages but showed an increase in permeabilization for three animals at 10 μM (Figure 11). For all eCAPs, there were differences in tolerance between individuals, with some more susceptible than others.

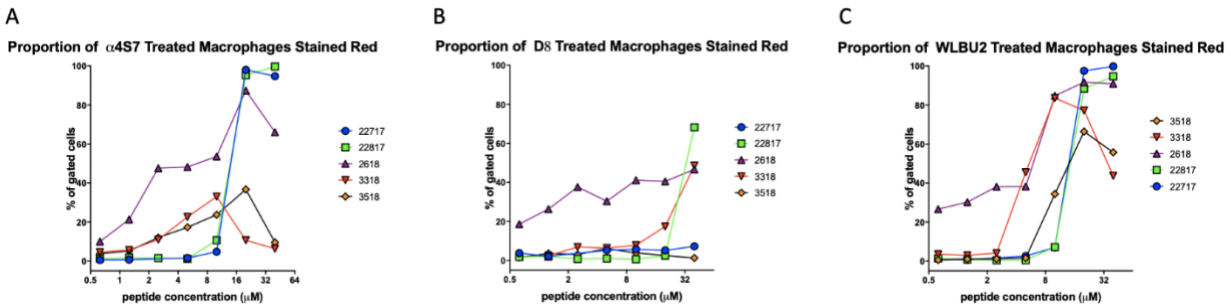

Figure 11: Permeabilization of monocyte-derived macrophages by eCAPs.

Proportion of permeabilized monocyte-derived macrophages after incubation with α4S7 (A), D8 (B), and WLBU2 (C). Proportion of cells was determined using flow cytometry.

4.2.2 Effect of eCAPs on Intracellular Infection Using Live/Dead Reporter Mtb

As an intracellular pathogen, Mtb infects and is found within macrophages, and can be found in the lumen between cells prior to infection of other cells. For eCAPs to be effective against Mtb, it needs to be determined whether they can prevent macrophage infection, addressing the question of whether an inhaled peptide could be effective at limiting bacterial viability and bacterial ability to infect new cells in the airway lumen, and whether they can attack and kill Mtb within a cell, addressing whether eCAPs can target bacteria in an intracellular environment. To address these questions, a live/dead reporter strain of Mtb was used. This strain intrinsically expresses mCherry fluorescence, and if the cell is transcriptionally active, it will express GFP upon induction with tetracycline. A cell that is transcriptionally active, or alive, will express both mCherry and GFP, and a cell that is not transcriptionally active, or dead, will only express mCherry (Figure 12).

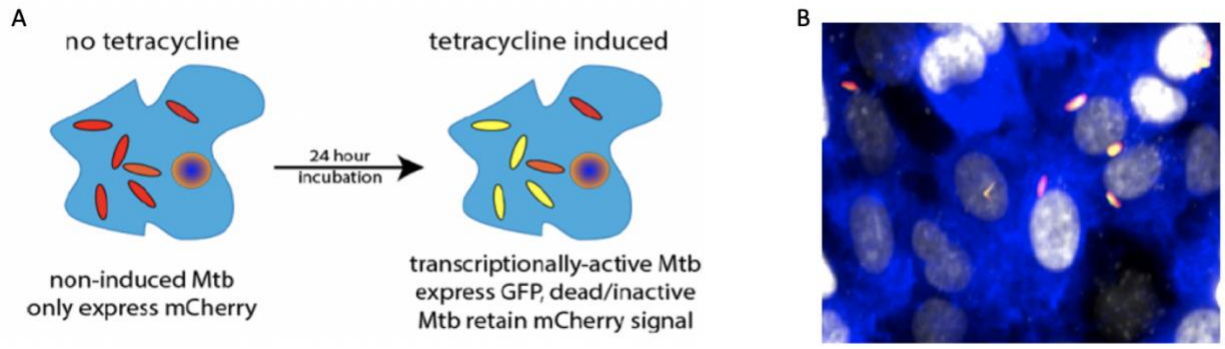

Figure 12: Schematic of live/dead Mtb reporter strain.

(A) Schematic of live/dead reporter strain mechanism. (B) A549 cells infected with live/dead reporter strain. Live cells are both red and yellow, while dead cells are only red.

To determine whether eCAPs can prevent and treat Mtb infection, monocyte-derived macrophages adhered to 12-well chamber slides were either treated with 10 μM of α4S7, D8, and

WLBU before infection (the pre-treatment group) or after infection (the post-treatment group) with the live/dead reporter strain of Mtb. After infection, the cells were treated with tetracycline to induce the live cells to produce GFP. The slides were analyzed using fluorescent microscopy to determine the proportion of Mtb infected macrophages after treatment with α4S7, D8, and WLBU in the pre-treatment and post-treatment groups. Macrophages treated with D8 and WLBU2 regardless of treatment group showed a decrease in Mtb compared to a no peptide treated control, but there was no significant difference between the pre-treatment cells and the post-treatment cells (Figure 13). Treatment with α4S7 before infection did not show a difference in the proportion of infected cells compared to the no treatment control, but there was a decrease in Mtb infected cells in the α4S7 post-treatment cells compared to the pre-treatment group, though none of the differences were significant compared to the no treatment control (Figure 13). The proportion of macrophages infected with live, viable Mtb was also determined. There appeared to be a decrease in live Mtb infected cells in the α4S7 pre-treatment group compared to the post-treatment group and no treatment control. There was no difference in viable Mtb infected cells between D8 treated groups and there appeared to be a decrease in live Mtb in the WLBU2 post-treatment group compared to the pre-treatment group (Figure 14). These differences were not significant compared to the no peptide treated control.

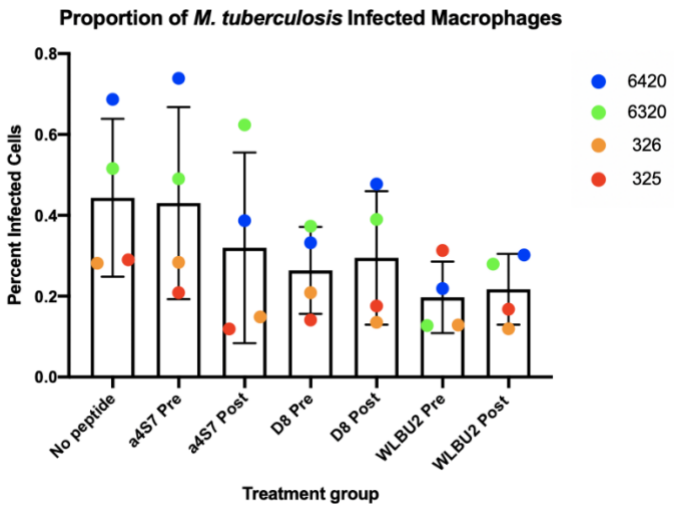

Figure 13: Proportion of Mtb infected monocyte derived macrophages after pre-treatment or post-treatment with eCAPs.

Proportion of macrophages infected with the live/dead reporter strain of Mtb after treatment with 10μM of α4S7, D8, and WLBU2 either before or after infection. Non-parametric Friedman Test was used to determine significance between no peptide control and each condition. $P<0.05$.

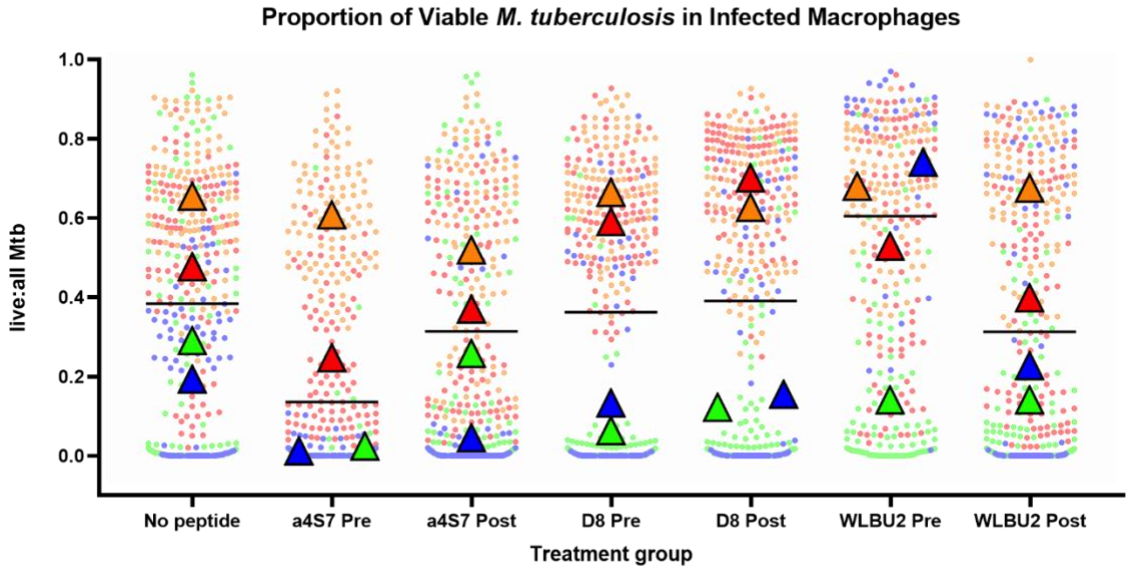

Figure 14: Proportion of monocyte-derived macrophages containing viable Mtb after pre-treatment or post-treatment with eCAPs.

Superplot of the proportion of macrophages infected with viable cells of the live/dead reporter strain of Mtb after treatment with 10μM of α4S7, D8, and WLBU2 either before or after infection. Non-parametric Friedman Test was used to determine significance between no peptide control and each condition. $P<0.05$.

4.2.3 Aim II Conclusions

In this aim, we observed human cell lines are permeabilized by eCAP. High concentrations of eCAPs are cytotoxic to human cell lines and can degrade THP-1s starting at 10 μM. Monocyte-derived macrophages are also permeabilized by eCAPs, but are more resistant to permeabilization and degradation than cell lines. These experiments also show that eCAP treatment has a statistically insignificant effect on Mtb intracellular infection of macrophages.

5.0 Discussion

5.1 eCAPs Do Not Inhibit Replication or Induce Bacterial Killing on Mtb and *M. bovis* BCG

Antimicrobial peptides have a well-established, potent activity against bacteria and other pathogens (23, 24). eCAPs have been engineered to have enhanced activity against bacterial pathogens and have shown great activity against gram positive organisms, but their function against mycobacteria has not been investigated (48, 49, 54). We incubated Mtb, *M. bovis* BCG, and *E. coli* with three eCAPs, α4S7, D8, and WLBU2, at several concentrations and plotted growth curves for each bacterium across several timepoints. While *E. coli* showed no replication after 4 hours at concentrations above 2.5 μM for all eCAPs, Mtb and *M. bovis* BCG only showed a slight reduction in replication after treatment with D8. Mtb and *M. bovis* BCG were more resistant to treatment with α4S7 and WLBU2. *E. coli* is more susceptible to eCAP treatment, which could be due to structure of the bacterium. Antimicrobial peptides and eCAPs function by targeting the cell wall or cell membrane and forming pores on the surface to kill the bacteria (25, 26). *E. coli's* cell wall is a less complex structure than the membrane of Mtb and *M. bovis* BCG, causing the eCAPs to be more effective against it than the mycobacteria.

Plating the bacteria after eCAP treatment allowed us to assess whether eCAPs were killing the bacteria in addition to inhibiting replication. Both α4S7 and D8 killed *E. coli* at 2.5μM and above or 20 μM, respectively. The CFU/mL of *M. bovis* BCG at any concentration for all eCAPs did not differ from the no peptide control, suggesting that bacterial killing was not occurring after

eCAP treatment. This could also be due to the differences in cell wall and cell membrane structure, preventing the eCAPs from attacking the *M. bovis* BCG as effectively as against *E. coli*.

5.2 eCAPs Do Not Permeabilize Mtb and *M. bovis* BCG As They Permeabilize *E. coli*

The bactericidal activity of antimicrobial peptides is accomplished by forming pores on the surface of target bacteria (26). We proposed that eCAPs function similarly against Mtb, *M. bovis* BCG, and *E. coli* and assessed this by using a membrane impermeable dye that would only stain cells that had been permeabilized. *E. coli* was permeabilized by all eCAPs and was significantly more permeabilized compared to the no peptide control at 2.5 μM for D8 and WLBU2 and 1.25 μM for α4S7. *E. coli* was completely degraded by α4S7 at all concentrations above 2.5 μM. The bacteria were completely degraded at higher concentrations of α4S7, leaving insufficient samples for flow cytometry analysis. While there appears to be an increase in permeabilization of Mtb and *M. bovis* BCG as the concentration of all eCAPs increase, we cannot confirm that the eCAPs are responsible for the permeabilization.

Morphology analysis of *E. coli* and *M. bovis* BCG after eCAP treatment showed that individual cells increased in length as the concentration of eCAP increased. The length increase of *E. coli* surpassed the range of size for the untreated control cells, while *M. bovis* BCG reached the upper limit of the size of untreated cells. Antimicrobial peptides can interfere with bacteria internally, in addition to targeting the cellular surface; several are involved in inhibiting DNA replication, protein synthesis, and cellular morphology (55). Some antimicrobial peptides have been observed elongate *E. coli* and inhibit cellular targets involved in cellular division, causing the *E. coli* to replicate despite the inability divide (56, 57). Human α-defensins have been observed to

traverse the outer and inner membranes of *E. coli*, inducing cellular damage and causing cellular elongation, blebbing, and clumping of cells (58, 59). The morphological changes induced by the eCAPs on *E. coli* and *M. bovis* BCG could be due to peptide interference with similar cellular damage.

5.3 eCAPs Synergize with Penicillin/Streptomycin to Improve Their Activity Against *M. bovis* BCG

Antimicrobial peptides and eCAPs function by forming pores in the bacterial cell wall and membrane to kill the bacteria (26). We proposed that the pores formed by eCAPs would allow antibiotics to attack the bacteria faster and would kill bacteria at a lower concentration than bacteria not treated with eCAPs. Incubation with eCAPs in *E. coli* resulted in an increase in antibiotic sensitivity, with samples incubated with α4S7 and WLBU2 having a lower concentration to inhibit growth than antibiotic only treated control. Similar to the lack of permeabilization by eCAPs, the lowest concentration to inhibit growth of *M. bovis* BCG did not decrease after incubation with D8 or WLBU2, and only decreased by tenfold after incubation with α4S7. These data suggest that eCAPs permeabilization of *M. bovis* BCG is not strong, but also suggest that eCAP treatment can synergize with antibiotics to increase mycobacterial antibiotic sensitivity. This is only a representation of a potential application of eCAPs. The concentration of eCAPs used was effective against the bacteria on its own and future experiments will be completed with different concentrations of eCAPs.

5.4 Human Cell Lines and Monocyte-Derived Macrophages are Degraded by High Concentrations of eCAPs

As a potential therapeutic, we need to understand how antimicrobial peptides and eCAPs are tolerated by mammalian cells. After incubation with eCAPs, U937, THP-1, and A549 cells were unaffected by concentrations below 5 μM but were significantly permeabilized when treated with eCAPs at higher concentrations. THP-1s appear to decrease in permeabilization between 10 μM and 40 μM and appear to not be significantly different from the untreated control cells. This is due to the cells being degraded at those concentrations and were unable to analyze using flow cytometry. To visualize what was happening to the cells after eCAP treatment, THP-1 cells were also stained and observed using microscopy. At 10 μM and above, the THP-1s were permeabilized and degraded. For eCAPs to have therapeutic potential, the concentration of peptide must be effective against bacteria, but not have any negative effects on the host cells. This data suggests that concentration below 5μM could be tolerated by host cells and effective as a therapeutic.

In addition to using cell lines, monocyte-derived macrophages were also used to investigate eCAP-mediated cytotoxicity in primary cells. While human cell lines are easy to use and are effective tools, they are derived from cancer cells and may have plasma membrane-specific defects that make them more or less susceptible to eCAPs than non-transformed cells (60-62). Monocyte-derived macrophages from cynomolgus macaques have normal cell functions and are more comparable to cells within a host, so we believed that we should test these as well. The macrophages were more resistant to the eCAPs than the cell lines. This could be due to the eCAPs targeting negatively charged structures that are present on bacteria and cancer cells, leading them to be more effective against cancer cell lines (63). Macrophages from most animals were resistant

to the eCAPs up to 10μM, but there were differences between individuals. The difference between individuals here recapitulates the differences between individuals who would receive these as a therapeutic, and highlight the need to consider this while developing a therapeutic.

5.5 eCAPs do not Prevent Intracellular Mtb Infection

As an intracellular pathogen, a therapeutic for Mtb must be able to treat intracellular infection and target extracellular bacteria, preventing infection. We performed experiments investigating the ability of eCAPs to both treat and prevent infection using a live/dead reporter strain of Mtb. Treatment with eCAPs before or after Mtb infection in macrophages did not significantly reduce the proportion of infected cells or the proportion of macrophages infected with viable Mtb. These experiments only used macrophages from four cynomolgus macaques, which aligned into two distinct populations, those with higher proportions of infected cells and those with lower. Due to laboratory shutdowns due to the SARS-CoV-2 pandemic, experiments with additional animal samples were unable to be completed. We believe with additional experiments with more samples, we will be able to see a trend correlating eCAP treatment and infection.

5.6 Public Health Significance

TB is a global health concern that affected nearly 10 million people in 2018 alone. Due to the burden of disease for the world's population, risk to compromised individuals, including people living with HIV, and the increase in incidence of MDR and XDR strains of Mtb, there is a need

for alternative treatments in addition to traditional antibiotics. eCAPs may be a promising alternative, targeting the cell surface to kill the bacteria rather than interfering with internal cell functions.

5.7 Future Directions

Further investigations on the effectiveness of eCAPs would focus on their toxicity to mammalian host cells of Mtb and their effects on treating and preventing intracellular infection. While human cell lines and monocyte-derived macrophages are good models of host cells, we would want to assess eCAP activity on lung cell populations, like alveolar macrophages or cells from bronchoalveolar lavage. Mtb typically infects the lungs, and understanding eCAP activity in that cellular environment is essential to understanding their therapeutic potential. We would also continue to assess the effect of eCAPs in our live/dead infection model using cells from additional animals, which would allow us to identify a trend in the data. Additionally, only three types of eCAPs were used here. eCAPs can be designed with different formulations to better target bacteria. We would want to test different types of eCAPs against Mtb and design eCAPs specifically for Mtb to determine if those are more effective than the ones tested here.

Bibliography

1. Chai Q, Zhang Y, Liu C. 2018. Mycobacterium tuberculosis: An Adaptable Pathogen Associated With Multiple Human Diseases. Front Cell Infect Microbiol 8.

2. WHO. 2019. Global tuberculosis report 2019. World Health Organization,

3. Barberis I, Bragazzi N, Galluzzo L, Martini M. 2017. The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. J Prev Med Hyg 58:E9-E12.

4. Diedrich CR, Flynn JL. 2011. HIV-1/mycobacterium tuberculosis coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun 79:1407-17.

5. Getahun H, Gunneberg C, Granich R, Nunn P. 2010. HIV infection-associated tuberculosis: the epidemiology and the response. Clin Infect Dis 50.

6. Sulis G, Roggi A, Matteelli A, Raviglione M. 2014. Tuberculosis: Epidemiology and Control. Mediterr J Hematol Infect Dis 6.

7. Shiloh M. 2016. Mechanisms of mycobacterial transmission: how does Mycobacterium tuberculosis enter and escape from the human host. Future Microbiol 11:1503-1506.

8. Turner R, Bothamley G. 2015. Cough and the transmission of tuberculosis. J Infect Dis 211:1367-1372.

9. Lee S. 2016. Tuberculosis Infection and Latent Tuberculosis. Tuberc Respir Dis 79:201-206.

10. Abrahams KA, Besra GS. 2018. Mycobacterial cell wall biosynthesis: a multifaceted antibiotic target. Parasitology 145:116-133.

11. Hansen J, Glochin S, Veyrier F, Domenech P, Boneca I, Azad A, Rajaram M, Schlesinger L, Divangahi M, Reed M, Behr M. 2014. N-Glycolylated Peptidoglycan Contributes to the Immunogenicity but Not Pathogenicity of Mycobacterium tuberculosis. J Infect Dis 209:1045-1054.

12. Houben R, Dodd P. 2016. The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. PLoS Med 13:e1002152.

13. Sandhu G, Battaglia F, Ely B, Athanasakis D, Montoya R, Valencia T, Gilman R, Evans C, Friedland J, Fernandez-Reyes D, Agranoff D. 2012. Discriminating Active from Latent Tuberculosis in Patients Presenting to Community Clinics. PLoS One 7.

14. Shea K, Kammerer S, Winston C, Navin T, Horsburgh C. 2014. Estimated rate of reactivation of latent tuberculosis infection in the United States, overall and by population subgroup. Am J Epidemiol 179:216-225.

15. Smith I. 2003. Mycobacterium tuberculosis Pathogenesis and Molecular Determinants of Virulence. Clin Microbiol Rev 16:463-496.

16. Ehlers S, Schaible U. 2012. The Granuloma in Tuberculosis: Dynamics of a Host–Pathogen Collusion. Front Immunol 3.

17. Lalvani A. 2007. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest 131:1898-1906.

18. dePerio M, Tsevat J, Roselle G, Kralovic S, Eckman M. 2009. Cost-effectiveness of interferon gamma release assays vs tuberculin skin tests in health care workers. Arch Intern Med 26:179-187.

19. Zulma A, Nahid P, Cole S. 2013. Advances in the development of new tuberculosis drugs and treatment regimens. Nat Rev Drug Discov 12:388-404.

20. Vernon A, Fielding K, Savic R, Dodd L, Nahid P. 2019. The importance of adherence in tuberculosis treatment clinical trials and its relevance in explanatory and pragmatic trials. PLoS Med 16.

21. Gandhi N, Nunn P, Dheda K, Schaaf H, Zignol M, Soolingen Dv, Jensen P, Bayona J. 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet 375:1830-1843.

22. Seung K, Keshavjee S, Rich M. 2015. Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. Cold Spring Harb Prospect Med 5.

23. Bahar AA, Ren D. 2013. Antimicrobial peptides. Pharmaceuticals (Basel) 6:1543-75.

24. Mahlapuu M, Hakansson J, Ringstad L, Bjorn C. 2016. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Front Cell Infect Microbiol 6:194.

25. Lei J, Sun L, Huang S, Zhu C, Li P, Mackey V, Coy D, He Q. 2019. The antimicrobial peptides and their potential clinical applications. Am J Transl Res 11:3919-3931.

26. Omardien S, Brul S, Zaat S. 2016. Antimicrobial Activity of Cationic Antimicrobial Peptides against Gram-Positives: Current Progress Made in Understanding the Mode of Action and the Response of Bacteria. Front Cell Dev Biol 14.

27. Schmitt P, Rosa RD, Destoumieux-Garzon D. 2016. An intimate link between antimicrobial peptide sequence diversity and binding to essential components of bacterial membranes. Biochim Biophys Acta 1858:958-70.

28. Jenssen H, Hamill P, Hancock R. 2006. Peptide antimicrobial agents. Clin Microbiol Rev 19:491-511.

29. Bolintineanu D, Kazessis Y. 2011. Computational studies of protegrin antimicrobial peptides: a review. Peptides 32:188-201.

30. Zhang L, Rozek A, Hancock R. 2001. Interaction of actionic antimicrobial peptides with model membranes. J Biol Chem 276:35714-35722.

31. Kai-Larson Y, Agerberth B. 2008. The role of the multifunctional peptide LL-37 in host defense. Front Biosci 13:3760-3767.

32. Torres-Juarez F, Cardenas-Vargas A, Montoya-Rosales A, Gonzalez-Curiel I, Garcia-Hernandez M, Enciso-Moreno J, Hancock R, Rivas-Santiago B. 2015. LL-37 Immunomodulatory Activity During Mycobacterium Tuberculosis Infection in Macrophages. Infect Immun 83:4495-4503.

33. Rivas-Santiago B, Hernandez-Pando R, Carranza C, Juarez E, Contreras JL, Aguilar-Leon D, Torres M, Sada E. 2008. Expression of cathelicidin LL-37 during Mycobacterium tuberculosis infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. Infect Immun 76:935-41.

34. Faridgohar M, Nikoueinejad H. 2017. New findings of Toll-like receptors involved in Mycobacterium tuberculosis infection. Pathog Glob Health 111:256-264.

35. Majewski K, Agier J, Kozlowska E, Brzezinska-Blaszczyk E. 2017. Serum level of cathelicidin LL-37 in patients with active tuberculosis and other infectious diseases. J Biol Regul Homeost Agents 31:731-736.

36. Jacobsen M, Repsilber D, Gutschmidt A, Neher A, Feldmann K, Mollenkopf H, Ziegler A, Kaufmann S. 2007. Candidate biomarkers for discrimination between infection and disease caused by Mycobacterium tuberculosis. J Mol Med 85:613-621.

37. Driss V, Legrand F, Hermann E, Loiseau S, Guerardel Y, Kremer L, Adam E, Woerly G, Dombrowicz D, Capron M. 2009. TLR2-dependent eosinophil interactions with mycobacteria: role of alpha-defensins. Blood 113:3235-3244.

38. Kisich K, Higgins M, Diamond G, Heifets L. 2002. Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils. Infect Immun 70:4591-4599.

39. Rivas-Santiago B, Schwander S, Sarabia C, Diamond G, Klein-Patel M, Hernandez-Pando R, Ellner J, Sada E. 2005. Human β-Defensin 2 Is Expressed and Associated with Mycobacterium tuberculosis during Infection of Human Alveolar Epithelial Cells. Infect Immun 73:4505-4511.

40. Moravej H, Moravej Z, Yazdanparast M, Heiat M, Mirhosseini A, Moghaddam MM, Mirnejad R. 2018. Antimicrobial peptides: features, action, and their resistance mechanisms in bacteria. Microb Drug Resist 24:747-767.

41. Starr C, Wimley W. 2017. Antimicrobial peptides are degraded by the cytosolic proteases of human erythrocytes. BBA-Biomembranes 1859:2319-2326.

42. Lee D, Hodges R. 2003. Structure-activity relationships of de novo designed cyclic antimicrobial peptides based on gramicidin S. Biopolymers 71:28-48.

43. Rathinakumar R, Walkenhorst W, Wimley W. 2009. Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: the importance of interfacial activity. J Am Chem Soc 131:7609-7617.

44. Miller M, Cloyd M, Liebmann J, Jr. CR, Islam K, Wang S, Mietzner T, Montelaro R. 1993. Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. Virology 196:89-100.

45. Tencza S, Creighton D, Yuan T, Vogel H, Montelaro R, Mietzner T. 1999. Lentivirus-derived antimicrobial peptides: increased potency by sequence engineering and dimerization. J Antimicrob Chemother 44:33-41.

46. Tencza S, Douglass J, Creighton D, Montelaro R, Mietzner T. 1997. Novel antimicrobial peptides derived from human immunodeficiency virus type 1 and other lentivirus transmembrane proteins. Antimicrob Agents Chemother 41:2394-2398.

47. Vogel H, Schibli D, Jing W, Lohmeier-Vogel E, Epand R, Epand R. 2002. Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. Biochem Cell Biol 80:49-63.

48. Deslouches B, Phadke S, Lazarevic V, Cascio M, Islam K, Montelaro R, Mistzner T. 2005. De Novo Generation of Cationic Antimicrobial Peptides: Influence of Length and Tryptophan Substitution on Antimicrobial Activity. Antimicrob Agents Chemother 49:316-322.

49. Deslouches B, Islam K, Craigo JK, Paranjape SM, Montelaro RC, Mietzner TA. 2005. Activity of the de novo engineered antimicrobial peptide WLBU2 against Pseudomonas aeruginosa in human serum and whole blood: implications for systemic applications. Antimicrob Agents Chemother 49:3208-16.

50. Kumagai A, Dupuy FG, Arsov Z, Elhady Y, Moody D, Ernst RK, Deslouches B, Montelaro RC, Di YP, Tristram-Nagle S. 2019. Elastic behavior of model membranes with antimicrobial peptides depends on lipid specificity and d-enantiomers. Soft Matter 15:1860-1868.

51. Chen C, Deslouches B, Montelaro R, Di Y. 2018. Enhanced efficacy of the engineered antimicrobial peptide WLBU2 via direct airway delivery in a murine model of Pseudomonas aeruginosa pneumonia. Clinical Microbiology and Infection 24:547.e1-547.e8.

52. Di Y, Lin Q, Chen C, Montelaro R, Doi Y, Deslouches B. 2020. Enhanced therapeutic index of an antimicrobial peptide in mice by increasing safety and activity against multidrug-resistant bacteria. Sci Adv 6.

53. Dulberger CL, Rubin EJ, Boutte CC. 2020. The mycobacterial cell envelope - a moving target. Nat Rev Microbiol 18:47-59.

54. Mandell JB, Deslouches B, Montelaro RC, Shanks RMQ, Doi Y, Urish KL. 2017. Elimination of Antibiotic Resistant Surgical Implant Biofilms Using an Engineered Cationic Amphipathic Peptide WLBU2. Sci Rep 7.

55. Le C, Fang C, Sekaran S. 2017. Intracellular Targeting Mechanisms by Antimicrobial Peptides. Antimicrob Agents Chemother 61:e02340-16.

56. Ishikawa M, Kubo T, Natori S. 1992. Purification and characterization of a diptericin homologue from Sarcophaga peregrina (flesh fly). Biochem J 287:573-578.

57. Salomon R, Farias R. 1992. Microcin 25, a novel antimicrobial peptide produced by Escherichia coli. J Bacteriol 174:7428-7435.

58. Moser S, Chileveru H, Tomaras J, Nolan E. 2014. A bacterial mutant library as a tool to study the attack of a defensin peptide. Chembiochem 15:2684-2688.

59. Wanniarachchi Y, Kaczmarek P, Wan A, Nolan E. 2011. Human defensin 5 disulfide array mutants: disulfide bond deletion attenuates antibacterial activity against Staphylococcus aureus. Biochemistry 50:8005-8017.

60. Alge C, Hauck S, Pringlinger S, Kampik A, Ueffing M. 2006. Differential protein profiling of primary versus immortalized human RPE cells identifies expression patterns associated with cytoskeletal remodeling and cell survival. J Proteome Res 5:862-878.

61. Lorsch J, Collins F, Lippincott-Schwartz J. 2014. Fixing problems with cell lines. Science 346:1452-1453.

62. Pan C, Kumar C, Bohl S, Klingmueller U, Mann M. 2009. Comparative Proteomic Phenotyping of Cell Lines and Primary Cells to Assess Preservation of Cell Type-specific Functions. Mol Cell Proteomics 8:443-450.

63. Deslouches B, Montelaro R, Urish K, Di Y. 2020. Engineered Cationic Antimicrobial Peptides (eCAPs) to Combat Multidrug-Resistant Bacteria. Pharmaceutics 12:E501.