Intranasal Administration of PcrV Conjugated to a Tobacco Mosaic Virus Delivery Platform Protects against *P. aeruginosa* in a Murine Model of Acute Pneumonia

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List of Abbreviation

AHR	Aryl Hydrocarbon Receptor
Alg	Alginate
APS	Antigen Presenting Cells
ATCC	American Type Tissue Culture Collection
BAL	Bronchoalveolar Lavage Fluid
COPD	Chronic Obstructive Pulmonary Disease
CF	Cystic Fibrosis
CFU	Colony Forming Units
CFTR	Cystic Fibrosis transmembrane Conductance regulator
DCs	Dendritic Cells
ELISA	Enzyme-Linked Immunosorbent Assay
HAP	Hospital-Acquired Pneumonia
IFN-γ	Interferon Gamma
Ig	Immunoglobin
IL	Interleukin
i.n.	Intranasal
KLH	Keyhole Limpet Hemocyanin
LD	Lethal Dose
LPS	Lipopolysaccharide
NK	Natural Killer Cells
NPs	Nanoparticles
OD	Optical Density
OMPs	Outer Membrane Proteins
PMA	Poly Mannuronic Acid
PMN	Polymorphonuclear Neutrophils
QS	Quorum Sensing
SDIC	Severe Combined Immunodeficiency
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide

	Gel Electrophoresis
TGF-β	Transforming Growth Factor Beta
TLR	Toll-Like Receptor
TMV	Tobacco Mosaic Virus
TNF-α	Tumor Necrosis Factor Alpha
T3SS	Type III Secretion System
VAP	Ventilator Acquired Pneumonia

Abstract:

Pseudomonas aeruginosa is a facultative anaerobic bacterium that is the most common type of Gram-negative bacteria causing nosocomial pneumonia in the United States. *P. aeruginosa* is an opportunistic pathogen ubiquitously found in the atmosphere that can colonize both plants and animals. It is known to cause a wide range of severe, persistent, and fatal infections ranging from acute and chronic pneumonia, skin and soft tissue infections to urinary tract infections, especially in immune-compromised individuals. Patients with cystic fibrosis are at high risk because of deficiencies in mucosal immunity and phagocytosis. Ultimately, *Pseudomonas* infections can be lethal. There is a very high level of antibiotic resistance in *P. aeruginosa* leading to many attempts to design a vaccine. However, no licensed vaccine has been approved or is commercially available. In this study we utilized the tobacco mosaic virus platform delivery system to develop a vaccine against *P. aeruginosa*.

The vaccine consists of the tip protein of the *Pseudomonas* type 3 secretion system, PcrV, conjugated to the coat protein of recombinant TMV. We vaccinated mice by administering 20µg of vaccine conjugate (TMV-PcrV) or 10µg of unconjugated rPcrV in PBS by intranasal droplet (IN) to anesthetized mice. In the first experiment mice were vaccinated on days 0, 14, and 28. In the second experiment, 2ug of di-cyclic-GMP adjuvant was included in the vaccine groups and were administered on days 0, 21, and 35. Vaccinated mice were divided into two groups of equal numbers, each challenged IN in a model of acute pneumonia with lethal doses of *P. aeruginosa* Boston 41510 ($1x10^7$ or $1x10^8$ CFU) seven days after the final boost. Mice were monitored daily for morbidity and mortality.

Strong total antibody response was detected in mice vaccinated with both TMV-PcrV vaccine and TMV-PcrV + di-c-GMP adjuvant compared to unvaccinated mice and

mice vaccinated with unconjugated rPcrV. All mice that were vaccinated without adjuvant that were challenged with $1x10^8$ CFU of *P. aeruginosa* succumbed to infection, though a 2-day right shift in mean-time-to death was observed in TMV-PcrV vaccinated mice compared to rPcrV vaccinated. TMV-PcrV vaccinated mice challenged with $1x10^7$ CFU had an 80% survival rate and regained their body weight by day 2.5, while all other mice succumbed. Challenge results with mice vaccinated in the presence of adjuvant enhanced the antibody production for both rPcrV and TMV-PcrV, consequently enhancing the survival rate. These results demonstrated the potential of TMV as a protein subunit vaccine delivery system for *P. aeruginosa* antigens. Collectively, TMV conjugation induced a strong humoral immune response and enhanced vaccine-mediated protection against *P. aeruginosa* in an acute pneumonia model, providing proof of principle for a TMV-mediated vaccine against this antibiotic-resistant nosocomial pathogen.

1.0 Introduction:

1.1 *Pseudomonas aeruginosa* and Respiratory Infection

P. aeruginosa is the most common type of gram-negative bacteria causing nosocomial pneumonia in the United States (Curran *et al.,* 2018). It has been reported that nosocomial pneumonia caused13% to 50% of mortality (Curran *et al.,* 2018). Pneumonia could be either hospital-acquired pneumonia (HAP) or Ventilator acquired pneumonia (VAP) (Curran *et al.,* 2018; Kalil *et al.,* 2016). Furthermore, *P. aeruginosa* is a ubiquitous pathogen in the environment that can colonize diverse life forms, including plants and animals (Tümmler & Klockgether, 2017). It is an opportunistic pathogen that survives harsh environmental conditions such as the lung. It infects airways and causes a respiratory infection that is classified as either acute or chronic (Gellatly & Hancock, 2013 ; Williams *et al*., 2010 ; Lavoie *et al.,* 2011). This bacterial infection generally does not infect healthy people and is only limited to patients with immune system defects or those who are immunocompromised (Williams *et al*., 2010).

1.1.1 Acute Pneumonia (Ventilator-Associated Pneumonia)

Ventilator-associated pneumonia is the most common infection that occurs in the intensive care unit among critical patients. It is attributed to a significant mortality rate of approximately 13.5%. *P. aeruginosa* is the prevalent causative agent of VAP. A recent study by Micek *et al.*(2012) reported that *Pseudomonas* VAP mortality has increased to around 41.9% in patients over the age of sixty-five (Ramírez-Estrada *et al.,* 2016). However, different serotypes of *Pseudomonas* behave differently: the percent mortality with O6 and O11, which are the common serotypes, were lower compared to the O1 and O2. Furthermore, the clinical resolution of O6 and O11 was 75% and 57%, respectively. On the other hand, in the less common stereotypes, O1 showed higher mortality than O2, which accounts for 40% of infection versus mortality; meanwhile, the clinical resolution appears to be significantly better with O2 (Lu *et al.,* 2014). Acute nosocomial pneumonia is associated with a VAP patient whose epithelial cells are damaged as a result of direct trauma due to the insertion of an endotracheal tube that can be a reservoir for *P. aeruginosa* and cause breached epithelium (Gellatly & Hancock, 2013). In this case, *P. aeruginosa* grows as biofilm, which increases its resistance to antibiotics and disinfectants (Gellatly & Hancock, 2013 ; Williams *et al.,* 2010). Acute pneumonia is more likely in those who lack an efficient immune response, including old age, or immunosuppression due to organ transplantation (Gellatly & Hancock, 2013). In the 2005 American Thoracic Society and Infectious Diseases Society of America guidelines, it was recommended to combine the antipseudomonal agents' cephalosporin (cefepime, ceftazidime) and fluoroquinolone (ciprofloxacin or levofloxacin) for treatment of *P. aeruginosa* VAP; however, this was inappropriate due to inadequate dosage that was determined by Therapeutic Drug Monitoring and suboptimal tolerance to antibiotics (Ramírez-Estrada *et al.,* 2016). In 2013, ceftazidime- avibactam showed efficient action in the treatment of nosocomial infection against *P. aeruginosa* in the murine model. It penetrated the epithelium lining fluid and

produced a reduction against *P. aeruginosa* with minimum inhibitory concentration (MIC) up to 32 µg/ml (Housman *et al.,* 2014).

1.1.2 Chronic Pneumonia (CF Patient)

Chronic pneumonia is known as non-acute pneumonia, but the characteristics that distinguish them are still unclear (Williams *et al.,* 2010). Over the last years, several studies have compared the detection of cystic fibrosis (CF) pathogens in lower airways and deep throat cultures (Lyczak *et al.,* 2002). A study that found a high percentage of oropharyngeal culture detected the presence of *Pseudomonas* in lower airways, which was determined by Bronchoalveolar Lavage (BAL) culture; a low negative predictive value detected *Pseudomonas* in their BAL with negative throat cultures (Lyczak *et al.,* 2002). CF lung has an abnormal composition of airway secretions that expose CF patients to chronic colonization by *P. aeruginosa.* These secretions may have aberrant chloride concentration due to a cystic fibrosis transmembrane conductance regulator (CFTR) mutation (Lyczak *et al.,* 2002).

Results obtained by Tager *et al.*(1998) demonstrated that in the presence of a significant level of chloride concentrations, the phagocytotic activity of neutrophils is impaired in CF patients (Tager *et al.,*1998). Neutrophil is considered one of the primary cells that produces antimicrobial peptides. Once it is defective, the composition of airway secretions consequently affects the action of antimicrobial peptides (Lyczak *et al.,* 2002).

In the lung, *P. aeruginosa* colonizes patients with CF and chronic obstructive pulmonary disease (COPD) (Gellatly & Hancock, 2013 ; Curran *et al.,* 2018). If *P. aeruginosa* is not eliminated during acute infection, it gets adapted to the lung environment and causes chronic infection (Gellatly & Hancock, 2013). In people who get CF via mutation in CFTR thick airway surface liquid (ASL) is formed that in turn

impairs immune response and resists its stimulation (Gellatly & Hancock, 2013 ; Williams *et al.,* 2010). CFTR mutation could inhibit mucociliary clearance by stimulating bacterial adhesion to airway epithelium cells (Lyczak *et al.,* 2002). Comparing the nasal epithelial culture of CF and non-CF patients, CF patients were found to have a higher level of the ganglioside asialo-GM1, an epithelial cell receptor that binds to *P. aeruginosa* pili (Saiman & Prince, 1993). In addition, another study examined the ability of the (CFTR) mutation to control bacterial inflammation (Blohmke *et al.,* 2012).

Furthermore, *P. aeruginosa* is associated with people who have COPD that occurs as a result of the stretching of bronchial airways due to the destruction of muscles and elastic tissues (Gellatly & Hancock, 2013). The influence of this chronic inflammation of the lung is narrowing of the airways and ultimately leads to restricted airflow (Gellatly & Hancock, 2013). Frequently, COPD patients are elderly, and generally, their lung function is declining due to age (Gellatly & Hancock, 2013). Ultimately, COPD can contribute to death if it is not treated, which is why it is the fourth cause of mortality in the United States (Hurd, 2000).

1.1.3 Importance in HAI, Antibiotic Resistance, and as an ESKAPE Pathogen

ESKAPE is a group of Gram-negative and Gram-positive bacteria that cause nosocomial infection which could be transmitted by either direct or indirect contact among healthcare workers, visitors, patients and contaminated objects. It is associated with significant mortality and morbidity in the United States. In 2011, there were around 722,000 cases of ESKAPE hospital-acquired infection (HAI) and 75,000 referred to nosocomial infection. Another survey conducted in 2002, reported approximately 1.7 million patients suffered from ESKAPE hospital-acquired infection (HAI) that contributed to a high percentage of death. Additionally, these ESKAPE bacteria are

characterized by potent antimicrobial resistance that highly correlates to nosocomial infection among immunocompromised patients (Santajit & Indrawattana, 2016).

ESKAPE pathogens show resistance to antimicrobials through several mechanisms which makes them difficult to control (Curran *et al.,* 2018 ; Livermore, 2002). *Pseudomonas* VAP isolates have been shown to be around 33% resistant to fluoroquinolone, 30% resistant to carbapenem, and 18% were multidrug resistance (Priebe & Goldberg, 2014). Firstly, many bacteria produce enzymes such as β-lactamase which is the most predominant bacteria defense that acts irreversibly and inactivates antibiotics by hydrolyzing the β-lactams ring present in all B-lactams antibiotics (Jacoby & Munoz-Price, 2005). PSE-1 and PSE-4 are the most frequent β-lactamase enzyme genes obtained by *P. aeruginosa* (Livermore, 2002). Secondly, some bacteria such as *P. aeruginosa* express the impermeability outer membrane protein function as exporters called efflux pumps; an example is the OprF gene that mediates cell-associated pores (Benz & Hancock, 1981; Santajit & Indrawattana, 2016). The impermeability mediated resistance is reflected by the upregulation of the efflux system such as MexAB-OprM, which is chromosomally encoded, and has a major role in the disorganized cytoplasmic membrane, which leads to increase resistance to many antibiotics, such as carbapenems and fluoroquinolones (Livermore, 2002; Santajit & Indrawattana, 2016).

Furthermore, the outer membrane of Gram-negative bacteria contains proteins known as porins that form a channel to allow flowing hydrophilic substances like antibiotics (Santajit & Indrawattana, 2016). In *P. aeruginosa* studies, it has been illustrated that reducing the amount of OprD proteins (porin) leads to decreased drug flow inside the cell, which gives resistance to bacteria against imipenem (Fukuoka *et al.,* 1993). Ultimately, the biofilm most likely contributes to drug-resistance by the formation matrix of biofilm, which provides conditions that weaken the activity of the drug (e.g., low pH, low O_2 and high CO_2) therefore, under these conditions it is

challenging to clear bacteria using an antibiotic. Moreover, exposure to environmental conditions such as biofilm growth may change gene expression resulting in the upregulation of genes that produce resistance (Gellatly & Hancock, 2013).

1.2 Genome and Structural Components of *Pseudomonas aeruginosa*

1.2.1 *P. aeruginosa* Genome

The completed genome of *P. aeruginosa* strain PAO1 has a 5.57 Mbp size and 75-67% guanine-cytosine contents. It is made of a circular chromosome plus different plasmids (Klockgether *et al.,* 2011). Genome sequencing was accomplished by whole-genome shotgun sequencing. *Pseudomonas* has the largest sequenced genome among the 25 completed sequenced bacterial genome with 6.3 million base pairs. The genome sequence for the bacteria gives insight into the versatility and potential drug resistance of *P. aeruginosa*, as well as its biology and mechanisms of pathogenesis (Stover *et al.,* 2000).

Furthermore, *P. aeruginosa* genome contains a high percentage of regulatory genes, transporters, transcriptional regulators, and two-component system regulatory systems, which is consistent with its large genome and environmental adaptability (Klockgether *et al.,* 2011; Stover *et al.,* 2000). More than 500 regulatory genes were specified in the *P. aeruginosa* strain PAO1 and around 8.4% of genes have a predominant role in regulation, such as the two components system and transcriptional regulators. However, the genome sequence of PA14, revealed more virulence factors than the PAO1 strain (Stover *et al.,* 2000). Approximately 58 regions of PA14 were not detected in the PAO1 strain including PA14 pathogenicity islands PAPI-1 and PAPI-2 (Klockgether *et al.,* 2011).

In a comparative analysis study, they compared the *Pseudomonas* genome with the *E. coli* genome. The study illustrated that the large genome of *Pseudomonas* is due to the complexity of genes and not to the differences in genome organization. Additionally, they identified the largest repeats in the *Pseudomonas* genome that appeared in four ribosomal DNA loci and one duplicated gene that extended through a thousand base pairs. Moreover, compared with other bacterial genomes, they found that the *Pseudomonas* genome contains more diverse paralogous genes, around 50% than other bacteria. Interestingly, 372 open reading frames (ORFs) were identified in the *Pseudomonas* genome as functional genes that primarily encode for exopolysaccharide enzymes, virulence factors, and other proteins involved in adhesion and motility (Stover *et al.,* 2000). Each exopolysaccharide (Psl, Pel, and Alginate) is encoded on a different region in the genome (Franklin *et al.,* 2011).

Around 150 genes encode for outer membrane proteins (OMPs), which is considered a large number compared to other genomes; 34 genes are involved in iron-siderophore uptake and ten genes encode for Efflux pumps. Furthermore, *Pseudomonas* has a large efflux system which accounts for ten genes as compared to *E. coli* that has only four genes. Exceptionally, the only known sequenced bacteria that has a large number of genes encoded for outer membrane proteins (OMPs) is H. *pylori*. The identification of OMP family could be a considerable impact on the vaccine and antimicrobial research (Stover *et al.,* 2000). In addition to these, the *Pseudomonas* genome has the most complicated chemosensory system among all sequenced bacterial genomes, which is illustrated in the four loci involved in chemotaxis signal transduction (Stover *et al.,* 2000).

1.2.2 *P. aeruginosa* Lipopolysaccharide

Lipopolysaccharide (LPS) is a complex glycolipid that makes up the outer membrane of Gram-negative bacteria. It is considered one of the most important virulence factors(Pier, 2007) that acts as a physical barrier by direct interaction with host immune response, antibiotics, and host cell receptors (King *et al.,* 2009). LPS is composed of three domains: Lipid A, Core oligosaccharide, and O-specific antigen polysaccharide (O-A)(King *et al.,* 2009; Gellatly & Hancock, 2013; Pier, 2007). Furthermore, *P. aeruginosa* produces two forms of O antigen. The first is a homopolymer of D-rhamnose (D-Rha), known as A band or common O polysaccharide, which is approximately 70 sugars long and elicits a weak antibody response (King *et al.,* 2009; Gellatly & Hancock, 2013). The second is a heteropolymer of different distinct sugars in its repeat unit that is called B band (King *et al.,* 2009; Lam *et al.,* 2011). Conversely, this O-specific antigen produces a strong antibody response (King *et al.,* 2009).

Lipid A and O polysaccharide are the two components of LPS that contributed the most to *P. aeruginosa* infection (Gellatly & Hancock, 2013). Lipid A is comprised of a diglucosamine biphosphate backbone with O- and N-linked primary and secondary fatty acids (Gellatly & Hancock, 2013). It has Penta or Hexa acylated form within 12-14 carbons in length (King *et al.,* 2009; Pier, 2007). During the *P. aeruginosa* infection, recognition of LPS by Toll-Like Receptor (TLR4) occurs via binding lipid A to the host cell coreceptors (LPS-binding protein (LBP), CD14, and MD-2).(Gioannini & Weiss, 2007) to initiate the NF-κB signaling pathway, which triggers pro-inflammatory cytokines and chemokines and eventually causes endotoxic shock (King et al., 2009; Gellatly & Hancock, 2013). It has been shown that lipid A elevates IL8 expression via recognition of LPS by TLR4 (Hajjar *et al.,* 2002). Compared to enterobacterial, the inflammatory response elicited by lipid A in *P. aeruginosa* is considered low (King *et al.,* 2009). On the other hand, the dominant role of O-specific antigen is to protect

bacteria from being engulfed by phagocytosis (Dasgupta *et al.,* 1994), as well as to protect against oxidative stress (Berry *et al.,* 2009). Moreover, it has been reported that O polysaccharide has a notable engagement in surface attachment (Abeyrathne *et al.,* 2005).

1.2.3 Alginate Capsule

Pseudomonas alginate is first synthesized as a linear of acidic polymer consisting of D-mannuronic acid (Franklin *et al.,* 2011).It is modified in the periplasm by the action of O- acetylation and other proteins (e.g. AlgJ, AlgI, AlgF), which have converted D-mannuronic acid to l-guluronic acid by AlgG at the polymer level. Alginate ultimately becomes a linear of acidic polymer consisting of D-mannuronic acid and l-guluronic acid (Rasamiravaka *et al.,* 2015).

Acetylation can occur at the hydroxyl groups of either the C2 and/or C3 positions. This modification distinguishes *Pseudomonas* alginate from other exopolysaccharides such as Psl and Pel as well as from the *E. coli* capsule (Franklin *et al., 2011)*. *In vitro* assays showed a high level of alginate exists in the cell envelope, which demonstrated that both the inner and outer membrane were essential for alginate biosynthesis (Remminghorst & Rehm, 2006). This result is evidence of the multiple protein interaction around the 13 alginate form a complex that extends through the inner membrane, periplasm and the outer layer (Franklin *et al.,* 2011).

More experiments have proved that a multi-protein biosynthetic system exists for alginate biosynthesis. In 1998, a study by Jain and Ohman studied the phenotypes of periplasmic proteins (AlgK, AlgX, and AlgG). They found that the deletion of one of these genes resulted in the release of unpolymerized uronic acid subunits, which are assumed to have been degraded by alginate AlgL (Franklin *et al.,* 2011; Flynn & Ohman, 1988). These genes are a part of the biosynthetic complex; otherwise, the

synthetic polymer is degraded by AlgL if the complex is not present (Franklin *et al.,* 2011). In 1993, another study concluded that the deletion of cytoplasmic proteins (Alg8 and Alg44) resulted in no alginate production, which is in contrast to periplasmic proteins. Therefore, Alg8 and Alg44 are required for alginate polymerization (Franklin *et al.,* 2011; Maharaj *et al.,* 1993).

Alginate is a high molecular weight protein (Franklin *et al.,* 2011). It has a predominant role in biofilm stability and protection. Alginate also aids in the maintenance of water and nutrients (Rasamiravaka *et al.,* 2015). The significance of alginate in the pathology of CF has resulted in being the first exopolysaccharide defined and it remains the best characterized *P. aeruginosa* exopolysaccharide (Franklin *et al.,* 2011). Moreover, alginate biosynthesis does not require lipid presence, which makes it similar to bacterial cellulose synthesis (Römling, 2002).

1.3 Lifecycle of *Pseudomonas aeruginosa*

Pseudomonas biofilm formation aggregates bacterial communities together in a matrix of extracellular polymeric substances (EPS) on a surface (Rasamiravaka *et al.,* 2015). The regulation of biofilm formation is controlled by the quorum-sensing system (Gellatly & Hancock, 2013; Moradali *et al.,* 2017) where different biofilm can be developed depending on the nutritional factors as well as *Pseudomonas* strains (Shrout *et al.,* 2006). Biofilm formation by *P. aeruginosa* (PAO1) is achieved through five stages. It starts with reversible adhesion, which occurs by planktonic bacterial cells attaching to a site to get ready for growth (stage I). This is followed by stage II: maturation and growth occur by irreversible attachment of bacteria and formation of microcolonies in the EPS matrix, which then mature to multi-layered cells. In stage III, microcolonies start to extend and form a more structured phenotype in a non-colonized space. Thereafter, the non-colonized space fills with bacteria until the total surface

area is covered (stage IV). Finally, in stage (V) the bacteria scatter and detach then reenter a planktonic state to settle other surfaces (Santajit & Indrawattana, 2016; Rasamiravaka *et al.,* 2015). Detachment can happen either actively, initiated by bacteria itself, or passively which is caused by outside forces (Santajit & Indrawattana, 2016).

Interestingly, *Pseudomonas* polysaccharides (Psl and Pel) contribute to biofilm stability and persistence (Ryder *et al.,* 2007) and act as a primary structure of biofilm development, which is involved in the early stage of biofilm formation (Colvin *et al.,* 2011). Besides polysaccharide, alginate takes part in holding biofilm cells together on the EPS matrix (Ryder *et al.,* 2007). Several studies have demonstrated the role of extracellular DNA in biofilm formation since bacteria that lack eDNA are more sensitive for degradation by detergent sodium dodecyl sulfate (SDS). eDNA plays an important role in protecting biofilm against DNase I and is a source of nutrients for bacteria during starvation *(Rasamiravaka et al.,* 2015).

1.4 Virulence Factors of *Pseudomonas aeruginosa*

A previous study revealed that *P. aeruginosa* isolated from acute infection has a different phenotype than chronic infection (Smith *et al.,* 2006). Moreover, genetic characteristics of the bacterial cell that presents in the early phase of chronic infection in CF patients are distinct from those that initiate the infection later (Hogardt & Heesemann, 2010; Smith *et al.,* 2006). What is clear is that virulence factors influence the severity of infection (Gellatly & Hancock, 2013). Several bacterial factors alter immune-inflammatory effects that are reduced during the switch from acute to chronic infection, such as pili and flagella (Smith *et al.,* 2006; Nguyen & Singh, 2006). The *P. aeruginosa* isolated from acute infection generally express more virulence factors than those isolated from chronic infection (Gellatly & Hancock, 2013).

The type III secretion system is the major virulence determinant in *Pseudomonas,* which impacts cell cytotoxicity. The PcrV is located at the tip of the T3SS needle, which resembles a molecular syringe. Upon contact with host cells it can transport multiple virulence factors directly into host cells. Developing antibodies against PcrV prevents T3S-mediated cytotoxicity (Tabor *et al.,* 2018)

Furthermore, the type III secretion system is downregulated in chronic CF *P. aeruginosa* infection (Hogardt & Heesemann, 2010). Effector proteins are secreted by the type III secretion system :ExoS, ExoT, and ExoU, which affect actin cytoskeletal, block reactive oxygen species and cause rapid death of host cell, respectively (Kipnis *et al.,* 2006; Tümmler & Klockgether, 2017; Hauser, 2009).

OprF is an outer membrane porin of *Pseudomonas aeruginosa*. Based on the comparative study of wild-type and isogenic oprF mutant of *P. aeruginosa*, OprF is required for *P. aeruginosa* virulence. The absence of OprF results in impaired adhesion to animal cells, secretion of ExoT and ExoS toxins through the T3SS, and production of the quorum-sensing-dependent virulence factors (Fito-Boncompte *et al.,* 2011). The small and abundant OprI lipoprotein was also shown to be involved in cell shape and maintain outer membrane stability. An oprI mutant produced a higher outer membrane vesicle than the wild-type strain, however its *Pseudomonas* quinolone signal (PQS) production was unaffected. This indicates that the absence of OprI stimulated OMV biogenesis by reducing the outer membrane tethering to peptidoglycan. Conversely, OprF mutant of PA14 led to the higher OMV biogenesis level as a result of an increase in PQS production(Chevalier *et al.,* 2017).

A recent study has shown that in chronic CF *P. aeruginosa* infection the bacteria has a deficiency in pili and flagella which are required to initiate colonization. This aids bacteria in avoiding immune recognition as well as clearing by phagocytosis (Hogardt & Heesemann, 2010). In mutagenesis studies, a mutation in the rpoN gene led to the loss of both flagella and pili (Hogardt & Heesemann, 2010).

Several proteases secreted by *P. aeruginosa* have a major role in degrading immunoglobin by cleavage the Fc region of antibodies,(Kipnis *et al.,* 2006), host complement protein(Gellatly & Hancock, 2013; Tümmler & Klockgether, 2017) (Laarman et al., 2012) and disrupt tight junction in epithelial cells (Kipnis *et al.,* 2006). Additionally, they inhibit recognition by alveolar macrophages, which promote intracellular survival (Gellatly & Hancock, 2013; Malloy *et al.,* 2005). Taken together, *P. aeruginosa* has diverse effector proteins that contribute to its pathogenicity.

1.5 *Pseudomonas aeruginosa* Vaccine Development

1.5.1 *P. aeruginosa* Vaccines for CF patients

P. aeruginosa appears in multiple infections, including CF patients (Gellatly & Hancock, 2013; Curran *et al.,* 2018; Pier *et al.,* 1987). CF is a recessive genetic disease that is susceptible to chronic lung infections by *P. aeruginosa* which is driven by multiple factors (Priebe & Goldberg, 2014). One factor is related to the CFTR mutation that enhances bacterial entry and causes bacterial clearance to fail (Priebe & Goldberg, 2014; Lyczak *et al.,* 2002). Others are disturbed in mucosal immunity and the defective mucociliary system, which is caused by forming odd viscous secretions (Priebe & Goldberg, 2014). In the mid-1980s, Pier and colleagues were exploiting resistant mechanisms of *P. aeruginosa* by studying serum from patients with CF and healthy adults. The sera were taken from different volunteers: CF patients colonized with mucoid *P. aeruginosa,* CF patients not colonized with mucoid *P. aeruginosa*, and healthy individuals (Pier *et al.,* 1987). They found that CF patients who were colonized with mucoid *P. aeruginosa* had a high level of opsonophagocytic antibodies against the alginate capsule compared to non-colonized patients (Pier *et al.*, 1987). The phenotype of *P. aeruginosa* is variable depending on the CF disease progression. A study that isolated *P. aeruginosa* from early and late CF patients concluded that isolates taken from early CF patients were characterized as non-mucoid with smooth

LPS, while samples isolated from late CF patients were mucoid with rough LPS (Hancock *et al.,* 1983). Furthermore, a study testing efficiency of opsonizing antibody by Pier, Small, and Warren in rodent models concluded that mice and rats immunized with mucoid exopolysaccharide (MEP) stimulated production of opsonizing antibodies. These immunized rodents had reduced *P. aeruginosa* bacteria after intratracheally challenge with mucoid *P. aeruginosa* compared to control groups (Pier *et al.,* 1990). However, the defense mechanism of opsonizing antibody is not obvious, especially in early CF chronic infections known as non-mucoid LPS (Priebe & Goldberg, 2014). Interestingly, the synthetic monoclonal antibody, which is designed specifically to carbon 6 of mannuronic acid of *Pseudomonas* alginate, confers protection in an acute pneumonia mice model and is accompanied by phagocyte killing mucoid and nonmucoid strains (Pier *et al.,* 2004).

There was a vaccine developed by Doering, Meisner, Stern, called bivalent flagella that was in phase III trials. Patients were given four intramuscular doses of bivalent flagella vaccine for a 14-month period. Then they evaluated the patient's sera for two years. During that time, patients developed a high and well-tolerated titer of antiflagellum IgG and other antibody types specific to flagella (IgA and secretory IgA). Subsequently, the bivalent flagella vaccine reduced *P. aeruginosa* by at least 66% in CF patients (Döring & Pier, 2008; Döring *et al.,* 2007). Unfortunately, the company stopped producing this vaccine before the end of the trial, and no other companies have taken over production of the flagella vaccine (Priebe & Goldberg, 2014).

Another trial of a flagella vaccine occurred in which, they conjugated the flagella vaccine with poly mannuronic acid (PMA) that has an epitope that could be recognized by a monoclonal antibody. This vaccine showed protection only against mucoid strains by producing opsonic antibodies (Priebe & Goldberg, 2014). However, the antibodies made by the flagella vaccine showed no neutralizing activity on Toll-Like Receptor 5 (TLR), which is an essential part of stimulating innate immune response to flagellated bacteria (Campodónico *et al.,* 2011).

1.5.2 Live Attenuated Vaccines

Research has focused on the mechanism of attenuation to develop robust vaccines against lethal pneumonia infections caused by *Pseudomonas aeruginosa*. In a mutagenesis study, *Pseudomonas* PA14 mutant *Aro* gene, which is required for aromatic amino acid synthesis, showed a highly immunogenic response and was attenuated for lung infections in a murine model (Priebe & Goldberg, 2014). A study that exploited a live attenuated vaccine based on the PA14 strain showed that *Aro* deleted PA14 or ∆*aroA*; immunized mice had high levels of early neutrophil recruitment to the airway post-infection compared to control mice. Moreover, T cells isolated from the immunized mice were proliferative and were accompanied by large quantities of IL-17 production in bronchoalveolar lavage fluid. Notably, the absence of IL-17 receptor abrogated this vaccine's protection (Priebe *et al.,* 2008).

Additionally, mice and rabbits vaccinated with PAO1 ∆ *aroA* intranasally produced a high level of IgG response. The rabbit antisera enhanced phagocytic killing of PA heterologous strains and O2\O5, strains whilst mouse antisera mediated killing of serotype O2\O5 strains (Priebe *et al.,* 2002). A different study used the neutropenic mouse model, which were immunized intranasally by live attenuated ∆ *aroA* vaccine against *P. aeruginosa*. Many macrophages and monocytes recruited post-infection served as potent effector innate cells after immunization. Additionally, this was accompanied by a high proportion of CD4 T cells in the lung and spleen, which enhanced clearance of *P. aeruginosa* from the lung. Thus, this study showed that mouse model immunity induced by mucosal *P. aeruginosa* live attenuated vaccines was protective even when neutrophils are deficient (Kamei *et al.,* 2013).

Another study by Kamei *et al.* (2011) approached multivalent live attenuated vaccines ∆*aroA* in a mouse model of acute pneumonia. The vaccine contains four

attenuated *Pseudomonas* strains with different LPS serogroups. This vaccine was efficacious to protect against *P. aeruginosa* lung infections and resulted in local CD4 T cells in the lung as well as an opsonic antibody against LPS core and other surface proteins (Kamei *et al.,* 2011).

1.5.3 Killed Whole Cell Vaccines

Pseudomonas vaccines using killed bacteria have been prepared by several techniques, such as treating *P. aeruginosa* cells with paraformaldehyde to inactivate the bacteria (Cripps *et al.,* 1994; Buret *et al.,* 1993). A study done in 1992 investigated the efficiency of immune clearance of bacteria in rat lung. Rats were immunized with killed a mucoid strain of *P. aeruginosa*. After challenging rats with live bacteria intratracheally, a high level of anti-*Pseudomonas* antibodies was present in the vaccinated rats' sera (IgG, IgA, IgM) and bronchoalveolar lavages (IgG and IgA). In addition, bacterial clearance was significantly accelerated in the lung of all immunized rat models. Moreover, this study found improvement in recruitment, chemotaxis, chemokinesis, and post-infection phagocytic activity of PMN. Notably, in the alveolar space of immunized rats, a high proportion of polymorphonuclear neutrophils (PMN) were observed. The increase of PMNs did not correlate with increased leukotriene B_4 (LTB_4), a pathway important in the pathogenesis of neutrophilic inflammation that coexists at sites of inflammation and tissue remodeling. In contrast the level of PMN recruitment was lower in unimmunized versus immunized rats and associated with elevating leukotriene B_4 in alveolar space (Cripps *et al.,* 1994).

Similarly, a study by Cripps *et al. (*1993), determined the efficiency of mucosal and systemic immunization with inactivated *Pseudomonas aeruginosa* against acute respiratory infections in rat models. The rats were immunized with paraformaldehyde killed *P. aeruginosa,* then were challenged with live *Pseudomonas* bacteria. The dose

was lethal to the unimmunized rats 12 hours post challenge. They determined the number of surviving bacteria in the immunized rats by analysis of bronchoalveolar lavage fluid (BAL) and lunge homogenate samples 4 hours after challenge. The results showed significant bacterial clearance in immunized rats compared to the bacterial burden in unimmunized models (Buret *et al.,* 1993).

This kind of vaccine may be a favorable approach to use as an oral formulation for infants with CF to avoid or postpone *P. aeruginosa* colonization (Cripps *et al.,*1994).

1.5.4 Subunit Vaccines

Ongoing studies of subunit vaccine development have identified many antigens that are able to induce partial protection against *P. aeruginosa*. Some vaccine strategies have focused on outer membrane proteins (OMPs), OprF and OprI, which are conserved among all *Pseudomonas* strains and required for adhesion to the host cells as well as OprF\OprI fusion or hybrid proteins (Priebe & Goldberg, 2014). In 1995, Hughes and Gilleland tested three different synthetic peptides presenting the epitope of OprF of Pseudomonas in acute murine models (Hughes & Gilleland, 1995). Synthetic peptides 9, 10 and 18 were each conjugated to keyhole limpet hemocyanin (KLH) which is highly immunogenic for T cell antigens. Mice immunized with peptide 9, 10, and 18 showed high levels of stimulating IgG and had a greater survival rate compared to control groups. Thus, these synthetic peptides showed a potential protection against acute pneumonia in murine models which could be considered for further studies (Hughes & Gilleland, 1995).

Recombinant OprF/I fusion protein has been tested and induces significant levels of specific opsonic IgG antibody (Westritschnig *et al.,* 2014) and induced IFN-γ CD4 and CD8 responses (Priebe & Goldberg, 2014). Likewise, Specht et al. showed that recombinant OprF/I hybrid protein protected immunodeficient mice against a 50%

lethal dose of *P. aeruginosa*. Additionally, OprF\OprI fusion protein was used separately to immunized rabbits and the immune rabbit sera protected severe combined immunodeficient (SCID) mice against fatal doses of *P. aeruginosa* (Von Specht *et al.,* 1995). Taken together, active and passive immunization can protect SCID and neutropenic mice against 50% lethal doses of *P. aeruginosa* (Priebe & Goldberg, 2014).

Wu *et al.* (2012) identified Th17 stimulatory proteins of *P. aeruginosa* and then tested their activity against lethal PA pneumonia. They identified four Th17 stimulating proteins and three His tagged proteins purified from protein libraries from splenocytes of mice immunized with live attenuated ∆ *aroA* vaccine with their protection based on Th17 production (Priebe *et al.,* 2008; Wu *et al.,* 2012). OprL, PopB, and FpvA enhanced the production of Th17 in PA14 Δ *aroA*-immune splenocytes (Priebe & Goldberg, 2014). Mice were immunized with PopB intranasally, a known virulence factor that is a highly conserved component of the type III secretion system. It elicited a strong IL-17 response when mixed with the beta-D-glucan curdlan a known Th17 eliciting adjuvant (Priebe & Goldberg, 2014; Wu *et al.,* 2012). The results showed the protection of immunized mice from PA pneumonia was associated with IL-17 in the absence of an opsonic antibody (Wu *et al.,* 2012*)*.

Recently in 2018, a study generated artificial PcrV derivative ($PcrV_{NH}$) by dividing full-length PcrV to four domains (Wan *et al.,* 2019). N-term (Met1-Lys127) and H12 (Leu251-Ile294) domains were linked together via a linker to generate artificial $PcrV_{NH}$. Immunizing mice with $PcrV_{NH}$ intramuscularly conferred a wide protection in the pneumonia of the acute models with the same functionality of full-length PcrV. Moreover, immunized mice with $PcrV_{NH}$ induced Th1, Th2, Th17 and high levels of IgG1, IgG2a, IgG2b in immunized mice sera where IgG2a or IgG2b induces Th1 immune response. In addition, IFN-γ and IL-17A were observed in the spleen, which suggests the induction of Th1 and Th17. IL-4 cytokines were detected in the spleen as

well with high levels that subsequently induce a Th2 immune response. Interestingly, $PcrV_{NH}$ immunized mice recover faster (within 120 hours post challenging) compared to unimmunized mice (Wan *et al.,* 2019).

Ultimately, a notable reduction of pro-inflammatory cytokines, such as TNF-α and IL1β were detected in $PcrV_{NH}$ immunized mice (Wan *et al.,* 2019).

1.5.6 Therapeutic antibody

It may not be possible to achieve the time or role of the immune system needed to develop an effective response to active vaccination. Thus, there have been multiple attempts to develop an antibody-based therapy for eliminating a *Pseudomonas* infection or preventing the disease.

Anti-*P. aeruginosa* IgY showed high activity in hindering *P. aeruginosa* attaching to epithelial cells (Priebe & Goldberg, 2014). Anti-*P. aeruginosa* IgY is generated by vaccination of laying hens that produce specific IgY transferred to egg yolk in large quantities (Priebe & Goldberg, 2014; Carlander *et al.,* 2000). It has been reported that laying hens can produce more than 20g of yolk antibodies per year. The advantage of using yolk antibodies is they cannot either bind to the Fc receptor or activate the human complement system which both lead to an inflammatory response. Importantly, many studies have shown the efficacy of yolk antibodies in protection against bacterial and viral infections (Carlander *et al.,* 2000).

Furthermore, Anti-O11 mAb was generated by vaccination of volunteers with early octavalent O-polysaccharide-toxin A conjugate vaccine. This human antibody was specific against *P. aeruginosa* serotype O11 O polysaccharide, which indicated the specificity of the response. In animal studies using *Pseudomonas* infected mouse models, this monoclonal antibody showed opsonophagocytic activities against *P. aeruginosa* serotype O11, protection against the bacteria in burn wounds, including

reduced numbers of bacteria and a notable reduction in lung injury; however, it did not provide complete protection in serotype O11. As expected, it showed no reduction against non-serotype O11 (Horn *et al.,* 2010). A study that generated an artificial PcrV derivative tested the efficiency of anti -$PcrV_{NH}$ antibodies against PA infections. The presence of anti -$PcrV_{NH}$ exhibited a potent opsonophagocytic activity, and the level of secreted ExoU was decreased by increasing anti -$PcrV_{NH}$ antibodies. In addition, this antibody has a significant effect on T3SS mediated cytotoxicity by inhibiting its action (Wan *et al.,* 2019).

1.6 Models for Cystic Fibrosis Studies and Chronic Infections with *P. aeruginosa*

1.6.1 Animal Model

In the past few decades, many mouse models have been created by using the targeted distruption of gene sequences within embryonic stem cells (Kukavica-Ibrulj & Levesque, 2008). Interestingly, mouse models are still different from humans due to several factors: mutation types, mRNA expression level, and the genetic background of the mouse strain. Eventually, a mixed genetic background including chromosomes from the mutant embryo stem cell line and the recipient blastocyst (FVB or C57BL/6) become available in CF mouse models today (Kukavica-Ibrulj & Levesque, 2008).

The first CF murine model was described by Snouwaert *et al.* (1992) at the University of North Carolina after the characteristics of CFTR gene were defined. They successfully generated a CFTR knockout ($CFTR^{tm1UNC}$) mouse from embryonic stem cells by insertion of a stop codon in exon 10 of the CFTR coding sequence, known as the S489X mutation. This model was used as a basis by other scientists to generate various knockout models, such as ($CFTR^{tm1CAM}$) and ($CFTR^{tm1HSC}$). In addition, knock-

in mutants were developed. Different mutations that causes CF were introduced to the endogenous mouse CFTR gene to produce mice carrying the Class II ΔF508 ($CFTR^{tm1EUR}$, $CFTR^{tm1KTH}$, and $CFTR^{tm2CAM}$) and G480C ($CFTR^{tm2HGU}$) mutations. However, it has been reported that there was intramutant variation in the disease severity, survival and pathology of these animals (Lavelle *et al.,* 2016).

Currently used mouse models contain various phenotypes of CF mutation including complete knock out (KO) of CFTR function, CFTR dysfunction, and the exact mutation found in human CF. The complete KO of CFTR and residual CFTR function mouse models resulted in total loss of CFTR function; with no CFTR protein produced examples are $CFTR^{tm1Unc}$ and $CFTR^{tm1Bay}$(Kukavica-Ibrulj & Levesque, 2008).

Agar-bead (chronic) pneumonia model used extensively in CF research and was originally developed in rats. They used agar or seaweed alginate beads as extracellular polymeric substances to mimic biofilm. The beads are loaded with bacteria in a process that requires mixing with mineral oil and emulsifying agent, sorbitan-monooleate, to increase the consistency of the beads (Bielen *et al.,* 2017). In 1987, Starke *et al.,* modified the first agar bead model to use in mice. Subsequently, this model has been used frequently to study CF lung disease and bacterial infections. Moreover, different CF mouse models were developed using gene targeting to disrupt murine CFTR locus through homologous recombination and to introduce a specific human mutation into mouse loci, such as ∆F508 (Kukavica-Ibrulj & Levesque, 2008).

On the other hand, the first rat model was designed by Cash in 1979 using *P. aeruginosa* embedded in agar beads for bronchopulmonary infections. The aim was to maintain the existence of bacteria in the airways to allow it to mimic bacterial biofilm in CF lungs. Recently, it has been proved that the presence of *P. aeruginosa* within agar beads is more characteristic of CF than the administration of *P. aeruginosa* alone. Interestingly, bacterial growth is shown to be slow within agar bead which is parallel to what is observed in biofilm formation and in the planktonic state. Moreover, a study

conducted in 2005 showed that an alginate capsule could be used as an alternative of an agar bead since it mimics normal conditions of CF in a lung where bacteria are embedded with alginate (Kukavica-Ibrulj & Levesque, 2008).

For acute pneumonia, a one hit acute pneumonia model is established by administering a bacterial inoculum into the lungs using different methods of bacterial delivery. Intratracheal installation is the most used method in HAP research, which involves injecting the bacterial suspension directly into the trachea or lungs followed by air for dispersion of the tablet. Second, endotracheal inoculation requires intubating the animal to facilitate the installation of the bacterial solution in the lungs. Ultimately, intranasal administration was done by administering bacterial dose in droplets through the nostrils (Bielen *et al.,* 2017)

Other animals are also used in various studies of *P. aeruginosa* virulence and in the CF area in general. An example is the cat model of chronic pulmonary infection with *P. aeruginosa*, which showed similar results to human CF macrophage stimulation and inflammation (Kukavica-Ibrulj & Levesque, 2008). Another model simulates an invasive infection of large-area burn wounds, where animals receive a burn injury on their back, followed by intradermal injection of the bacterial inoculum. This model has been used widely in burn infection studies, such as bacterial translocation, gene therapy, and antibiotic efficacy studies (Maura *et al.,* 2018). Additionally, sheep, pigs and cats could be used as models to understand the role of submucosal glands found only in the proximal trachea in mice (Joo *et al.,* 2001).

1.6.2 Non-Mammalian Models

Alternative, easy, ethical and less costly experimental models are still needed to understand the virulence of bacterial lung diseases. Non-mammalian models would be appropriate to explore the pathogen-host interaction, such as the vertebrate zebrafish (Daniorerio) and non-vertebrate insects and nematodes (e.g. Caenorhabditis elegans). In 2009, the zebrafish embryo model was developed by Brannon *et al.,* to explore systemic *P. aeruginosa* infection as well as evaluate the type III secretion system (T3SS) mutant. The results showed that T3SS and quorum sensing is required for lethal infection with *P. aeruginosa*. Interestingly, T3SS was associated with the initiation of infection in CF patients and elevated risk in hospitalized patients. Another study using zebrafish as infectious models by Phennicie *et al.* in (2010) studied the role of CFTR in the innate immune response to acute *Pseudomonas* infection (López Hernández *et al.,* 2015).

Using zebrafish as a model has various characteristics. Firstly, when embryo zebrafish are used, a short time is needed because they develop very rapidly. Secondly, only a single bacterial dose is required to initiate the pathogen population, which is like an infection in mice models. Thirdly, the time frame of zebrafish infection proceeds within days rather than weeks to months. In addition, the zebrafish model allows for the chemical screening of antimicrobial compounds. However, adult fish are less susceptible hosts for bacterial infection, so a higher dose of live bacteria 5 x 10^7 is required (López Hernández *et al.,* 2015).

In 1999, Tan *et al.* used C. elegans for the first time to explore *Pseudomonas* pathogenesis. They illustrated that the accumulation of *P. aeruginosa* cells in intestines is important in clarifying killing mechanisms. Moreover, insects were used as host models to identify *Pseudomonas* virulence factors in mammalians in this previous study. Boman *et al.* (1972) concluded that *P. aeruginosa* is virulent in fruit flies. Eventually, using D. melanogaster as a model helped to identify the human anti-

microbial peptide that inhibited the initiation of biofilm formation, which could be used to develop new therapeutics for *P. aeruginosa* infections in the future (López Hernández *et al.,* 2015).

1.7 Immune Response Following *P. aeruginosa* Infection

1.7.1 Innate Immune Response

During the development of a pulmonary *P. aeruginosa* infection, bacteria evade innate immune cells in order to colonize and infect the host successfully. The first line of defense that counters microbes are the epithelial cells, which perform a major role in innate immune response against infectious pathogens and serve as a physical barrier (Gellatly & Hancock, 2013; Perl *et al.,* 2002). Furthermore, these cells act as sentinels to alert the innate and adaptive immune system about the infection by expressing a number of immunoregulatory membrane receptors, and intracellular danger sensors and their downstream effectors (Perl *et al.,* 2002). Ciliated epithelial cells constitute around 50% of the epithelial surface in the trachea; they push the infectious agent out of the lung to the throat by using their cilia (Gellatly & Hancock, 2013; Knight & Holgate, 2003). Mucin one of the secreted epithelium surface components, is located on the airway epithelium to trap and eliminate inhaled foreign microorganisms. Depending on the variety of its structure and physical properties, mucin has two distinct classes that perform different functions (Williams *et al.,* 2006). Other molecules are secreted by the epithelial cells, such as cytokines and chemokines upon activation via Toll-like receptors (TLRs), to recruit and activate the innate and adaptive immune cells (Gellatly & Hancock, 2013). In mutagenesis studies, it has been reported that a deficiency in the CD59\CD59 ligand, mediated-cell death, inhibit apoptosis of lung epithelial cells *in vivo* and *in vitro* (Grassme *et al.,* 2000).

During the infection, phagocytic cells, either neutrophils or macrophages, play a prominent role in clearing a *P. aeruginosa* infection (Curran *et al.,* 2018). Massive recruitment of neutrophils into the infected airway occurs by the production of chemokines (Lavoie *et al.,* 2011) IL8 in human and KC in mice which is a member of CXCR family (Gellatly & Hancock, 2013). Upon the binding of chemokines to the CXCR2 receptor, neutrophils migrates into the site of inflammation and the chemokines' receptor CXCR1 induces reactive oxygen species to clear bacteria (Tümmler & Klockgether, 2017). CXCR1 is a novel non-canonical chemokine receptor that regulates the response of anti-*Pseudomonas* neutrophils by modulating reactive oxygen species and interfering with TLR5 expression (Carevic *et al.,* 2016). A previous study revealed that mice, which were administrated with a monoclonal antibody (anti CXCR), inhibited neutrophils recruitment by 50% (Gellatly & Hancock, 2013; Curran *et al.,* 2018). Neutrophils recruitment depends on β2 integrin (e.g. CD11b/CD18, CD3) (Lavoie *et al.,* 2011).

Macrophages are important during *Pseudomonas* infection (Gellatly & Hancock, 2013). MCP-1 cytokines are secreted by alveolar epithelial cells which in turn stimulate macrophage migration and phagocytosis (Lavoie *et al.,* 2011). Macrophage phagocytize microbes and sequester antigen, (Gellatly & Hancock, 2013) then secrets cytokines (TNF-α and IL-6) and chemokines (KC) (Lavoie *et al.,* 2011) which enhance neutrophil recruitment (Lavoie *et al.,* 2011). Additionally, macrophages opsonize dead neutrophils (Lavoie *et al.,* 2011). One study illustrated that MCP-1 deficient mice had reduced bacterial clearance and elevated lung tissue-damage (Kannan *et al.,* 2009). Upon infection, *P. aeruginosa* induces (NLRP3) inflammasome, which leads to macrophage secretion of caspase-1 and interleukin 1β (Deng *et al.,* 2015).

During bacterial clearance, inflammation should be controlled and limited or otherwise it causes organ damages (Tümmler & Klockgether, 2017). Cytokine IL-17 serves as a balance between host response and the inflammation that triggers organ

injuries. In the lung IL-17 A is released by many T-cell subtypes, innate lymphoid cells, and macrophages. Once released, it activates airway epithelium to release IL-17C, which in turn produces neutrophilic cytokines from the alveolar epithelium and expand inflammation. Proinflammatory neutrophilic cytokines act as priming agents for neutrophils, e.g., (IL-1 and TNF-α). These cytokines have been shown to expand neutrophil function. IL-17C production is directly induced upon *P. aeruginosa* infection (Wolf *et al.,* 2016). The NKGD2 receptor is expressed by variety of immune cells, including natural killer cells (NK), which prime the immune system's responses to infectious particles (Lavoie *et al.,* 2011).

1.7.1.1 Cytokines and Chemokines in Host Defense

Many cytokines are produced in response to a *Pseudomonas* infection; the major cytokines produced by macrophages and epithelial cells in the lung is TNF-α, which favors the elimination of *P. aeruginosa*. In a mutagenesis study, mice lacking TNF-α had difficulty eradicating a *Pseudomonas* infection (Williams *et al.,* 2010). TNF-α is primarily produced by bone marrow-derived cells in the MyD88-dependent pathway following *P. aeruginosa* acute pulmonary infection. Moreover, TNF-α can produce anti-inflammatory cytokines, such as IL-10. IL-10 is produced late (24 h) after acute *P. aeruginosa* pulmonary infection, at a time when the levels of pro-inflammatory cytokines and chemokines are reduced (Williams *et al.,* 2010; Lavoie *et al.,* 2011*)*. Also, TNF-α positively regulates anti-inflammatory molecules through TNFR1, such as Muc1, that has a role in suppressing TLR. Muc1$^{-/-}$ mice have a high inflammatory response to *Pseudomonas aeruginosa* compare to wild type (Ueno *et al.,* 2008). Additionally, Muc1 is induces apoptosis in neutrophils (Williams *et al.,* 2010; Lavoie *et al.,* 2011). A study on Muc1 knock out mice showed increases in the recruitment of neutrophils in airways and accelerated bacterial clearing (Lavoie *et al.,* 2011).

The role of IFN-γ is less clear, when administrated exogenously mice had enhanced bacterial clearance; however in mice deficient with IFN-γ receptor also showed enhanced bacterial clearance (Williams *et al.,* 2010). IL-4 is a product of the Th2 lymphocyte population, which stimulates B cell activation and antibody production against *P. aeruginosa* (Williams *et al.,* 2010). Mice with overexpression of Interleukin 4 eliminate bacteria in a short period of time post-infection (Jain-Vora *et al.,* 1998). In addition to these, Interleukin 8 is a human chemokine (CXCL8), and (KC and MIP2 in mice) are activated by TLR upon stimulation with *Pseudomonas*. It plays a primary role in the migration of neutrophil and otherwise causes a defect if there is no neutrophil response (Williams *et al.,* 2010).

Finally, IL-1 and IL-18 tend to be more harmful cytokines than beneficial in an acute pneumonia model. Mice deficient in IL-1 and\or IL-18 were protected against a large challenge dose of PA. However, when a small number of bacteria were used to infect IL-1 knock out mice, they became more susceptible to chronic infection. Besides the role of IL-17 in modulation of innate immune responses, it also modulates many components of the adaptive immune system (Williams *et al.,* 2010).

Th17 $CD4^+$ cells are formed in the presence of IL-6 and TGF-β cytokines and are maintained by the presence of IL-23, which is produced by alveolar macrophages. These cells recruit neutrophils to the site of infection through the actions of IL-17 (Williams *et al.,* 2010).

1.7.2 Adaptive Immune Response

The activation of adaptive immune response starts when antigen presenting dendritic cells migrate to the lymph node where they react with naive T cells to induce antigen specific proliferation and differentiation of effector T cells (Worbs *et al.,* 2017). Multi subunit types of T cells have been identified including CD4, CD8, Th17, Treg and NKT cells (Williams *et al.,* 2010). A study done by Worgall *et al.,* concluded that dendritic cells pulsed with *P. aeruginosa* elicit an efficient immune response against pulmonary infection. They Incubated mice bone marrow-derived DC with *P. aeruginosa* in vitro, and administered them to syngeneic mice, then the mice were infected with *P. aeruginosa* (5×10^4 CFU/mouse) intrapulmonary. 45% of mice receiving *P. aeruginosa*-pulsed DC showed sustained survival for more than 14 days. However, when they pulsed DCs with heat-killed or heat-inactivated bacterium; there was a protection of $CD8^{-\backslash-}$ mice, but not $CD4^{-\backslash-}$ mice. They concluded that CD4 t cells are required to induce immunity against pulmonary infection (Worgall *et al.,* 2001).

Another study proved the role of CD4 in eliminating *P. aeruginosa* from the lung. Based on a cell transfer experiment, they transferred lymphocytes or CD4 cells from mesenteric lymph node (MLN) from orally immunized or unimmunized rats with either killed or activated bacteria to a naïve rat then challenge them with *P. aeruginosa*. The study found that the immunized rats only stimulated clearance of bacteria from airways by the transferred T cell (CD4) from immunized mice (Dunkley *et al.,*1994).

Furthermore, the C3H/HeN mice strain, known as Th1 responders were more efficient at eliminating *P. aeruginosa* from lung than BALB/c mice which are Th2 responders. Protection was accompanied with high levels of interferon (IFN-γ) and low levels of IL-4 cytokines in the C3HIHeN strain (Moser *et al.,* 1997). Th17 and Th23 have also been implicated in *P. aeruginosa* response (Williams *et al.,* 2010). Mice

deficient in either Th17 or Th23 have increased lung tissue damage and mortality (Curran *et al.,* 2018).

Treg cells play a role in the generation of an immunosuppressive environment. Treg cells are increased in chronic infection (Williams *et al.,* 2010). Quorum sensing (QS) systems play an important role in regulating biofilm formation. In a previous study, QS molecules had complex impacts on the host immune response (Feng *et al.,* 2016). An experiment on rat models infected with *P. aeruginosa* found the percentage of suppressive cytokines (IL-10, TGF-β) and Treg cells were higher in wild type bacteria compared to QS mutants. This indicates that QS affects the production of some T cell populations (Treg) and modulation of Tregs in biofilm-associated infections may be altered by bacterial QS systems. (Feng *et al.,* 2016). NKT cells or CD1d also have a role in the regulation of *P. aeruginosa* clearance. These cell populations account for a small percentage of total T cell population (Williams *et al.,* 2010; Lavoie *et al.,*2011).

On the other hand, humoral immunity is also required for prolonged protection against *P. aeruginosa*. A high level of the aryl hydrocarbon receptor (AHR) is induced during B cell activation. This has a negative feedback on class switch recombination and differentiation of B cells to plasma cells that lead to the formation of effector of B cells and antibody-secreting cells (Vaidyanathan *et al.,* 2017). *P. aeruginosa* produces pyocyanin, a phenazine, which binds to the aryl hydrocarbon receptor (AhR), causing neutrophilic inflammation, which is a part of the host defense response. TLR and AhR pathways both participate in IL-8 synthesis, known as a neutrophil chemotactic factor. The AhR played a major role when planktonic *P. aeruginosa* was grown in a media favoring phenazine synthesis. However, when *P. aeruginosa* was grown as a biofilm, the TLR pathway showed no contribution to biofilm-IL-8 synthesis and AhR was found to partially contribute to IL-8 synthesis, suggesting the involvement of other unknown signaling pathway (Roussel *et al.,* 2016).

A previous study tested the level of IgA in sera and sputa of different groups. The group infected with *P. aeruginosa* showed higher levels of IgA titers than the healthy group and those infected with bacteria other than *Pseudomonas*. This increase was due to the presence of monomeric IgA and polymeric IgA in sera and sputa respectively (Noda *et al.,* 1992). Nevertheless, it is not sufficient to clear *P. aeruginosa* infection (Williams *et al.,* 2010).

Several studies have addressed the role of Ig against *P. aeruginosa* which could be used to either prevent the infection or clear *Pseudomonas* bacteria *aeruginosa* (Williams *et al.,* 2010; Priebe & Goldberg, 2014). It has been demonstrated that PcrV+ strains cause a high rate of mortality even in isolates deficient in ExoU and ExoS cytotoxin proteins. Based on this, PcrV could be a potent therapeutic target against T3SS *Pseudomonas* infection (Sato & Frank, 2011; El Solh *et al.,* 2008). Previous studies have shown the role of polyclonal anti-PcrV antibody in blocking the translocation of T3SS effector proteins, consequently inhibiting bacterial interaction with the host cell (Priebe & Goldberg, 2014). An *in vitro* study showed also blocked killing of macrophages (Sato *et al.,* 2011). In addition, there was significant protection in many acute and chronic pneumonia mouse models (Priebe & Goldberg, 2014) and decreased lung tissue damage and inflammation. Unfortunately, they failed in human clinical trials (Sawa *et al.,* 1999).

Moreover, an anti-Psa monoclonal antibody (Cam-003) showed effective protection by minimizing *Pseudomonas* bacterial growth (Priebe & Goldberg, 2014). Besides the role of IL-17 in eliminating *Pseudomonas* during innate immunity, a recent study illustrated its action in humoral immunity as well (Pan *et al.,* 2016). IL-17 is produced by T cells during adaptive immune responses. The level of immunoglobin (IgA, IgG, IgM) was significantly increased in wild type as compared to γδ $TCR^{-/-}$ mice and improved after IL17-γδ T cell transfusion. Additionally, IL-17 took part in B cell activation during acute *Pseudomonas* infection (Pan *et al.,* 2016).

1.8 Vaccine Design

The termed adjuvants provide the “help” (from adjuvare, to help) needed to enhance and boost immunity to vaccines and antigens (Coffman *et al.,* 2010). In the era of the development of novel vaccines, adjuvants have been used successfully to boost the host immune response to poorly immunogenic purified antigens and subunit vaccines (Di Pasquale *et al.,* 2015; Coffman *et al.,* 2010). Additionally, adjuvants could improve immune responses in those who respond poorly to vaccination (Di Pasquale *et al.,* 2015). In 1932, Alexander Glenny exploited the effects of immune response on aluminum salt at a time when aluminum was the only licensed adjuvant. Aluminum acts by elevating antibody production therapy; it is an efficient adjuvant used in human vaccines against pathogens which are killed mainly by antibodies (Di Pasquale *et al.,* 2015). However, aluminum fails to eliminate intracellular bacteria (Coffman *et al.,* 2010). In the mid- 1990s, a virosome adjuvant system was used in the Hepatitis B vaccine. This triggered the initiation of adaptive T cell responses and B cell activation. Scientists have also designed a combination of adjuvants, known as an adjuvant system, to enhance T cell immune response (Di Pasquale *et al.,* 2015).

In this study, we aim to exploit the Tobacco Mosaic Virus (TMV) based delivery system due to its ability to serve as an adjuvant and deliver multiple protective *Pseudomonas* antigens into a novel vaccine formulation (Banik *et al.,* 2015; Arnaboldi *et al.,* 2016; Kernan *et al.,* 2017). TMV has a simple functional structure, a 6.4 kb positive-strand RNA core surrounded by 2130 copies of an identical coated protein to prevent RNA degradation by enzymes (Stubbs, 1999; Simmons & Blout, 1960; McCormick & Palmer, 2008). Viral RNA is infectious by itself to a wide range of plants and tobacco; however, it is not infectious for humans or animals (McCormick & Palmer, 2008). Recently, TMV has appeared in vaccine development due to its ability to

function as nanoparticles (McCormick & Palmer, 2008; Kernan *et al.,* 2017; McCormick & Palmer, 2008).

Recombinant TMV was developed in the lab of Dr. Alison McCormick and contains a surface exposed, reactive lysine residue incorporated into the coat protein allowing for the chemical conjugation of antigens to the viral surface. Besides its ability to deliver proteins via conjugation (Kemnade *et al.,* 2014), TMV has many characteristics that potentiate vaccine formulation. First is the ability to enhance antigen specific T cell immunity (Kemnade *et al.,* 2014) and generate vaccine-specific antibodies (Banik *et al.,* 2015; Arnaboldi *et al.,* 2016) due to its nonfunctional RNA and repetitive antigens on its surface that mimic an effective virus surface (McCormick & Palmer, 2008). Second, no evidence has shown that TMV induces neutralizing antibodies that block its function as an antigen delivery system (Kemnade *et al.,* 2014). TMV is very stable at different temperatures, which allows it to be used repetitively for boosting (McCormick & Palmer, 2008).

Several studies have used TMV as a carrier to deliver multi-subunit vaccines (Banik *et al.,* 2015). In tularemia studies, the administration of subunit vaccines conjugated to TMV showed significant enhancement of humoral immune response compared to the proteins alone. This, induced a high level of antibody production therapy that protected mice from respiratory infections (Banik *et al.,* 2015). Our group utilized TMV to intranasally deliver a subunit vaccine against *Yersinia pestis* (the causative agent of plague). Administration of a subunit vaccine for *Y. pestis* (F1 and LcrV) intranasally induced a significant production of antibody (IgG) and protected mice from lethal challenge with pneumonic plague (Arnaboldi *et al.,* 2016). Moreover, an experiment in a murine model, used virus-like particles (TMV) containing the hybrid-coating protein 13 amino acid sequence of the murine zona pellucida ZP3 protein. Immunizing mice with this hybrid protein resulted in the production of specific antibodies, ZP3 peptides (Fitchen *et al.,* 1995) and breaking B cell tolerance since B

cells reactive to antigens have been shown to be tolerized (McCormick & Palmer, 2008). Ultimately, TMV could be a potential adjuvant for novel vaccines to induce complete protection against pulmonary infection (Arnaboldi *et al.,* 2016) and develop subunit vaccines for other bacteria in the future (Banik *et al.,* 2015).

2.0 Hypothesis and Specific Aims:

2.1 Hypothesis:

Pseudomonas aeruginosa (PA) is an opportunistic human pathogen that causes a range of severe and fatal infections, especially in critically ill and immunocompromised patients. Cystic Fibrosis patients, for example, are at a high risk of *P. aeruginosa*. *P. aeruginosa* is the most common Gram-negative bacterium that causes nosocomial pneumonia (chronic or acute) in the United States. In addition, it is a ubiquitous pathogen found throughout the atmosphere that can colonize a wide range of organisms (Lyczak *et al.,* 2002; Tümmler & Klockgether, 2017; Gellatly & Hancock, 2013).

P. aeruginosa has been studied intensively. Several candidate antigens were identified for use in subunit vaccines: LPS, flagella, alginate, and a variety of surface proteins and virulence factors. While previous studies made many attempts to design vaccines, the lack of an adequate delivery system has been a limiting factor in the development of a safe and effective PA vaccine (Priebe & Goldberg, 2014). As *P. aeruginosa* is resistant to multiple antibiotics (a number that is constantly growing)(Curran *et al.,* 2018), the goal of this study is to explore a potential vaccine using a tobacco mosaic virus- based delivery platform that provides optimal protection against *P. aeruginosa* respiratory infections and generates protective immune responses. Ultimately, the aim is to find alternatives to prevent and/or treat *P. aeruginosa* infection.

In this study, **we hypothesized that conjugating the tobacco mosaic virus (TMV) chemically to *Pseudomonas* antigens protects against the development of pneumonia respiratory infections**. Two specific aims were designed for this study.

2.2 Specific Aim 1: Produce a functional TMV-conjugate vaccine for *P. aeruginosa*

PcrV is highly conserved among all *Pseudomonas* strains and is the tip protein of type 3 secretion system. We cloned and expressed recombinant PcrV protein. The protein was conjugated to TMV to develop a vaccine that is ready for mice testing. In addition, we cloned and expressed two *P. aeruginosa* proteins (OprI and OprF) that will be conjugated to TMV and tested as a vaccine in mice. Unfortunately, the extended troubleshooting prevented the study of the efficacy of OprI and OprF.

2.3 Specific Aim 2: Evaluate the effectiveness of TMV-PcrV in a model of acute pneumonia.

The hypothesis was tested using intranasal administration to immunize mice with PcrV-TMV vaccine. Antibody production was assessed after each booster as well as morbidity and mortality in mouse models of acute (nonmucoid) PA pneumonia. In addition, we assessed the effectiveness of TMV-PcrV with di-c-GMP adjuvant for enhancing a systemic immune response.

3.0 Materials and methods:

In Vitro Assay:

3.1 Bacterial Strains:

P. aeruginosa Boston 41510 was used in all experiments for this study. It was obtained from the American Type Tissue Culture Collection (ATCC). *E. coli* TOP10 and *E. coli* BL21(DE3) (Invitrogen, Grand Island, NY) were used for cloning and expression of recombinant PcrV proteins, respectively. *E. coli* BL21 PlySE (DE3) (Invitrogen) was used for the expression of recombinant OprI and OprF proteins.

3.2 Expression and Purification of Recombinant Proteins:

3.2.1 Cloning, Expression and Purification of Recombinant PcrV Protein of *P. aeruginosa:*

P. aeruginosa was cultivated in Trypticase Soy Broth (TSB) overnight at 37°C in a shaking incubator (225 rpm). After culture, *P. aeruginosa* bacterial DNA was isolated using Wizard Plus SV minipreps DNA purification system according to the manufacturer's instructions (Promega). DNA concentration was quantitated using a Denovix microvolume spectrophotometer. Bacterial DNA was stored at -80°C for further use. Bacterial DNA was isolated using Wizard Plus SV minipreps total DNA purification system according to the manufacturer's instructions (Promega). PcrV gene was amplified using forward (F_pcvR_NdeI): CTGAGGAATCATGCATATGGAAGTCAGAAACCTT and reverse (R_pcrvR_EcoRI): CAGTTGCATGCTGAATTCTAGATCGCGCTGAGAATGTC primers.

PCR reaction was performed using Thermo Fisher Master Mix and gradient PCR where the PCR Conditions for PcrV were 36 cycles of 95°C 5 min, 95°C 30sec, gradient

30sec, 72°C 1:15min, 72°C 10min. PCR products were checked on a 1% agarose gel. pET28a vector and PcrV PCR product were digested by NdeI and EcoRI at 37°C overnight. All digested DNA were cut and purified from a 0.7% agarose gel, using the Wizard SV Gel and PCR clean-up system according to manufacturer's instructions (Promega) and ligated overnight at 4°C. The ligated product was transformed into *E. coli* BL21(DE3) (Invitrogen) in LB agar plates containing kanamycin (50 μg/ml) overnight at 37℃. Sequence verification was confirmed using Sanger sequencing. Bacteria transformant was grown and bacteria was grown on LB broth containing kanamycin (50 μg/ml) at 37 °C to an OD_{600} of 0.4. Expression was induced by the addition of 0.75 mM of Isopropyl β- d-1-thiogalactopyranoside (IPTG) and grown in shaker incubation at 37°C for an additional 5 hours. Cells were pelleted at 5000 rpm for 10 min. Pellets were stored at -20℃ until protein purification. The protein was purified at 4°C using a nickel nitrilotriacetic acid (Ni-NTA) column chromatography (2-ml bed volume), equilibrated with 3 volumes of binding buffer containing 20 mM imidazole (pH 7.5). The column was washed with 2 volumes of the same buffer 2x, followed by the second wash with 3 volumes of washing buffer 5x containing 40 mM imidazole (pH 7.5). Bound protein was eluted in 50 mM Tris-HCl (pH 7.5)-150 mM NaCl with either 250 mM or 500 mM imidazole (pH 4). The purity of the protein was confirmed by SDS-PAGE and Western blot analysis. Protein concentration was determined by RC DCTM Protein Assay (Bio-Rad), and protein was stored at -20°C. PcrV cloning and purification were performed by Christina Toumanios in the lab.

3.2.2 Expression and Purification of Recombinant OprI and OprF Protein of *P. aeruginosa:*

The OprI and OprF genes were obtained from Invitrogen*,* (Grand Island, NY) in a pET100/D-Topo expression vector. The plasmids were transformed into *E. coli* PlySE(DE3) (Invitrogen). Single colony transformants were grown in LB containing carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml) overnight at 37°C in a shaking incubator at 225RPM, and plasmids were purified using the PureYieldTM Plasmid Midiprep System according to the manufacturer's instructions (Promega, Madison, WI, USA). The sequences were verified by Sanger sequencing (Genewiz, South Plainfield, NJ). Bacterial glycerol stocks in 25% glycerol were generated for each sequence confirmed colony and stored at -80 °C for further use. Bacteria transformants were grown in LB broth containing carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37°C in a shaking incubator at 225RPM overnight and were used to streak LB agar plates (containing the same antibiotics). Single colonies were used to inoculate 10ml of LB media and bacteria was grown overnight as described above. The next morning a larger culture was inoculated with a 1:20 dilution of the overnight culture and grown until it reached an OD_{600} of 0.4. Expression was induced by the addition of 0.75 mM of IPTG and bacteria grown in a shaker incubator (225RPM) at 37°C for an additional 3 hours. Cells were pelleted by centrifugation at 5000 rpm for 10 min and then store at -20°C. Protein was purified using nickel-NTA column chromatography (Sigma, St Louis, MO) according to the manufacturer's instructions. All buffers contained 8M Urea, 50 mM sodium phosphate, and 300 mM NaCl. The column (2-ml bed volume) was equilibrated with 3 volumes of binding buffer containing 8M Urea, 5mM Imidazole, 50 mM sodium phosphate, and 300 mM NaCl (pH 8). The lysis buffer contains 25% Triton X-100, 25% Tween 20, protease tablet, 125µg of lysosome, DNase I RNase-free, and RNase

A. The column was washed with 2 volumes of the same buffer 2x, followed by the second wash with 2 volumes of washing buffer 5x containing 8M Urea, 20Mm Imidazole, 150 mM sodium phosphate, and 300 mM NaCl (pH 7). Bound protein was eluted in 50 mM Tris-HCl (pH 7.5)-150 mM NaCl with 250 mM imidazole, and 8M Urea (pH 4). The purity of the protein was confirmed by SDS-PAGE and Western blot analysis. Protein concentration was determined by RC-DC Protein Assay (Bio-Rad), and stored at -20°C.

3.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Two 10% and 15% SDS-PAGE gels were prepared. Proteins samples were mixed with 6x of loading dye, then heated at 95°C for 5 minutes to denature proteins. 10µl of protein samples and 2µl of Page Ruler Pre-Stained Protein Ladder (Thermo Scientific) were loaded into wells of gels. The samples were run at 120 volts for around 2 hours or until the dye front reached the bottom of gels. The two gels were removed and one stained with the Coomassie blue dye and the second used for western blot analysis.

3.2.4 Western Blot Analysis:

The proteins on the gel were transferred onto polyvinylidene difluoride membranes nitrocellulose membrane (PVDF) using a Semi-Dry transfer apparatus (Bio-Rad) according to the manufacturer’s instruction. After transfer, the membrane was washed three times with 1x of Tris-Buffered Saline and Tween 20 (TBST) buffer containing 0.05%TWEEN 20 for 3 minutes. Then, the membrane was blocked with 1%BSA in 1x TBST of blocking buffer for 1 hour at room temperature with gentle shaking. This was followed by incubation of the membrane for 2 hours at room

temperature with Monoclonal-PolyHistidine as primary antibody obtained from (Sigma, St. Louis, MO) and diluted in blocking buffer (1:3000). The primary antibody was removed by washing membrane with 1x TBST buffer containing 0.05%TWEEN 20 3x for 5 minutes each at room temperature. After washing, the membrane was incubated with secondary antibody Alkaline Phosphate Affinity-Purified Goat Anti-Mouse IgG (H+L)(Jackson ImmunoResearch) (1:5000) dilution for 1 hour with gentle shaking at room temperature. After the incubation, the membrane was washed again with 1x TBST buffer containing 0.05%TWEEN 20 three times for 5 minutes, and the fourth washing with water for 2 minutes at room temperature with gentle shaking. Antibody binding was detected using Alkaline-Phosphatase substrate (NBT/BCIP) for up to 15 minutes, resulting in the appearance of a dark-colored band.

3.3 Generation and Purification of TMV:

TMV purification and conjugations were done by our collaborator Alison McCormick at Touro University, California.

3.3.1 Conjugation of *P. aeruginosa* Proteins to TMV:

Conjugation of *P. aeruginosa* proteins to TMV was performed by Dr. Alison McCormick at Touro University to design Pseudomonas proteins onto the TMV surface.

5mg of PcrV was conjugated to 5mg of single TMV virion. Successful conjugation of recombinant protein of *P. aeruginosa* to TMV virion was determined by SDS-PAGE.

In Vivo Assay:

3.4 Immunogenicity and Protective Efficacy of TMV Conjugated Vaccine:

3.4.1 Mice

C57BL/6 mice were purchase from Jackson Laboratories (Bar Harbor, ME). Six-week-old male and female mice were used in all experiments. Mice were maintained by the Department of Comparative Medicine of New York Medical College in specific-pathogen-free environment. All animal experiments were performed according to the New York Medical College Animal Care and Use Committee guidelines using approved protocols.

3.4.2 Vaccine Formulation:

Two different vaccine formulations were used. First, PcrV protein was conjugated with single TMV separately, which is designed as TMV- conjugated vaccine. The second formulation was TMV-PcrV vaccine mixed with di-c-GMP adjuvant **(Figure 1)**.

3.4.3 Immunization Schedule:

Schedule I: C57BL/6 mice were immunized intranasally (i.n.) with 20µg of TMV-PcrV vaccine and 10µg of rPcrV protein in PBS. TMV-PcrV consists of 10µg of TMV and 10µg of rPcrV, thus control mice receive 10µg of rPcrV to equivocate the amount of antigen used. Mice were given doses on day 0, 14, and 28. On day 0 the mice were immunized i.n. with 50µl volume of the vaccine in one nostril. For booster vaccinations, days 14 and

28 after the first immunization, mice were immunized i.n with 40µl volume of the vaccine formulation in one nostril. A group of mice that received no vaccine was used as a control. All mice were bled on days 21 and 35 and sera were analyzed to determine total antibody response. All mice were challenged with *P. aeruginosa* Boston 41510 on day 42 after primary immunization **(Figure 2A)**.

Schedule II: C57BL/6 mice were immunized intranasally (i.n.) on days 0, 21, and 35. They received 20µg of TMV-PcrV vaccine mixed with 2µg of di-c-GMP adjuvant and 20µg of rPcrV protein mixed with 2µg of di-c-GMP adjuvant in the volume of 40µl of the vaccine formulation in one nostril. A group of mice that received no vaccine were used as a control. All mice were bled on days 14, 28 and 42 and blood sera were collected from immunized and control mice to determine total and isotype specific antibody response. All mice were challenged with *P. aeruginosa* Boston 41510 on day 49 after primary immunization **(Figure 2B)**.

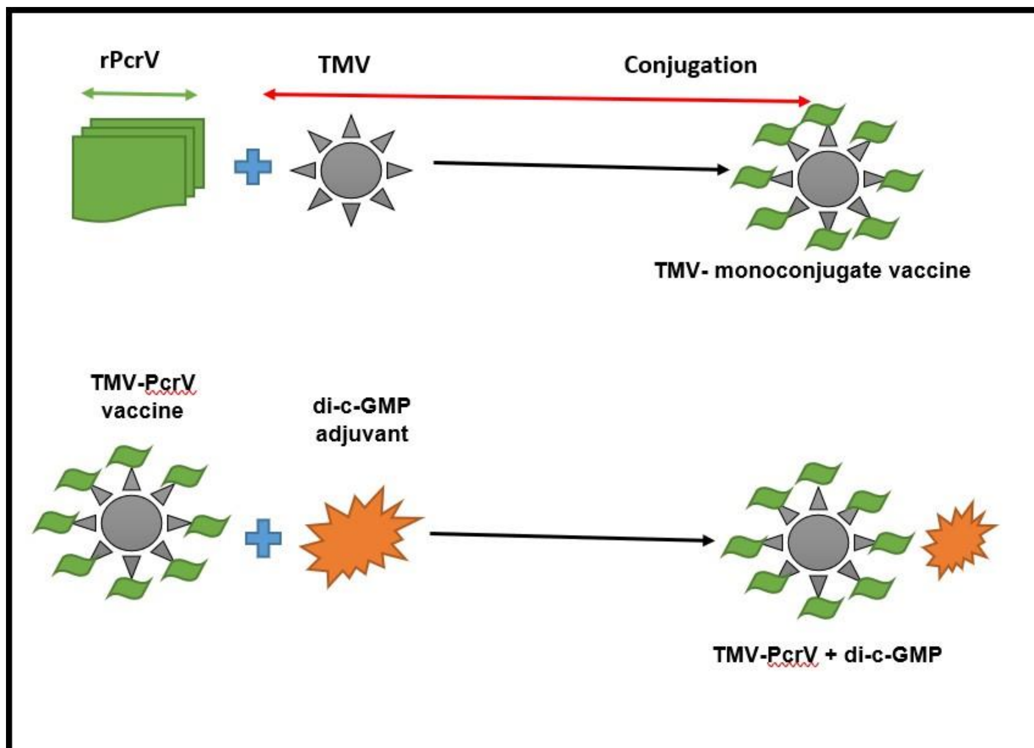

Figure 1: Vaccine formulation. Two different formulations were used. First, PcrV protein was conjugated with single TMV separately, which is designed as TMV-conjugated vaccine. The second is TMV-PcrV vaccine mixed with di-c-GMP adjuvant.

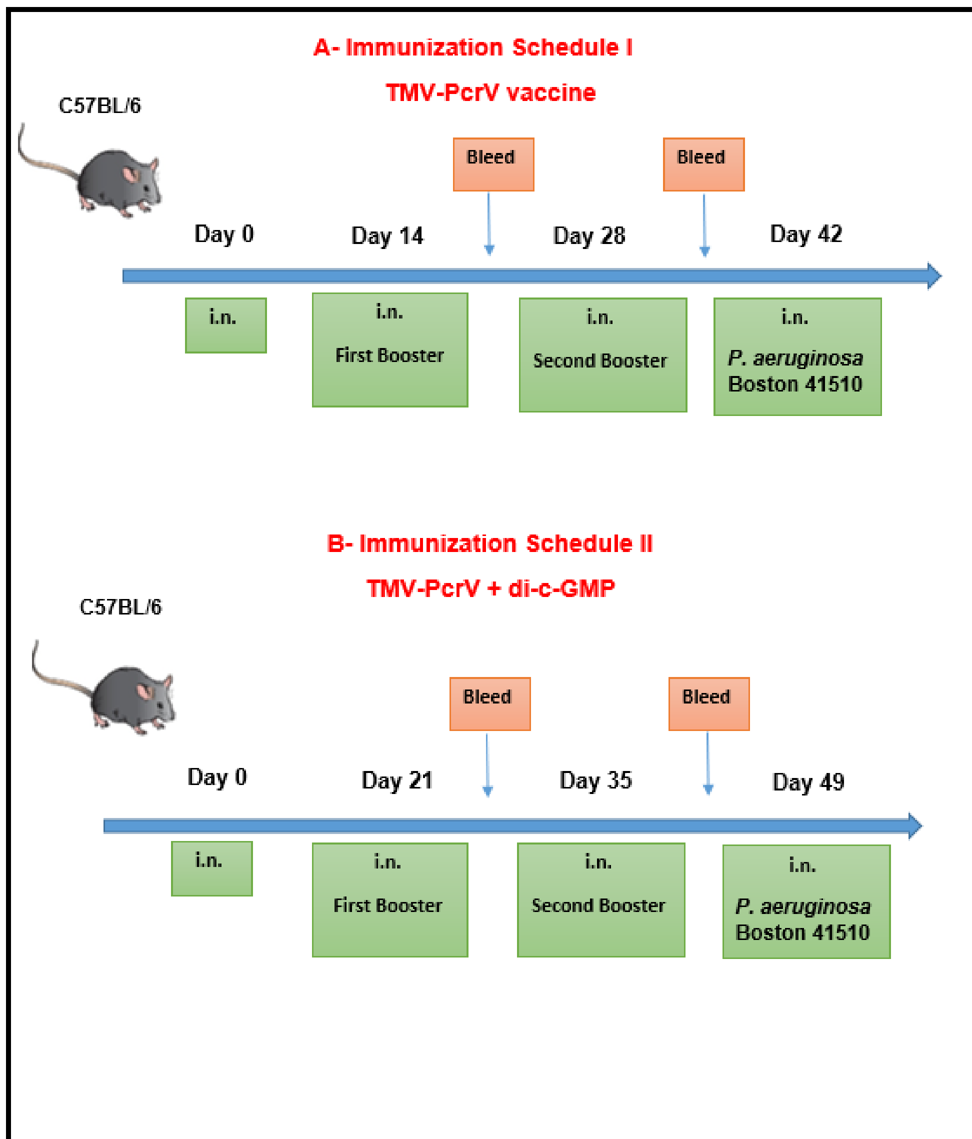

Figure 2: Time points of the immunization schedules. A) C57BL/6 mice were immunized intranasally with 20µg of TMV-PcrV vaccine or 10µg of rPcrV protein and booster vaccinations were administrated i.n. using similar doses on days 14 and 28. **B)** C57BL/6 mice were immunized intranasally with the same formula included 2µg of di-c-GMP adjuvant. Two boosters were administered with a similar dose on days 21 and 35.

3.4.4 Immunogenicity of TMV Vaccine:

For the detection of *P. aeruginosa* specific antibody induced in vaccinated mice, ELISA was performed using rPcrV protein to determine total anti-PcrV specific antibody levels. Blood was collected from mice immunized with TMV-PcrV, rPcrV, and control mice, and centrifuged at 5000 rpm at room temperature for 15 minutes to collect the sera. Sera were stored at -20°C until analysis. Ninety-six wells microtiter plates (Thermofisher- Scientific) were coated with 3µg/ml of rPcrV diluted in coating buffer (0.1 M of Sodium Carbonate, pH9.4) for 1 hour at room temperature. Plates were washed three times with washing buffer (1xPBS with 0.05% TWEEN20). After washing, blocking was performed by adding 250µl/well of blocking buffer (1%BSA in PBS) then the plates were incubated at room temperature for 1 hour. The plates were washed again to remove the blocking buffer. The sera were diluted in blocking buffer (1%BSA in PBS), and added to each well then the plates incubated at room temperature for 1 hour. Plates were washed three times with washing buffer (1xPBS with 0.05% TWEEN20). Goat Anti-Mouse Ig (H+L)-HRP (Southern Biotech Birmingham, AL) was diluted at 1:5000 in blocking buffer (1%BSA in PBS) and, added to each well and plates incubated for 1 hour at room temperature. Plates were washed again three times with washing buffer (1xPBS with 0.05% TWEEN20). TMB substrate (KPLSeraCare) was added to plates 50µl/well volume and plates were incubated in the dark for 30 minutes at room temperature. Finally, 50µl/well of 2N sulfuric acid was added as stop solution, and the color density was read at 450nm and 570nm. The data were plotted as absorbance vs. reciprocal serum dilution, and the 50% maximal binding titers were interpolated from the plotted curves using Prism 7.0. Interpolations were only performed, from curves that had at a maximal binding absorbance of greater than $OD_{450-570}$ of 0.200.

3.4.5 Mice Challenge Studies:

P. aeruginosa Boston 41510 was cultivated in Trypticase Soy Broth (TSB) overnight at 37°C in a shaking incubator (225 rpm). The following morning, this culture was used to seed another broth culture (1:20 diluting) which was grown until the OD_{600} was between 0.6 and 0.9. The culture medium was removed by washing 3x with normal saline and centrifugation at 5000 rpm for 10 minutes at room temperature. The culture was resuspended in normal saline to an OD_{600} of ~1.25. This corresponds to ~2 x 10^9 CFU/ml. The lethal dose 50 of *P. aeruginosa* Boston 41510 was previously determined to be $2x10^{7-6}$ CFU in an acute pneumonia model. Groups of immunized and control mice received $1x10^8$ or $1x10^7$ CFU for the challenge. 2-3 doses are commonly used for challenge studies of Pseudomonas. Mice were anesthetized by intramuscular injection with a cocktail of ketamine/xylazine (100mg/10mg/kg). All mice groups (TMV, rPcrV, and control) were split into two groups for the bacterial challenge. The first group was challenged i.n. with $1x10^8$ CFU in one nostril (50µl) and the second group was challenged i.n. with $1x10^7$ CFU in one nostril with (50µl) volume. Challenges were performed in a BLS2 biosafety cabinet. The number of bacteria inoculated into mice on the day of the challenge was confirmed by plating serial dilutions on TSB agar plates incubated for 24 hours at 37°C.

3.4.5.1 Post-Challenge Studies:

Challenged mice were monitored daily for morbidity (hunched posture, loss of activity, weight loss) and mortality for four days post-infection. A previous study has demonstrated that mice surviving greater than 4 days do not succumb to infection. Mice were weighed every day until they regained their pre-infection weight. Mice that lost

more than 20 % of their original body weight were euthanized. Experiments continued until all mice recovered and regained their original weight before infection.

3.4.6 Statistical Analysis:

Antibody levels of immunized mice are presented as mean absorbance (450-570nm) ± Standard Deviation (SD) or mean ± Standard Error of Mean (SEM). Antibody titers were compared between TMV-PcrV and rPcrV by Student's T-test. Survival data were presented by Kaplan-Meier Survival curves, and were compared by Mantel-Cox Test. Data were statistically analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc, USA).

4.0 Results:

4.1 Expression and Purification of Recombinant PcrV, OprI and OprF Proteins of *P. aeruginosa*:

The *P. aeruginosa* PcrV, OprI, and OprF proteins were expressed in bacterial expression system *E. coli* BL21(DE3), *E. coli* PlySE(DE3), and *E. coli* PlySE(DE3), respectively, tagged with N-terminal 6X-His protein then purified by nickel nitrilotriacetic acid (Ni-NTA) column chromatography. The purity of proteins was confirmed by SDS-PAGE and Western blot analysis using Monoclonal-PolyHistidine as the primary antibody. Bands of 37kDa, 12kDa, and 42kDa confirmed the identity of PcrV, OprI and OprF proteins, respectively **(Figure3).**

OprI and OprF expression and purification required substantial troubleshooting. The optimizing conditions for OprI and OprF protein expression and purification were totally different from PcrV protein. In the first attempt, we followed the same growth conditions as we did with PcrV. We used *E. coli* BL21(DE3) to express our proteins, 100µg of ampicillin antibiotic, 0.75mM of IPTG with 5 hours induction time. Unfortunately, no protein bands were seen on the polyacrylamide gel; however, a very low expression was detected by western blot using Monoclonal-PolyHistidine. In the second attempt, we replaced the 100µg of ampicillin with 100µg of carbenicillin, which is more stable than ampicillin and helps to increase the expression levels by preventing loss of the pET TOPO® plasmid. A high expression level was detected on western blot only. Next, we purified our proteins using the same PcrV protocol, where it has done at 4°C. After protein purification, no bands were seen on the polyacrylamide gel. A band was observed only on western blot using Monoclonal-PolyHistidine. We discovered that we lost substantial protein during the wash steps in the protocol. At this point, we figured out the problem was not only associated with protein expression, but also with the purification method. We decided to perform cell lysis for inclusion body as well to see if

our proteins were there. As we expected, most of our proteins were in the inclusion body. This result showed us that the purification methods for OprI and OprF are different. We also decided to use urea buffer. In the final attempt, we changed the IPTG stimulation time as well as the bacterial expression system. We expressed our proteins in *E. coli* PlySE(DE3) and the induction time was reduced from 5 hours to 3 hours with the same IPTG concentration and 100µg of carbenicillin. Finally, clear bands were observed on both the polyacrylamide gel and western blot analysis. For this attempt, we purified our proteins using a buffer containing 8M of urea, 50 mM sodium phosphate, and 300 mM NaCl.

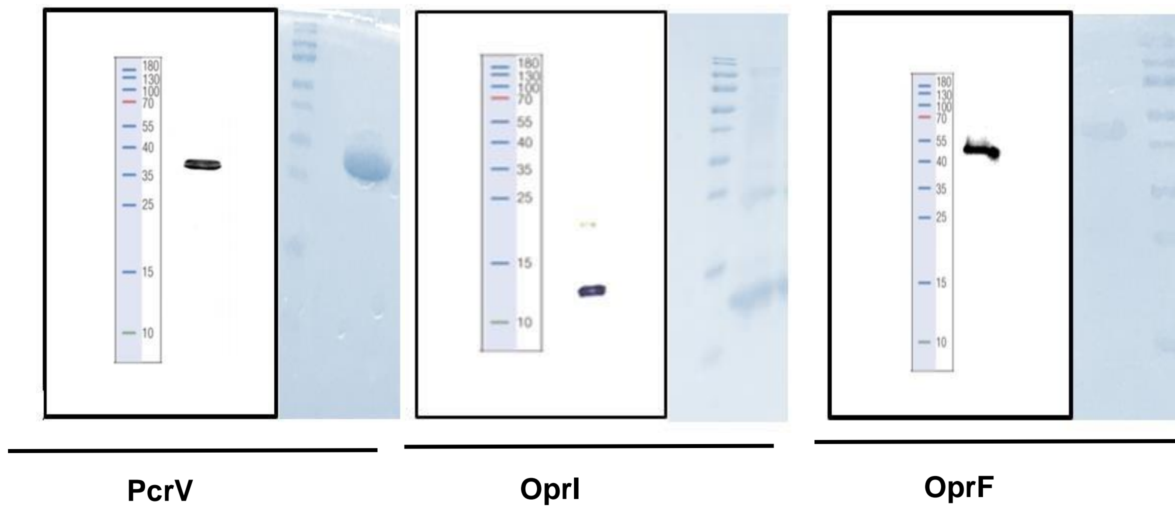

Figure 3: Expression and purification of recombinant PcrV, OprI, OprF proteins of *P. aeruginosa*. Purification of recombinant PcrV OprI, OprF proteins was confirmed by SDS-PAGE and Western blot analysis using anti-PolyHistidine Monoclonal antibodies.

4.2 Immunization with TMV-PcrV Vaccine Generates a Strong Antibody Response:

We examined the level of total antibodies generated in response to TMV-PcrV. We determined antibody response in mice that were vaccinated with 20µg of vaccine conjugate (TMV-PcrV) or 10µg of unconjugated rPcrV and that received two boosters on days 14 and 28. Mice were bled on day 21 and day 35 post-primary immunization and total antibody response was detected using an ELISA assay coated with the rPcrV protein.

A stronger antibody response was detected for the TMV conjugate vaccine compared to the recombinant PcrV protein and unvaccinated group (**Figure 4).** The level of total antibodies observed on day 35 for the TMV-PcrV vaccine were much higher than those observed on day 21 **(Figure 4).** The antibody response was still detectable at a dilution of 25,600 in TMV-PcrV vaccine on both days 21 **(Figure 4.A**) and 35 **(Figure 4.B)**.

Vaccine-specific antibody titers were elevated in the mice that received TMV-conjugated vaccine protein compared to the recombinant protein alone **(Figure 5.A)**. However, the average level of vaccine-specific antibody titers with TMV-conjugated vaccine on day 21 was higher than the mean of antibody titers on day 35 **(Figure 5.B).**

Total Ig

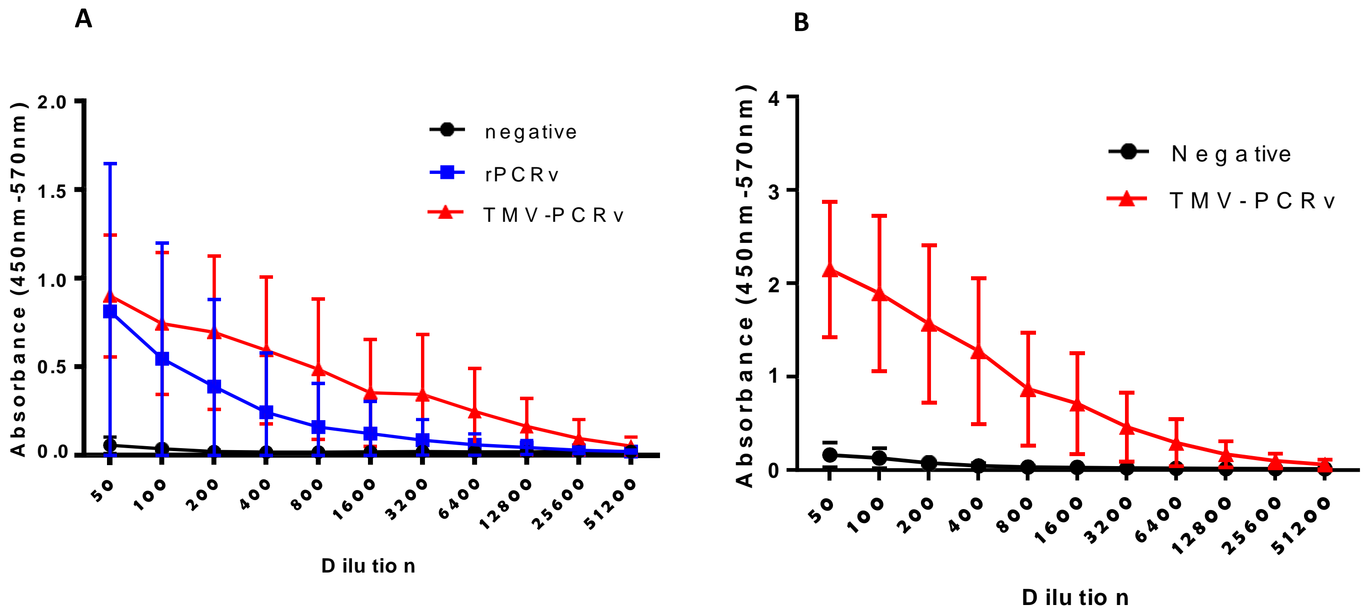

Figure 4: Immunized mice with TMV-PcrV vaccine generate a strong antibody response. C57BL/6 mice were immunized i.n. with TMV-PcrV vaccine (n=10/group). On days 21 **(A)** and 35 **(B)** post-primary immunization, anesthetized mice were bled retro-orbitally to obtain serum. *P. aeruginosa* specific total antibodies levels in serum samples were determined using an ELISA plates coated with *P. aeruginosa* rPcrV protein. The data are presented as Mean ± S.D.

50% Maximum Binding Titer

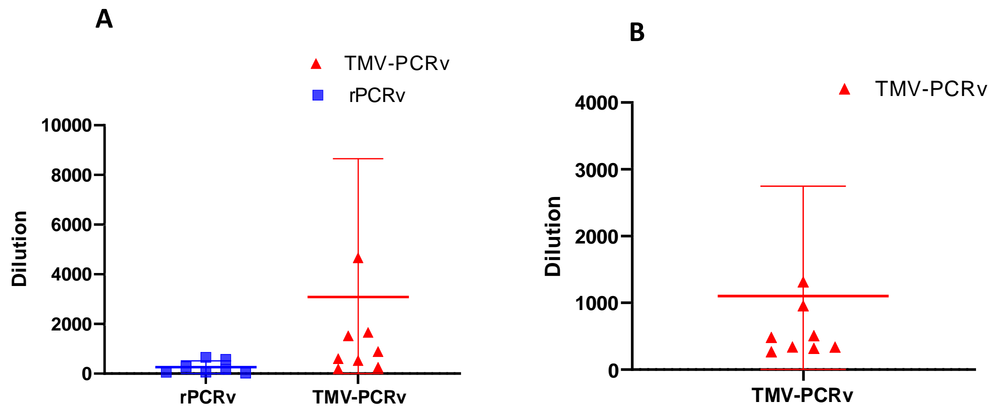

Figure 5: Immunized mice with TMV-PcrV vaccine generate a strong antibody response. 50% maximum binding titers were calculated for total PcrV specific antibodies levels in mice vaccinated and boosted with TMV-PcrV (n = 10) and recombinant PcrV (n = 10) on days 21 **(A)** and 35 **(B). P-value= 0.1570** was determined by Paired test.

4.3 Immunization with TMV-PcrV including di-c-GMP Generates Stronger Antibody Response than rPcrV+ di-c-GMP Alone:

We next examined the total antibody response generated in mice where 2ug of the di-cyclic-GMP adjuvant were included in the vaccine formulation. The second vaccine schedule included 2 boosters on days 21 and 35 post-primary immunization and mice were bled retro-orbitally on days 14, 28, and 42. As we expected the total Ig response observed on days 28 and 42 were significantly higher than those observed with mice that were vaccinated with the rPcrV vaccine only **(Figure 6)**.

The total level of antibody responses observed in the most diluted sera (51200) with TMV-PcrV+ di-c-GMP was notably higher than the rPcrV+ di-c-GMP vaccine after the two boosters **(Figure 6.B and 6.C)**. Furthermore, many mice vaccinated with the TMV vaccine formulation, including di-c-GMP generated a higher level of PcrV-specific antibody than immunized mice with the rPcrV+ di-c-GMP **(Figure 6.B and 6.C)**.

Vaccine-specific antibody titers were elevated in the mice that received TMV-PcrV+ di-c-GMP compared to the rPcrV protein+ di-c-GMP **(Figure 7.B and 7.C)**. Interestingly, the average level of total PcrV specific Ig titers with the TMV-conjugated vaccine that included the di-c-GMP adjuvant was elevated significantly compared to the mean of antibody titers after two boosters (P-value <0.0001) **(Figure 7.B and 7.C)**.

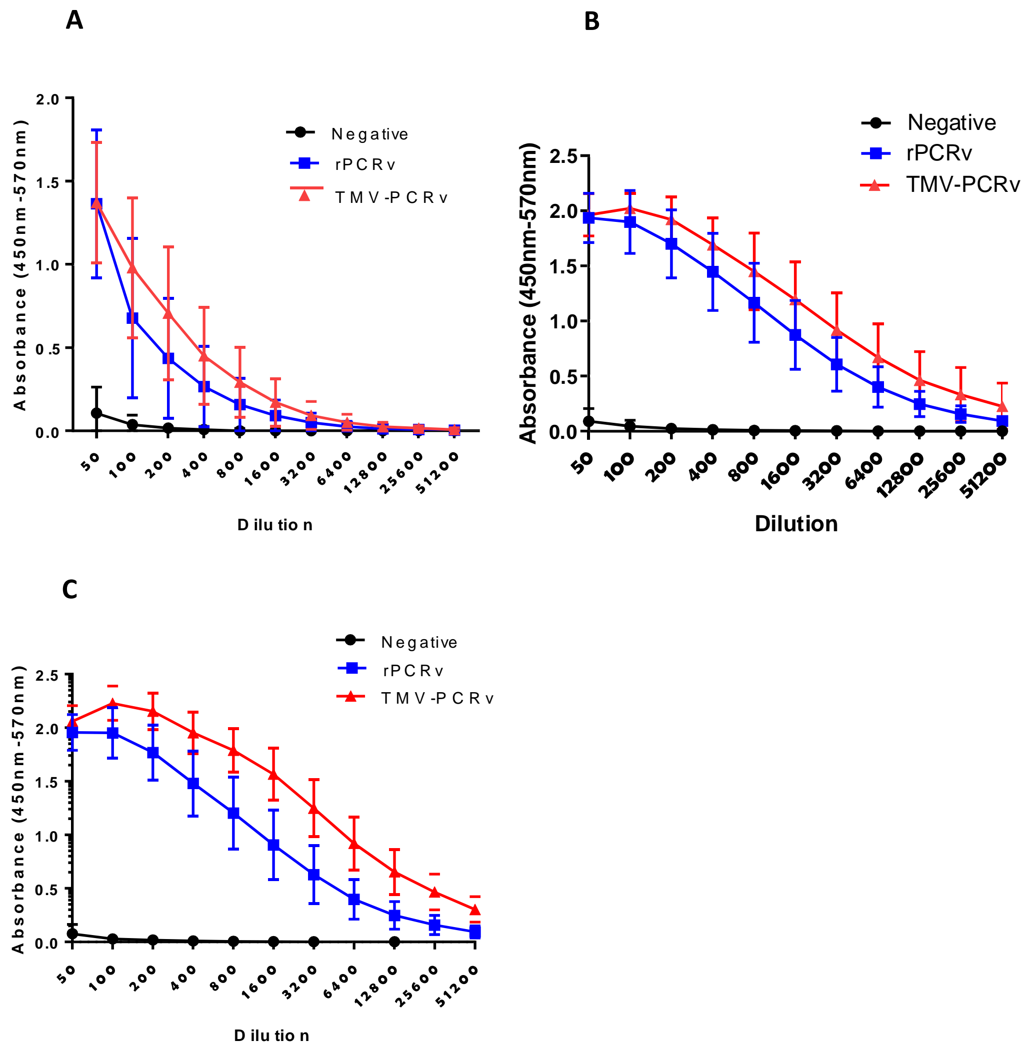

Figure 6: Immunization with TMV-PcrV including di-c-GMP generates stronger antibody response than rPcrV+ di-c-GMP alone. C57BL/6 mice were immunized i.n. with TMV-PcrV vaccine (n=20/group). On days 14 **(A)** 28 **(B)** and 42 **(C)** post-primary immunization, anesthetized mice were bled by retro-orbitally to obtain serum. *P. aeruginosa* specific total antibodies levels in serum samples were determined using an ELISA plates coated with *P. aeruginosa* rPcrV protein. The data are presented as Mean ± S.D.

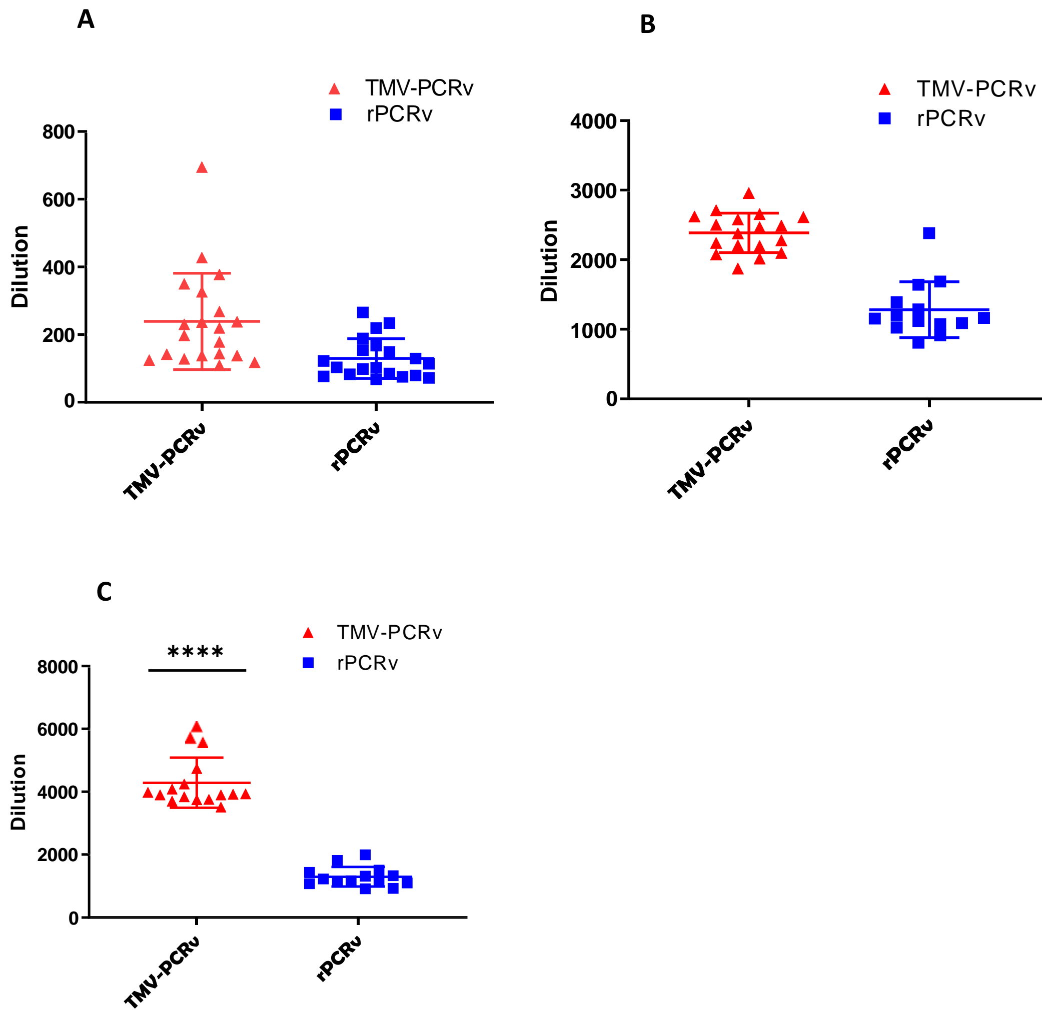

Figure 7: Immunization with TMV-PcrV including di-c-GMP generates stronger antibody response than rPcrV+ di-c-GMP alone. 50% maximum binding titers were calculated for total PcrV specific antibodies levels in mice vaccinated and boosted with TMV-PcrV (n = 20) and recombinant PcrV (n = 20) on days 14 **(A)** 28 **(B)** and 42 **(C).** Asterisk (*) illustrates the significance. **P-value <0.0001** was determined by Paired t test.

4.4 Protective Efficacy of the TMV-Conjugate Vaccine against Acute Pneumonia:

We tested the efficiency of the TMV-conjugate vaccine by performing the two schedules described in the methods section. Mice were challenged intranasally with two lethal doses of *P. aeruginosa* $1x10^8$ and $1x10^7$ seven days after the second booster. Infected mice were then monitored for morbidity and mortality 2x per day until they regained pre-challenge weight.

In the first vaccine experiment (schedule I), 100% of the control mice that were challenged with $1x10^8$ CFU bacteria and received no vaccine succumbed to infection by day 1. All mice immunized with TMV-PcrV that received the same dose died by day 2, demonstrating a right shift in MTD **(Figure 8)**. In the second group, challenged with $1x10^7$ CFU bacteria, all unvaccinated mice succumbed to infection by Day 2.5 (the afternoon of Day 2), while 80% of mice vaccinated with TMV-PcrV survived the infection **(Figure 8)**.

In the second vaccine experiment (schedule II), 100% of unvaccinated mice challenged with $1x10^8$ CFU bacteria succumbed to infection by the morning of day 1. 50% of rPcrV + di-c-GMP receiving the same dose died by the afternoon of day 1 post-infection (day 1.5) and the rest succumbed to infection by the afternoon of day 2 post-infection (day 2.5). A similar response was observed in the TMV+ di-c-GMP group; 60% of the mice died by the afternoon of the first-day post-challenge (day 1.5), while the remaining 40% succumbed to infection by the afternoon of the second day post-challenged (day 2.5) **(Figure 9).** In the group that received $1x10^7$ CFU bacteria, all unvaccinated mice succumbed by the afternoon of day 2 post-infection (day 2.5). Conversely, 60% of TMV-PcrV+ di-c-GMP mice and 50% of rPcrV+ di-c-GMP survived the infection. The death occurred at a more rapid rate in mice vaccinated with rPcrV compared to mice vaccinated with TMV-PcrV **(Figure 9)**.

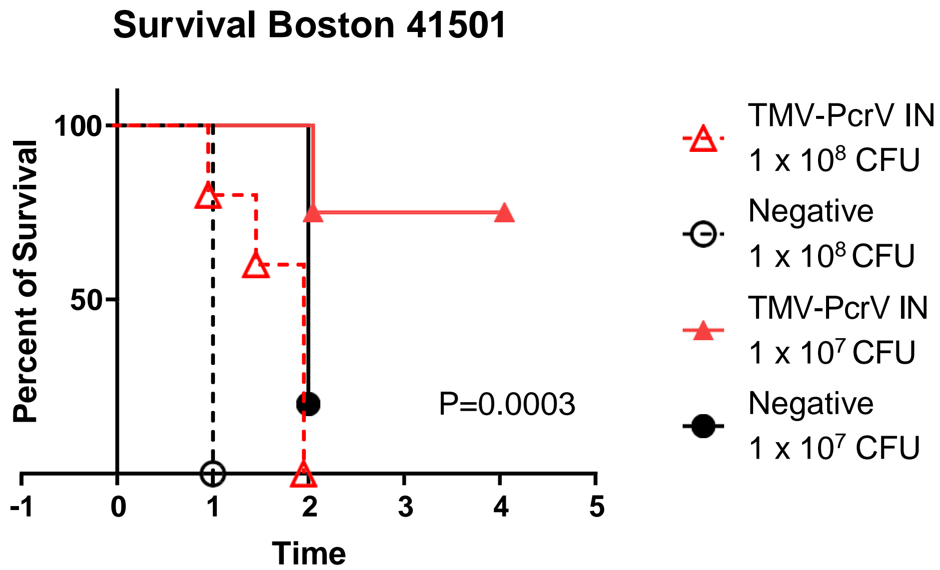

Figure 8: Protective efficacy of TMV-vaccine against an acute pneumonia model. Survival of mice vaccinated (n=10) i.n. with TMV-PcrV. Immunized mice (n=5/group) were challenged i.n. with $1x10^8$ or $1x10^7$ CFUs of *P. aeruginosa* on day 42 post-primary immunization. Mice (n=10) received no vaccine served as controls. Challenged mice were monitored for morbidity and mortality for four days post-challenging. **P-value= 0.0003** was determined by Log-rank (Mantel-Cox) test.

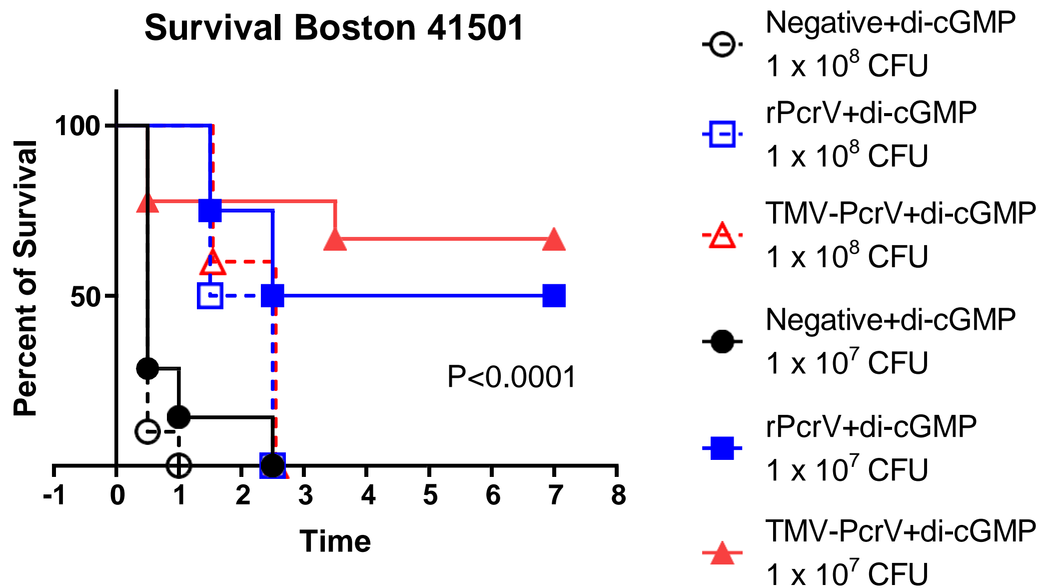

Figure 9: Protective efficacy of TMV-vaccine against an acute pneumonia model. Survival of mice vaccinated (n=20) i.n. with TMV-PcrV+ di-c-GMP. Immunized mice (n=10/group) were challenged i.n. with $1x10^8$ or $1x10^7$ CFUs of *P. aeruginosa* on day 49 post-primary immunization. Mice (n=20) received no vaccine served as controls. Challenged mice were monitored for morbidity and mortality for seven days post-challenging. **P value <0.0001** was determined by Log-rank (Mantel-Cox) test.

5. Discussion:

Pseudomonas aeruginosa is an opportunistic pathogen. It is responsible for a wide range of diseases, mostly in hospitalized, immunocompromised patients, patients with burn wounds, and patients with cystic fibrosis (Williams *et al.,* 2010). The increasing antibiotic resistance of *P. aeruginosa* creates an urgent need for vaccines, yet there is no FDA-approved *P. aeruginosa* vaccine and recent promising candidates have failed in large clinical trials.

Research on *Pseudomonas* has been focused mainly on developing an LPS-based vaccine, a live attenuated vaccine, a killed whole-cell vaccine, and subunit vaccines. However, some vaccines were limited by toxicity, inefficient protection, and lack of appropriate delivery system, which halted progress (Priebe & Goldberg, 2014).

Once developed, the pulmonary disease is difficult to treat; therefore, effective prophylaxis against *Pseudomonas aeruginosa* would be highly significant (Kalil *et al.,* 2016; Gellatly & Hancock, 2013). There are many trials to develop antibody-based therapy against *Pseudomonas* infections, but unfortunately, none of these has been approved by U.S Food and Drug Administration. Most fail in phase 2 or 3 clinical trial development (Priebe & Goldberg, 2014).

Recently, developing the subunit vaccines using the adenoviral vector and keyhole limpet hemocyanin (KLH) delivery platform showed great promise in animal models and significant protection against acute pneumonia since adenoviral vectors display an OprF peptide that has both B and T cell epitope (Priebe & Goldberg, 2014).

Other proteins including outer membrane proteins (OprF and OprI), were investigated as a subunit vaccine that showed a well-tolerated reaction in a human trial as well. However, despite different subunit components providing good protection, they either failed or protected only against 50% of lethal doses of *P. aeruginosa* (Priebe & Goldberg, 2014). A reasonable explanation of the limited protection of these subunit

vaccines, even though they are potently immunogenic, is the lack of an adequate delivery platform or adjuvant that can deliver antigen in a consistent way.

The purpose of this study was to explore and generate proof of concept data for a potential vaccine using a TMV delivery platform that offers adequate protection against respiratory *P. aeruginosa* infections. TMV is a potent antigen carrier that has been found to provide a robust humoral response since it has repetitive antigens that mimic the virus's surface. This is essential in generating a strong antibody response (McCormick & Palmer, 2008). In addition, TMV's nonfunctional RNA only in animals is significant for inducing Th1 cellular immunity (McCormick & Palmer, 2008; Kemnade *et al.,* 2014).

In our study, we used the purified proteins of *P. aeruginosa* for TMV conjugation studies and tested the efficiency of TMV *Pseudomonas* protein conjugates to prove the concept and ability of TMV as a carrier for *Pseudomonas* antigens. For our ultimate study, three genes of *P. aeruginosa* encoding for PcrV, OprI, and OprF were cloned in *E. coli* expression vector pET28a*,* Pet100/D-TOPO*,* and Pet100/D-TOPO, respectively and expressed in *E. coli* BL21(DE3), *E. coli* PlySE(DE3), *and E. coli* PlySE(DE3) competent cells, respectively. They were then purified using metal affinity chromatography.

These three genes were selected based in part on a previous study done in our lab with *Y. pestis* LcrV, which is an analogue of PcrV, that showed a high level of protection (Arnaboldi *et al.,* 2016). Similarly, these outer membrane proteins were used in other studies as subunit vaccines and showed high levels of stimulating IgG responses as well as inducing IFN-γ CD4 and CD8 responses (Priebe & Goldberg, 2014; Yang *et al.,* 2017). PcrV was chemically conjugated to the TMV surface using (EDC) and (NHS) protocol by Dr. Alison McCormick and her lab members at Touro University in California. Our initial intention for this thesis was to conjugate and test OprF and OprI as well. However, extensive troubleshooting was required to produce

these two proteins. While both proteins were ultimately produced and purified in sufficient quantities, we were unable to have them conjugated in time for *in vivo* studies.

Two vaccine formulations were used in this study consisting of the vaccine conjugate (TMV-PcrV) or unconjugated protein (rPcrV) and di-cyclic-GMP adjuvant were included with the vaccine group.

Anti-PcrV antibody was generated in both the presence and absence of TMV but was enhanced by chemical conjugation of PcrV to TMV. In our LcrV studies, chemical conjugation between the protein antigen and TMV has been shown to be critical for enhancing antibody production. This indicates that PcrV is strongly immunogenic and that antigenicity is enhanced by TMV.

Additionally, in the first experiment with the PcrV-TMV, an 80% of survival rate was observed after mice received 3 doses of vaccine on days 0, 14 and 28 then were challenged with $1x10^7$ CFU of *Pseudomonas* bacteria **(Figure 8)**. Recombinant protein alone was absent from this challenge experiment because the group was accidentally euthanized by Dr. Arnaboldi during a scheduled retro-orbital bleed. This result proves the efficiency of TMV as a potent delivery platform of *Pseudomonas* antigens and stimulates a robust protective immune response.

Furthermore, when we compared the humoral immune response of two immunization schedules, di-c-GMP adjuvant appeared to greatly enhance the anti-PcrV antibody response, when comparing the magnitude of the antibody response in TMV-PcrV or rPcrV mice to that of unvaccinated mice **(Figure 4 and 6)**. Collectively, these results indicate that a TMV-PcrV+ di-c-GMP adjuvant vaccine is an efficient vaccine formulation that can generate a potent humoral immune response. Despite this, vaccine-mediated protection was not enhanced by the addition of a di-cyclic-GMP adjuvant.

In the second experiment (schedule II), where di-c-GMP was included with the vaccine formulation, more than 60% of survival rate was observed after TMV mice received 3 doses of vaccine on days 0, 21 and 35 then were challenged with $1x10^7$ CFU of *Pseudomonas* bacteria. In contrast, 50% of mice vaccinated with rPcrV survived **(Figure9)**. Compared to the survival rate in the first experiment, these results indicate that the inclusion of di-c-GMP in the vaccine formulation can enhance antibody production for both TMV and rPcrV groups **(Figure6)**, but enhanced antibody production does not translate to enhanced survival.

The inability of TMV-PcrV to completely protect against lethal challenge with *P. aeruginosa* suggests that additional antigens are needed for full protection. In addition, it is not clear that all strains of *Pseudomonas* will utilize the same spectrum of virulence factors providing further support for the inclusion of additional vaccine proteins for a multi-subunit vaccine.

In conclusion, our vaccine showed partial efficacy and provided proof of the principle that a TMV-based multi-subunit vaccine can be effective. Our results show that a TMV-based vaccine could be administered with multiple doses without concerns about side effects and toxicity. The TMV can have a dual function as antigen carrier as well as an enhancer of immune response. Taken together, TMV can serve as a suitable platform to deliver *Pseudomonas aeruginosa* proteins.

List of Reference

Abeyrathne, P. D., Daniels, C., Poon, K. K. H., Matewish, M. J., & Lam, J. S. (2005). Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of Pseudomonas aeruginosa lipopolysaccharide. *Journal of Bacteriology*, *187*(9), 3002–3012.

Arnaboldi, P. M., Sambir, M., D'Arco, C., Peters, L. A., Seegers, J. F. M. L., Mayer, L., McCormick, A. A., Dattwyler, R. J. (2016). Intranasal delivery of a protein subunit vaccine using a Tobacco Mosaic Virus platform protects against pneumonic plague. *Vaccine*, *34*(47), 5768–5776.

Banik, S., Mansour, A. A., Suresh, R. V., Wykoff-Clary, S., Malik, M., McCormick, A. A., & Bakshi, C. S. (2015). Development of a multivalent subunit vaccine against tularemia using tobacco mosaic virus (TMV) based delivery system. *PLoS ONE*, *10*(6).

Benz, R., & Hancock, R. E. W. (1981). Properties of the large ion-permeable pores formed from protein F of Pseudomonas aeruginosa in lipid bilayer membranes. *BBA - Biomembranes*, *646*(2), 298–308.

Berry, M. C., Mcghee, G. C., Zhao, Y., & Sundin, G. W. (2009). Effect of a waaL mutation on lipopolysaccharide composition, oxidative stress survival, and virulence in Erwinia amylovora: RESEARCH LETTER. *FEMS Microbiology Letters*, *291*(1), 80–87.

Bielen, K., Jongers, B., Malhotra-Kumar, S., Jorens, P. G., Goossens, H., & Kumar-Singh, S. (2017). Animal models of hospital-acquired pneumonia: Current practices and future perspectives. *Annals of Translational Medicine*, *5*(6).

Blohmke, C. J., Mayer, M. L., Tang, A. C., Hirschfeld, A. F., Fjell, C. D., Sze, M. A.,

Falsafi, R., Wang, S., Hsu, K., Chilvers, M. A., Hogg, J. C., Hancock, R. E., Turvey, S. E. (2012). Atypical Activation of the Unfolded Protein Response in Cystic Fibrosis Airway Cells Contributes to p38 MAPK-Mediated Innate Immune Responses. *The Journal of Immunology*, *189*(11), 5467–5475.

Buret, A., Dunkley, M., Clancy, R. L., & Cripps, A. W. (1993). Effector mechanisms of intestinally induced immunity to Pseudomonas aeruginosa in the rat lung: role of neutrophils and leukotriene B4. *Infection and Immunity*, *61*(2), 671–679.

Campodónico, V. L., Llosa, N. J., Bentancor, L. V., Maira-Litran, T., & Pier, G. B. (2011). Efficacy of a conjugate vaccine containing polymannuronic acid and flagellin against experimental Pseudomonas aeruginosa lung infection in mice. *Infection and Immunity*, *79*(8), 3455–3464.

Carevic, M., Öz, H., Fuchs, K., Laval, J., Schroth, C., Frey, N., Hector, A., Bilich, T., Haug, M., Schmidt, A., Autenrieth, S. E., Bucher, K., Beer-Hammer, S., Gaggar, A., Kneilling, M., Benarafa, C., Gao, J. L., Murphy, P. M., Schwarz, S., Moepps, B., Hartl, D. (2016). CXCR1 Regulates Pulmonary Anti-Pseudomonas Host Defense. *Journal of Innate Immunity*, *8*(4), 362–373.

Carlander, D., Kollberg, H., Wejåker, P. E., & Larsson, A. (2000). Peroral immunotheraphy with yolk antibodies for the prevention and treatment of enteric infections. *Immunologic Research*, *21*(1), 1–6.

Chevalier, S., Bouffartigues, E., Bodilis, J., Maillot, O., Lesouhaitier, O., Feuilloley, M. G. J., Orange, N., Dufour, A., Cornelis, P. (2017). Structure, function and regulation of Pseudomonas aeruginosa porins. *FEMS Microbiology Reviews*, *020*, 698–722.

Coffman, R. L., Sher, A., & Seder, R. A. (2010). Vaccine adjuvants: Putting innate immunity to work. *Immunity*, Vol. 33, pp. 492–503.

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G.

C. L., & Parsek, M. R. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. *PLoS Pathogens*, *7*(1).

Cripps, A. W., Dunkley, M. L., & Clancy, R. L. (1994). Mucosal and systemic immunizations with killed Pseudomonas aeruginosa protect against acute respiratory infection in rats. *Infection and Immunity*, *62*(4), 1427–1436.

Curran, C. S., Bolig, T., & Torabi-Parizi, P. (2018). Mechanisms and targeted therapies for pseudomonas aeruginosa lung infection. *American Journal of Respiratory and Critical Care Medicine*, Vol. 197, pp. 708–727.

Dasgupta, T., De Kievit, T. R., Masoud, H., Altman, E., Richards, J. C., Sadovskaya, I., Speert, D. P., Lam, J. S. (1994). Characterization of lipopolysaccharide-deficient mutants of Pseudomonas aeruginosa derived from serotypes O3, O5, and O6. *Infection and Immunity*, *62*(3), 809–817.

Deng, Q., Wang, Y., Zhang, Y., Li, M., Li, D., Huang, X., Wu, Y., Pu, J., Wu, M. (2015). Pseudomonas aeruginosa triggers macrophage autophagy to escape intracellular killing by activation of the NLRP3 inflammasome. *Infection and Immunity*, *84*(1), 56–66.

Di Pasquale, A., Preiss, S., Da Silva, F. T., & Garçon, N. (2015). Vaccine adjuvants: From 1920 to 2015 and beyond. *Vaccines*, Vol. 3, pp. 320–343.

Döring, G., Meisner, C., & Stern, M. (2007). A double-blind randomized placebo-controlled phase III study of a Pseudomonas aeruginosa flagella vaccine in cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(26), 11020–11025.

Döring, G., & Pier, G. B. (2008). Vaccines and immunotherapy against Pseudomonas aeruginosa. *Vaccine*, Vol. 26, pp. 1011–1024.

Dunkley, M. L., Clancy, R. L., & Cripps, A. W. (1994). A role for CD4+ T cells from

orally immunized rats in enhanced clearance of Pseudomonas aeruginosa from the lung. *Immunology*, *83*(3), 362–369.

El Solh, A. A., Akinnusi, M. E., Wiener-Kronish, J. P., Lynch, S. V., Pineda, L. A., & Szarpa, K. (2008). Persistent infection with Pseudomonas aeruginosa in ventilator-associated pneumonia. *American Journal of Respiratory and Critical Care Medicine*, *178*(5), 513–519.

Feng, L., Xiang, Q., Ai, Q., Wang, Z., Zhang, Y., & Lu, Q. (2016). Effects of Quorum Sensing Systems on Regulatory T Cells in Catheter-Related Pseudomonas aeruginosa Biofilm Infection Rat Models. *Mediators of Inflammation*, 4012912.

Fitchen, J., Beachy, R. N., & Hein, M. B. (1995). Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. *Vaccine*, *13*(12), 1051–1057.

Fito-Boncompte, L., Chapalain, A., Bouffartigues, E., Chaker, H., Lesouhaitier, O., Gicquel, G., Bazire, A., Madi, A., Connil, N., Véron, W., Taupin, L., Toussaint, B., Cornelis, P., Wei, Q., Shioya, K., Déziel, E., Feuilloley, M., Orange, N., Dufour, A., Chevalier, S. (2011). Full virulence of Pseudomonas aeruginosa requires OprF. *Infection and Immunity*, *79*(3), 1176–1186.

Flynn, J. L., & Ohman, D. E. (1988). Cloning of genes from mucoid Pseudomonas aeruginosa which control spontaneous conversion to the alginate production phenotype. *Journal of Bacteriology*, *170*(4), 1452–1460.

Franklin, M. J., Nivens, D. E., Weadge, J. T., & Lynne Howell, P. (2011). Biosynthesis of the Pseudomonas aeruginosa extracellular polysaccharides, alginate, Pel, and Psl. *Frontiers in Microbiology*, Vol. 2, 167.

Fukuoka, T., Ohya, S., Narita, T., Katsuta, M., Iijima, M., Masuda, N., Yasuda, H., Trias, J., Nikaido, H. (1993). Activity of the carbapenem panipenem and role of

the OprD (D2) protein in its diffusion through the Pseudomonas aeruginosa outer membrane. *Antimicrobial Agents and Chemotherapy*, *37*(2), 322–327.

Gellatly, S. L., & Hancock, R. E. W. (2013). Pseudomonas aeruginosa: New insights into pathogenesis and host defenses. *Pathogens and Disease*, *67*(3), 159–173.

Gioannini, T. L., & Weiss, J. P. (2007). Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunologic Research*, Vol. 39, pp. 249–260.

Grassme, H., Kirschnek, S., Riethmueller, J., Riehle, A., Von Kurthy, G., Lang, F., Weller, M., Gulbins, E. (2000). CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. *Science*, *290*(5491), 527–530.

Hajjar, A. M., Tsai, J. H., Wilson, C. B., & Miller, S. I. (2002). Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nature Immunology*, *3*(4), 354–359.

Hancock, R. E., Mutharia, L. M., Chan, L., Darveau, R. P., Speert, D. P., & Pier, G. B. (1983). Pseudomonas aeruginosa isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infection and Immunity*, *42*(1), 170–177.

Hauser, A. R. (2009). The type III secretion system of Pseudomonas aeruginosa: Infection by injection. *Nature Reviews Microbiology*, Vol. 7, pp. 654–665.

Hogardt, M., & Heesemann, J. (2010). Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. *International Journal of Medical Microbiology*, Vol. 300, pp. 557–562.

Horn, M. P., Zuercher, A. W., Imboden, M. A., Rudolf, M. P., Lazar, H., Wu, H., Hoiby, N., Fas, SC., Lang, A. B. (2010). Preclinical in vitro and in vivo

characterization of the fully human monoclonal IgM antibody KBPA101 specific for Pseudomonas aeruginosa serotype IATS-O11. *Antimicrobial Agents and Chemotherapy*, *54*(6), 2338–2344.

Housman, S. T., Crandon, J. L., Nichols, W. W., & Nicolau, D. P. (2014). Efficacies of ceftazidime-avibactam and ceftazidime against Pseudomonas aeruginosa in a murine lung infection model. *Antimicrobial Agents and Chemotherapy*, *58*(3), 1365–1371.

Hughes, E. E., & Gilleland, H. E. (1995). Ability of synthetic peptides representing epitopes of outer membrane protein F of Pseudomonas aeruginosa to afford protection against P. aeruginosa infection in a murine acute pneumonia model. *Vaccine*, *13*(18), 1750–1753.

Hurd, S. (2000). The impact of COPD on lung health worldwide: epidemiology and incidence. *Chest*, *117*(2 Suppl), 1S-4S.

Jacoby, G. A., & Munoz-Price, L. S. (2005). The New β-Lactamases. *New England Journal of Medicine*, *352*(4), 380–391.

Jain-Vora, S., LeVine, A. M., Chroneos, Z., Ross, G. F., Hull, W. M., & Whitsett, J. A. (1998). Interleukin-4 enhances pulmonary clearance of Pseudomonas aeruginosa. *Infection and Immunity*, *66*(9), 4229–4236.

Joo, N. S., Krouse, M. E., Wu, J. V, Saenz, Y., Jayaraman, S., Verkman, A. S., & Wine, J. J. (2001). HCO3- transport in relation to mucus secretion from submucosal glands. *JOP : Journal of the Pancreas*, *2*(4 Suppl), 280–284.

Kalil, A. C., Metersky, M. L., Klompas, M., Muscedere, J., Sweeney, D. A., Palmer, L. B., Napolitano, L. M., O'Grady, N. P., Bartlett, J. G., Carratalà, J., El Solh, A. A., Ewig, S., Fey, P. D., File, T. M.Jr., Restrepo, M. I., Roberts, J. A., Waterer, G. W., Cruse, P., Knight, S. L., Brozek, J. L. (2016). Management of Adults With Hospital-acquired and Ventilator-associated Pneumonia: 2016 Clinical Practice

Guidelines by the Infectious Diseases Society of America and the American Thoracic Society.
Clinical Infectious Diseases, Vol. 63, pp. 61–111.

Kamei, A., Coutinho-Sledge, Y. S., Goldberg, J. B., Priebe, G. P., & Pier, G. B. (2011). Mucosal vaccination with a multivalent, live-attenuated vaccine induces multifactorial immunity against Pseudomonas aeruginosa acute lung infection. *Infection and Immunity*, *79*(3), 1289–1299.

Kamei, A., Wu, W., Traficante, D. C., Koh, A. Y., Van Rooijen, N., Pier, G. B., & Priebe, G. P. (2013). Collaboration between macrophages and vaccine-induced CD4+ T cells confers protection against lethal pseudomonas aeruginosa pneumonia during neutropenia. *Journal of Infectious Diseases*, *207*(1), 39–49.

Kannan, S., Huang, H., Seeger, D., Audet, A., Chen, Y., Huang, C., Gao, H., Li, S., Wu, M. (2009). Alveolar epithelial type II cells activate alveolar macrophagesand mitigate P. aeruginosa infection. *PLoS ONE*, *4*(3).

Kemnade, J. O., Seethammagari, M., Collinson-Pautz, M., Kaur, H., Spencer, D. M., & McCormick, A. A. (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine*, *32*(33), 4228–4233.

Kernan, D. L., Wen, A. M., Pitek, A. S., & Steinmetz, N. F. (2017). Featured Article: Delivery of chemotherapeutic vcMMAE using tobacco mosaic virus nanoparticles. *Experimental Biology and Medicine*, *242*(14), 1405–1411.

King, J. D., Kocíncová, D., Westman, E. L., & Lam, J. S. (2009). Lipopolysaccharide biosynthesis in Pseudomonas aeruginosa. *Innate Immunity*, Vol. 15, pp. 261–312.

Kipnis, E., Sawa, T., & Wiener-Kronish, J. (2006). Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Medecine et Maladies Infectieuses*,

Vol. 36, pp. 78–91.

Klockgether, J., Cramer, N., Wiehlmann, L., Davenport, C. F., & Tümmler, B. (2011). Pseudomonas aeruginosa genomic structure and diversity. *Frontiers in Microbiology*, *2*, 150.

Knight, D. A., & Holgate, S. T. (2003). The airway epithelium: Structural and functional properties in health and disease. *Respirology*, Vol. 8, pp. 432–446.

Kukavica-Ibrulj, I., & Levesque, R. C. (2008). Animal models of chronic lung infection with Pseudomonas aeruginosa: Useful tools for cystic fibrosis studies. *Laboratory Animals*, Vol. 42, pp. 389–412.

Laarman, A. J., Bardoel, B. W., Ruyken, M., Fernie, J., Milder, F. J., van Strijp, J. A. G., & Rooijakkers, S. H. M. (2012). Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways . *The Journal of Immunology*, *188*(1), 386–393.

Lam, J. S., Taylor, V. L., Islam, S. T., Hao, Y., & Kocíncová, D. (2011). Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide. *Frontiers in Microbiology*, *2,* 118.

Lavelle, G. M., White, M. M., Browne, N., Mcelvaney, N. G., & Reeves, E. P. (2016). Animal Models of Cystic Fibrosis Pathology: Phenotypic Parallels and Divergences. *BioMed Research International*, 5258727.

Lavoie, E. G., Wangdi, T., & Kazmierczak, B. I. (2011). Innate immune responses to Pseudomonas aeruginosa infection. *Microbes and Infection*, Vol. 13, pp. 1133–1145.

Livermore, D. M. (2002). Multiple Mechanisms of Antimicrobial Resistance in Pseudomonas aeruginosa: Our Worst Nightmare? *Clinical Infectious Diseases*, *34*(5), 634–640.

López Hernández, Y., Yero, D., Pinos-Rodríguez, J. M., & Gibert, I. (2015). Animals

devoid of pulmonary system as infection models in the study of lung bacterial pathogens. *Frontiers in Microbiology*, Vol. 6, 38.

Lu, Q., Eggimann, P., Luyt, C. E., Wolff, M., Tamm, M., François, B., Mercier, E., Garbino, J., Laterre, P. F., Koch, H., Gafner, V., Rudolf, M. P., Mus, E., Perez, A., Lazar, H., Chastre, J., & Rouby, J. J. (2014). Pseudomonas aeruginosa serotypes in nosocomial pneumonia: prevalence and clinical outcomes. *Critical care (London, England)*, *18*(1), R17.

Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews*, Vol. 15, pp. 194–222.

Maharaj, R., May, T. B., Wang, S. K., & Chakrabarty, A. M. (1993). Sequence of the alg8 and alg44 genes involved in the synthesis of alginate by Pseudomonas aeruginosa. *Gene*, *136*(1–2), 267–269.

Malloy, J. L., Veldhuizen, R. A. W., Thibodeaux, B. A., O'Callaghan, R. J., & Wright, J. R. (2005). Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *288*(2), L409-18.

Maura, D., Bandyopadhaya, A., & Rahme, L. G. (2018). Animal models for pseudomonas aeruginosa quorum sensing studies. In *Methods in Molecular Biology* (Vol. 1673, pp. 227–241).

McCormick, A. A., & Palmer, K. E. (2008). Genetically engineered Tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines*, Vol. 7, pp. 33–41.

Moradali, M. F., Ghods, S., & Rehm, B. H. A. (2017). Pseudomonas aeruginosa lifestyle: A paradigm for adaptation, survival, and persistence. *Frontiers in Cellular and Infection Microbiology*, Vol. 7, 39.

Moser, C., Johansen, H. K., Song, Z., Hougen, H. P., Rygaard, J., & Høiby, N.

(1997). Chronic Pseudomonas aeruginosa lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, *105*(11), 838–842.

Nguyen, D., & Singh, P. K. (2006). Evolving stealth: Genetic adaptation of Pseudomonas aeruginosa during cystic fibrosis infections. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 103, pp. 8305–8306.

Noda, Y., Yasuoka, S., Inoue, I., Tani, K., Fujisawa, K., & Ogura, T. (1992). Analysis of IgA Antibody to Pseudomonas aeruginosa in Sera and Sputa of Patients with Chronic Airway Diseases. *Internal Medicine*, *31*(5), 575–582.

Pan, T., Tan, R., Li, M., Liu, Z., Wang, X., Tian, L., Liu, J., & Qu, H. (2016). IL17-Producing γδ T Cells May Enhance Humoral Immunity during Pulmonary *Pseudomonas aeruginosa* Infection in Mice. *Frontiers in Cellular and Infection Microbiology*, *170*(6).

Perl, A. K. T., Tichelaar, J. W., & Whitsett, J. A. (2002). Conditional gene expression in the respiratory epithelium of the mouse. *Transgenic Research*, *11*(1), 21–29.

Pier, G. B. (2007). Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. *International Journal of Medical Microbiology*, Vol. 297, pp. 277–295.

Pier, G. B., Boyer, D., Preston, M., Coleman, F. T., Llosa, N., Mueschenborn-Koglin, S., Theilacker, C., Goldenberg, H., Uchin, J., Priebe, G. P., Grout, M., Posner, M., Cavacini, L. (2004). Human Monoclonal Antibodies to Pseudomonas aeruginosa Alginate That Protect against Infection by Both Mucoid and Nonmucoid Strains. *The Journal of Immunology*, *173*(9), 5671–5678.

Pier, G. B., Saunders, J. M., Ames, P., Edwards, M. S., Auerbach, H., Goldfarb, J.,

Speert, D. P., Hurwitch, S. (1987). Opsonophagocytic Killing Antibody to Pseudomonas aeruginosa Mucoid Exopolysaccharide in Older Noncolonized Patients with Cystic Fibrosis. *New England Journal of Medicine*, *317*(13), 793–798.

Pier, G. B., Small, G. J., & Warren, H. B. (1990). Protection Against Mucoid Pseudomonas aeruginosa in Rodent Models of Endobronchial Infections. *Science*, Vol. 249, pp. 537–540.

Priebe, G. P., Brinig, M. M., Hatano, K., Grout, M., Coleman, F. T., Pier, G. B., & Goldberg, J. B. (2002). Construction and characterization of a live, attenuated aroA deletion mutant of Pseudomonas aeruginosa as a candidate intranasal vaccine. *Infection and Immunity*, *70*(3), 1507–1517.

Priebe, G. P., & Goldberg, J. B. (2014). Vaccines for pseudomonas aeruginosa: A long and winding road. *Expert Review of Vaccines*, Vol. 13, pp. 507–519.

Priebe, G. P., Walsh, R. L., Cederroth, T. A., Kamei, A., Coutinho-Sledge, Y. S., Goldberg, J. B., & Pier, G. B. (2008). IL-17 Is a Critical Component of Vaccine-Induced Protection against Lung Infection by Lipopolysaccharide-Heterologous Strains of Pseudomonas aeruginosa. *The Journal of Immunology*, *181*(7), 4965–4975.

Ramírez-Estrada, S., Borgatta, B., & Rello, J. (2016). Pseudomonas aeruginosa ventilator-associated pneumonia management. *Infection and Drug Resistance*, Vol. 9, pp. 7–18.

Rasamiravaka, T., Labtani, Q., Duez, P., & El Jaziri, M. (2015). The formation of biofilms by Pseudomonas aeruginosa: a review of the natural and synthetic compounds interfering with control mechanisms. *BioMed Research International*, 759348.

Remminghorst, U., & Rehm, B. H. A. (2006). In vitro alginate polymerization and the functional role of Alg8 in alginate production by Pseudomonas aeruginosa. *Applied and Environmental Microbiology*, *72*(1), 298–305.

Römling, U. (2002). Molecular biology of cellulose production in bacteria. *Research in Microbiology*, Vol. 153, pp. 205–212.

Roussel, L., LaFayette, S., Nguyen, D., Baglole, C. J., & Rousseau, S. (2016). Differential Contribution of the Aryl-Hydrocarbon Receptor and Toll-Like Receptor Pathways to IL-8 Expression in Normal and Cystic Fibrosis Airway Epithelial Cells Exposed to *Pseudomonas aeruginosa*. *Frontiers in Cell and Developmental Biology*, *4*, 148.

Ryder, C., Byrd, M., & Wozniak, D. J. (2007). Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Current Opinion in Microbiology*, Vol. 10, pp. 644–648.

Saiman, L., & Prince, A. (1993). Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *Journal of Clinical Investigation*, *92*(4), 1875–1880.

Santajit, S., & Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Research International*, 2475067.

Sato, H., & Frank, D. W. (2011). Multi-Functional Characteristics of the Pseudomonas aeruginosa Type III Needle-Tip Protein, PcrV; Comparison to Orthologs in other Gram-negative Bacteria. *Frontiers in Microbiology*, *142*(2).

Sato, H., Hunt, M. L., Weiner, J. J., Hansen, A. T., & Frank, D. W. (2011). Modified needle-tip PcrV proteins reveal distinct phenotypes relevant to the control of type III secretion and intoxication by Pseudomonas aeruginosa. *PLoS ONE*, *6*(3).

Sawa, T., Yahr, T. L., Ohara, M., Kurahashi, K., Gropper, M. A., Wiener-Kronish, J. P., & Fkank, D. W. (1999). Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury. *Nature Medicine*, *5*(4), 392–398.

Shrout, J. D., Chopp, D. L., Just, C. L., Hentzer, M., Givskov, M., & Parsek, M. R. (2006). The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. *Molecular Microbiology*, *62*(5), 1264–1277.

Simmons, N. S., & Blout, E. R. (1960). The Structure of Tobacco Mosaic Virus and Its Components: Ultraviolet Optical Rotatory Dispersion. *Biophysical Journal*, *1*(1), 55–62.

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S. M., Burns, J. L., Kaul, R., Olson, M. V. (2006). Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(22), 8487–8492.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., Olson, M. V. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959–964.

Stubbs, G. (1999). Tobacco mosaic virus particle structure and the initiation of disassembly. *Philosophical Transactions of the Royal Society B: Biological*

Sciences, *354*(1383), 551–557.

Tabor, D. E., Oganesyan, V., Keller, A. E., Yu, L., McLaughlin, R. E., Song, E., Warrener, P., Rosenthal, K., Esser, M., Qi, Y., Ruzin, A., Stover, C. K., DiGiandomenico, A. (2018). Pseudomonas aeruginosa PcrV and Psl, the Molecular Targets of Bispecific Antibody MEDI3902, Are Conserved Among Diverse Global Clinical Isolates*. The Journal of Infectious Diseases, 218*(12):1983-1994.

Tager, A. M., Wu, J., & Vermeulen, M. W. (1998). The effect of chloride concentration on human neutrophil functions: Potential relevance to cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology*, *19*(4), 643–652.

Tümmler, B., & Klockgether, J. (2017). Recent advances in understanding Pseudomonas aeruginosa as a pathogen. *F1000Research*, Vol. 6, 1261.

Ueno, K., Koga, T., Kato, K., Golenbock, D. T., Gendler, S. J., Kai, H., & Kim, K. C. (2008). MUC1 mucin is a negative regulator of toll-like receptor signaling. *American Journal of Respiratory Cell and Molecular Biology*, *38*(3), 263–268.

Vaidyanathan, B., Chaudhry, A., Yewdell, W. T., Angeletti, D., Yen, W. F., Wheatley, A. K., Bradfield, C. A., McDermott, A. B., Yewdell, J. W., Rudensky, A. Y., Chaudhuri, J. (2017). The aryl hydrocarbon receptor controls cell-fate decisions in B cells. *Journal of Experimental Medicine*, *214*(1), 197–208.

Von Specht, B. U., Knapp, B., Muth, G., Broker, M., Hungerer, K. D., Diehl -, K. D., Massarrat, K., Seemann, A., Domdey, H. (1995). Protection of immunocompromised mice against lethal infection with Pseudomonas aeruginosa by active or passive immunization with recombinant P. aeruginosa outer membrane protein F and outer membrane protein I fusion proteins. *Infection and Immunity*, *63*(5), 1855–1862.

Wan, C., Zhang, J., Zhao, L., Cheng, X., Gao, C., Wang, Y., Xu, W., Zou, Q., Gu, J. (2019). Rational Design of a Chimeric Derivative of PcrV as a Subunit Vaccine Against Pseudomonas aeruginosa. *Frontiers in Immunology*, *10*, 781.

Westritschnig, K., Hochreiter, R., Wallner, G., Firbas, C., Schwameis, M., & Jilma, B. (2014). A randomized, placebo-controlled phase i study assessing the safety and immunogenicity of a Pseudomonas aeruginosa hybrid outer membrane protein OprF/I vaccine (IC43) in healthy volunteers. *Human Vaccines and Immunotherapeutics*, *10*(1), 2846–2859.

Williams, B. J., Dehnbostel, J., & Blackwell, T. S. (2010). Pseudomonas aeruginosa: Host defence in lung diseases. *Respirology*, Vol. 15, pp. 1037–1056.

Williams, O. W., Sharafkhaneh, A., Kim, V., Dickey, B. F., & Evans, C. M. (2006). Airway mucus: From production to secretion. *American Journal of Respiratory Cell and Molecular Biology*, Vol. 34, pp. 527–536.

Wolf, L., Sapich, S., Honecker, A., Jungnickel, C., Seiler, F., Bischoff, M., Wonnenberg, B., Herr, C., Schneider-Daum, N., Lehr, C. M., Bals, R., Beisswenger, C. (2016). IL-17A-mediated expression of epithelial IL-17C promotes inflammation during acute Pseudomonas aeruginosa pneumonia. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *311*(5), L1015–L1022.

Worbs, T., Hammerschmidt, S. I., & Förster, R. (2017). Dendritic cell migration in health and disease. *Nature Reviews Immunology*, Vol. 17, pp. 30–48.

Worgall, S., Kikuchi, T., Singh, R., Martushova, K., Lande, L., & Crystal, R. G. (2001). Protection against pulmonary infection with Pseudomonas aeruginosa following immunization with P. aeruginosa-pulsed dendritic cells. *Infection and Immunity*, *69*(7), 4521–4527.

Wu, W., Huang, J., Duan, B., Traficante, D. C., Hong, H., Risech, M., Priebe, G. P.

(2012). Th17-stimulating protein vaccines confer protection against Pseudomonas aeruginosa pneumonia. *American Journal of Respiratory and Critical Care Medicine*, *186*(5), 420–427.

Yang, F., Gu, J., Yang, L., Gao, C., Jing, H., Wang, Y., Zhang, J. (2017). Protective Efficacy of the Trivalent Pseudomonas aeruginosa Vaccine Candidate PcrV-OprI-Hcp1 in Murine Pneumonia and Burn Models. *Scientific Reports*, *7*(1), 3957.