ENHANCEMENT OF THE HUMORAL IMMUNE RESPONSE TO PSEUDOMONAS AERUGINOSA FLAGELLIN

DIISSERTATION

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By

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ABSTRACT

The goal of this project was to develop a vaccination strategy that would enhance the protective humoral immune response against the flagellin protein of *Pseudomonas aeruginosa*. DNA vaccine constructs encoding the type A flagellin gene were tested for protective efficacy in animal studies. Even though high titers of anti-flagellar antibodies were produced in mice injected with these constructs, no protection was seen. Further analyses suggested that type A flagellin expressed by eukaryotic cells was not glycosylated, while type A flagellin expressed by *P. aeruginosa* is, and that antibodies against these glycosyl groups may be needed for protection.

The ability of a T cell binding ligand to promote a Th2 response and enhance the production of IgG1 antibodies upon intramuscular injection of DNA was tested. The DNA encoding amino acids 135-149 of the β2 domain of MHCII was cloned in front of the type B flagellin gene. After injection into mice, no enhancement or directing of the humoral immune response was seen, suggesting that this T cell binding ligand did not direct a Th2 response when it was fused to the whole flagellin protein in this DNA vaccine.

The mechanism behind the enhancement of the humoral immune response seen with dendritic cell targeting was also examined. Purified flagellin cross-linked to N418, a hamster monoclonal antibody specific for CD11c on dendritic cells, induced high titers

of anti-flagellar antibodies 7 days after injection into mice. This rapid response depended on the presence of the N418 antibody. The optimum boost schedule that resulted in the highest titers of antibody was determined to be two injections of N418 conjugate one week apart. Using this schedule, mice were significantly protected after challenge. It was determined that binding of the N418 conjugate to dendritic cells activated them causing an increase in the number of cells expressing MHCII and CD86. This correlated with the ability of the dendritic cells to stimulate primed T cells to secrete IL-2. These experiments also indicated that conjugate immunized mice produced primed T cells one week after injection.

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INTRODUCTION

1.1 *Pseudomonas aeruginosa*

1.1.1 *The organism: Pseudomonas aeruginosa* is a Gram negative bacillus belonging to the γ-Proteobacteria group. Like most pseudomonads, *P. aeruginosa* is motile, oxidase positive, catalase positive, mesophilic and has great nutrient diversity. What distinguishes it from other pseudomonads is that it possesses a single polar flagellum, it can grow at 42°C, and it produces both pyocyanin and pyoverdin (115,186). It is an obligate respirer, but it can use nitrate as a final electron acceptor. *P. aeruginosa* has a large genome, over 6.2 Mb, with 67% GC content (179).

P. aeruginosa is a ubiquitous organism found mainly in soil and water. It is also a significant opportunistic pathogen that infects people with cancer, cystic fibrosis (CF), severe burns and immunosuppressed individuals. It is also a major nosocomial pathogen accounting for 17% of hospital acquired pneumonias, 11% of urinary tract infections and 3.8% of bloodstream infections (115). Contributing to pathogenesis are several virulence factors including but not limited to pili, flagella, lipopolysaccharide, alginate and biofilm production, the secretion of several toxins, type III secretion and quorum sensing.

Along with these virulence factors, *P. aeruginosa* is highly resistant to several antibiotics such as cephalosporins, aminoglycosides and quinolones and disinfectants especially chlorinated phenols and quartenary ammonium compounds (22). This

resistance is due to several factors. First, the low permeability of the outer membrane excludes these agents from the cell (16). Second, the presence of up to 12 multi-drug efflux systems pump several types of antibiotics as well as biocides, dyes, detergents, metabolic inhibitors and organic solvents out of the cell (142, 169). Third, the microbe possesses R plasmids that encode proteins capable of inactivating many antibiotics (186). Fourth, the ability to degrade organic compounds may contribute to its resistance to disinfectants (16).

1.1.2 *Virulence factors:* To aid in adherence to host tissues *P. aeruginosa* possesses several adhesins. One major adhesin is the pilus. *P. aeruginosa* produce polar pili, classified as N-methylphenylalanine pili or type 4 pili. These appendages average 2-5 um in length, 5 nm in diameter and are retractable. The pilus filament is composed of only pilin protein subunits, arranged helically and has a base-tip differentiation where only the tip is capable of receptor binding (66). The N-terminus of the pilin subunit is probably involved in subunit-subunit interactions while the C-terminus, which is exposed at the tip of the pilus, is where receptor binding occurs (33, 44, 72). The minimum receptor for pilin binding is the disaccharide GalNAcβ(1-4)Gal found in asialo-GM1 and asialo-GM2 and lactosylceramide (11, 101, 105, 163, 170).

The role of pili in pathogenesis was demonstrated both *in vitro* and *in vivo*. *In vitro* adherence assays have found that pili are responsible for 90% of the adherence to host cells and antibodies against pili can inhibit binding to epithelial cells (28, 44, 154, 200). In AB.Y/SnJ mice, non-piliated mutants had a 10 fold higher LD_{50} than the wild-type strain (66). Pili also aid in virulence in burn infections and corneal infections (166, 211). In both instances, non-piliated mutants did not colonize the host tissues as well as

the piliated wild-type strains. Interestingly, it has been noted that *P. aeruginosa* preferentially adheres to injured epithelial cells (150, 152, 155). Scanning electron microscopic studies found that piliated strains bind injured exfoliating tracheal cells and not healthy cells, suggesting that pili are an important virulence factor in the initial colonization of the immunocompromised host (212).

A second major virulence factor in *P. aeruginosa* pathogenesis, especially in CF patients, is alginate. This molecule is an O-acelylated linear polymer of mannuronic acid and guluronic acid that is secreted from the cell and gives colonies a mucoid appearance when it is produced in large amounts. Alginate functions in the attachment to solid surfaces and in protection. Alginate binds to tracheal epithelial cells, and this binding can be decreased by the presence of antibodies against alginate or by the administration of sublethal concentrations of aminoglycosides (45, 63, 120, 151). Mucoid strains are also able to adhere to mucin in the respiratory tract (148, 153). In the host, alginate confers protection to the microbe from host defenses and antimicrobial therapy. A study by Marrie showed that even though antibodies against *P. aeruginosa* were present they were unable to coat the infecting mucoid strain (121). Then Baltimore and Mitchell showed that a higher titer of opsonizing antibody was required to get PMN killing of mucoid strains suggesting that the alginate glycocalyx prevented the antibodies from binding to the cells (12). Alginate also inhibits non-opsonic phagocytosis by neutrophils and macrophages (51). There is evidence that the presence of large quantities of alginate interferes with neutrophil chemotaxis and oxidative burst, as well as the induction of lymphocyte proliferation (88, 116). *P. aeruginosa* cells embedded in alginate biofilms are more resistant to antimicrobial agents requiring higher concentrations of the agent to

mediate killing (14, 23). The exact environmental signals that lead to the conversion to a mucoid phenotype are still unknown but nutrient deprivation, high osmolarity and dehydration seem to contribute (17, 40, 183, 184, 207). This suggests that in hostile environments, such as the CF lung, *P. aeruginosa* is induced to produce large quantities of alginate that allow it to survive and evade host immune responses.

Another major virulence factor located at the cell's surface is the flagellum. *P. aeruginosa* possesses a single polar flagellum that functions in motility and attachment. The flagellum is made up of a basal body that is anchored in the cytoplasmic membrane and cell wall with its associated motor proteins, a hook region that connects the flagellar filament to the basal body, and the flagellar filament composed mostly of the flagellin protein. Two types of flagellins are produced by *P. aeruginosa*, type A and type B. These two groups were first distinguished serologically then by molecular size (4) and finally by genetic analysis (177, 198). The type A group is a heterogeneous group with molecular weights ranging from 45-52 kDa while the type B group is more homogeneous with a molecular weight of 53 kDa (4). At the genetic level flagellin is encoded by the *fliC* gene. PCR analysis revealed that the type A strains yielded a 1.02 kb PCR product and the type B strains yielded a 1.25 kb PCR product, indicating that each strain only possess one copy of the *fliC* gene and does not undergo antigenic variation (198). A more thorough analysis by Spangenberg et al showed that the type B flagellin gene is more conserved than housekeeping genes and the amino acid sequence was identical among all the strains analyzed. However, the type A strains showed more diversity at the nucleotide and amino acid level with the most diversity occurring in the central portion of the gene (177). Comparisons between type A and B flagellin amino acid sequences

showed 63-65% identity. The most variable regions are in the central portion of the protein (177). Even with this variability, the deduced secondary and tertiary structures were remarkably similar suggesting that constraints on flagellar filament assembly limit the structural diversity of these proteins (177).

Both type A and B flagellins are post-translationally modified. Phosphorylated tyrosine residues are found in both type A and B flagellins (89, 90). In addition the type A flagellins are glycosylated which accounts for the majority of the molecular size variation seen between these flagellins (26). Recently, a glycosylation island that is responsible for flagellar glycosylation has been found in type A strains(6). In *P. aeruginosa* strain PAK this island contains 14 genes; however, several type A strains contain a shorter island that is missing several genes (8). This polymorphism is associated with different degrees of glycosylation. The longer island allowed for the addition of longer carbohydrate chains (168). In all instances though, the first sugar residue, rhamanose, was O-linked to either serine or threonine and each flagellin subunit had two possible glycan binding sites located in the central portion of the protein (168). There was no obvious sequence specificity to these sites suggesting that a structural motif, based on the surface accessibility of serine or threonine, may exist (168).

The synthesis of flagella is a tightly regulated process. More than 40 genes are involved in the expression of flagella and motility. Dasgupta et al proposed a 4-tiered hierarchy of regulation involving two alternate sigma factors and a two-component regulatory system (38). In this hierarchy the class I genes, *fleQ* and *fliA*, are constitutively expressed. FleQ along with RpoN (σ^{54}) then activate the transcription of the class II genes which encode proteins associated with the basal body, motor and switch

proteins, flagellar export apparatus, the filament cap protein, FliD, and the two-component system, FleSR. FleR and RpoN then activate the transcription of the class III genes. The signal that FleS senses to then activate FleR is unknown at this time (38). The class III genes encode the rest of the basal body and hook structures. Upon the completion of this structure the anti-sigma factor, FlgM, is exported from the cell, and the class IV genes, which includes *fliC* and *flgM*, are transcribed with the aid of FliA (σ^{28}). This allows the filament to form and the subsequent shut down of *fliC* transcription when FlgM levels increase again (38). Flagella synthesis is also controlled by other systems in the cell. The alginate alternate sigma factor, AlgT, negatively modulates the expression of flagella. The exact interactions that occur for this regulation are unknown (62).

The function of flagella in virulence was implicated by the use of flagella negative (Fla^-) mutants. In the burn mouse model, Fla- mutants showed an increase in the LD_{50} as compared to the Fla^+ wild-type strain (48). This decrease in virulence was correlated with the decreased ability of the mutant to invade deeper tissues. In a neonatal mouse model of pneumonia, infection with *fliC* mutants resulted in the development of pneumonia in 25% of the mice and no deaths, whereas infection with the parental wild-type strain resulted in the development of pneumonia in 80% of the mice and a 30% death rate. As in the burn mouse model, the decrease in virulence was linked to the inability of the *fliC* mutant to spread throughout the lung (54). These studies demonstrate the importance of motility in the invasive virulence of *P. aeruginosa*. In support of this, others studies have demonstrated that antibodies against flagella, that inhibit motility, are protective after passive transfer in animal challenge models (49, 132, 158). Along with motility, flagella also aid in the adherence to host tissues. Several receptors for flagellar

adherence have been identified. These include the glycosphingolipids, GM1 and asialo-GM1, and mucin (7, 54, 111). Flagella are also potent stimulators of the immune response. The binding of flagellin to mucosal epithelial cells induces the expression of IL-8, mucin and matrilysin (1, 112, 124).

Bacterial lipopolysaccahride (LPS) is a potent virulence factor for many Gram negative pathogens and *P. aeruginosa* is no exception. The LPS of *P. aeruginosa* is very similar to, yet distinctive from, the LPS from *Enterobacteriaceae*. Like *Enterobacteriaceae* LPS, *P. aeruginosa* LPS is a tripartite molecule composed of a hydrophobic lipid A portion embedded in the outer membrane, a polysaccharide core region that contains ketodeoxoctonate, and the hydrophilic O-polysaccharide, which is highly antigenic. Unlike *Enterobacteriaceae*, *P. aeruginosa* produces 2 forms of LPS, A band and B band. In A band LPS, the hydrophilic O-polysaccharide is composed of repeating trisaccharide D-rhamnose units (156). In B band LPS, the O-polysaccharide is a heteropolymer of repeating di- to pentasaccharide units. B band polysaccharides are much longer than A band polysaccharides, and they are the basis for serotyping (156). Currently 20 different serogroups are recognized (64). Unlike B band polysaccharides, A band polysaccharides are common in several serogroups (64, 156). Also unlike *Enterobacteriaceae* LPS, P. *aeruginosa* LPS has a different lipid A structure. Here lipid A is penta-acylated instead of hexa-acylated (102).

All three regions of the LPS molecule have a function in virulence. Rough mutants, which do not have long chain O-polysaccharide, are less virulent in animal infection models (140). These mutants are also sensitive to complement-mediated lysis of the cell, unlike smooth variants, which are resistant because they possess the long

chain O-polysaccharide. The LPS core region also plays a role in virulence by functioning as an adhesin. This region binds to the cystic fibrosis transmembrane conductance regulator (CFTR) (139). Upon binding to the receptor, the bacteria are ingested by the cells. In eye infections this may protect and hide the bacteria from the immune system, and in acute lung infections this may aid in the dissemination of the organism. A study by Priebe et al demonstrated that *galU* mutants, which are unable to make up a complete LPS core, were less virulent in an eye model of *P. aeruginosa* infection (146). They also showed that the mutants were less virulent in an acute pneumonia mouse model which correlated with a hindered ability to spread to the spleen (146). Lastly, lipid A functions in virulence as an endotoxin. Upon release from the outer membrane of the cell, lipid A is detected by host cells via CD14 and TLR4 (10, 110, 147). This recognition causes the release of several pro-inflammatory mediators, including the cytokines IL-1, IL-6, IL-8, IL-10, MCP-1, GM-CSF and TNFα (99, 156). While the lipid A from *P. aeruginosa* is not as potent of a stimulator as the lipid A from enteric bacteria, due to its penta-acylation, it is still capable of over-stimulating the immune system and causing septic shock and death (10, 156).

Siderophores and their outer membrane receptors (IROMPs) are another important virulence factor in *P. aeruginosa*. Two distinct siderophores are made by *P. aeruginosa*. Pyoverdin is responsible for the fluorescence that is characteristic of this organism. This siderophore consists of a dihydroxyquinolone chromophore with an attached peptide chain of varying length depending on the particular pyoverdin produced (141). This peptide also contains the unusual amino acids, ornithine and diaminobutyric acid (27). The second major siderophore produced is pyochelin, which is derived from

salicyclic acid and two cysteine residues (27). The receptors for ferri-pyoverdin and ferri-pyochelin are the outer membrane proteins FpvA and FptA, respectively (67). Both of these siderophores and their receptors are produced by clinical isolates of *P. aeruginosa* and are needed for full virulence of the organism (141). Meyer et al demonstrated that pyoverdin deficient mutants were severely attenuated in a burn mouse model of infection (127). Then, Takase et al determined that both pyoverdin and pyochelin were needed for full virulence in immunosuppressed mice (181). This study also indicated that the ability to produce pyoverdin was probably more important for growth and dissemination *in vivo* (181). This may be due to pyoverdin's higher iron-binding constant, 10^{32}, versus pyochelin, 10^{5} (203). Both pyoverdin and pyochelin, however, are capable of acquiring iron from transferrin and lactoferrin, two important iron chelators in humans, indicating this is their primary role in virulence (141, 203). Another role in virulence is the induction of synthesis of other virulence factors, specifically exotoxin A and protease IV, by the binding of ferri-pyoverdin to this receptor, FpvA (15). In this system the binding of ferri-pyoverdin to FpvA sends a signal to FpvR, an integral cytoplasmic membrane anti-sigma factor protein, to release its cognate sigma factors. FpvR is capable of binding two sigma factors, PvdS and PvdI. The release of PvdS activates the transcription of pyoverdin synthesis genes, the exotoxin A gene and the PrpL endoprotease (protease IV) gene. The release of PvdI activates the transcription of the FpvA gene (15).

Like the IROMPs, other outer membrane proteins may play a role in virulence. OprM, OprJ and OprN all function in multi-drug resistance efflux pumps which actively remove various antibiotics and disinfectants from the cell (142, 169). While other outer

membrane proteins, such as OprF and OprI, do not seem to aid in pathogenesis they do induce protective antibodies against them (144). Overall, the exact function of many outer membrane proteins in virulence is still largely unknown.

Along with all its cell surface associated virulence factors, *P. aeruginosa* also secretes a variety of toxins and degradative enzymes that aid in its pathogenesis. The most toxic of these factors is exotoxin A (ETA). This toxin is 10,000 times more toxic than *P. aeruginosa* LPS (61). ETA is an adenosine-diphosphate (ADP)-ribosyl transferase that covalently links ADP- ribose from NAD to elongation factor-2 (EF-2) in eukaryotic cells. This modification inhibits protein synthesis and inevitably kills the eukaryotic cell. ETA seems to aid in localized colonization and dissemination and it is particularly lethal to hepatocytes causing liver necrosis in intoxicated and infected mice (133). *P. aeruginosa* also secretes several degradative enzymes, including two phospholipases, PLC-H and PLC-N, two elastases, LasA and LasB, alkaline protease and protease IV. The two phospholipases are produced under phosphate-limiting conditions and are thought to function as phosphate scavengers (193). PLC-H is hemolytic where PLC-N is not. This is due to the ability of PLC-H to cleave spingomyelin, which is abundant in the outer sheath of eukaryotic membranes, along with phosphatidyl-choline (PC). PLC-N, which also cleaves PC, cleaves phosphatidyl-serine instead of spingomyelin. Since phosphatidyl-serine is present in the inner sheath of eukaryotic membranes PLC-N does not lyse cells. The ability to cleave PC to diacylglycerol and choline may contribute to pathogenesis by inducing the production of arachidonic acids and eicosinoids, which are potent inflammatory mediators (193). Also, cleavage of PC may help to protect the bacteria from osmotic stress via the production of

osmoprotectants, such as choline, betaine and dimethyl-glycine (193). The two elastases are structurally different proteins that have elastolytic activity. LasA is a metallo-endopeptidase that cleaves the pentaglycine bridge of *Staphylococcus aureus* peptidoglycan (122). LasA also enhances the elastolytic activity of LasB. LasB is a zinc metalloprotease. Along with its ability to degrade elastin it also degrades several other host proteins including type III and IV collagens, laminin, immunoglobulins, complement proteins, coagulation factors and serum protease inhibitors (61, 79, 122, 178). It is hypothesized that the degradation of these host factors aids in the dissemination of the bacteria. LasB also degrades pulmonary surfactant protein D (SP-D) which resulted in the inability of this protein to opsonize bacteria (3). Alkaline protease has similar activities to LasB, however it can not cleave elastin (61). These two proteins can inactivate IFN-γ, IL-2, RANTES, MCP-1, and epithelial-neutrophil activating protein-78 (108). They can also interfere with neutrophil, monocyte, NK cell, and lymphocyte functions, which allows *P. aeruginosa* to evade the immune response (122). Protease IV is a serine protease that is capable of digesting immunoglobulins, complement proteins, fibrinogen and plasminogen. It was also implicated as an important virulence factor in *P. aeruginosa* keratitis (122). Recently, protease IV was shown to degrade pulmonary surfactant proteins A, B and D. This degradation resulted in the loss of the biophysical functions of the surfactant proteins as well as their innate immune functions which may aid in the initial colonization of the lungs (117). With all that is know about the functions of these toxins and enzymes, their exact contribution to pathogenesis is unclear.

Accompanying these secreted virulence factors, *P. aeruginosa* also possesses a type III secretion system that allows it to inject toxins directly into eukaryotic cells.

There are over 30 genes which are coordinately regulated that encode this secretion system (189). These genes fall into three categories: components of the secretion apparatus, components of the translocation apparatus, and the type III secreted effectors and their chaperones (75). The expression of these genes is tightly regulated, and they are only efficiently expressed and secreted at low calcium concentrations or when the bacterium comes into contact with eukaryotic host cells (190). The exact mechanism of how these conditions are sensed is unknown. Wolfgang et al recently identified a unique adenylate cyclase (CyaB) that is activated under calcium limited conditions. The subsequent increase in cAMP leads to the activation of transcription of the type III secretion genes (199). The type III secretion genes are also regulated by the transcription factor ExsA, which is only capable of binding to its promoters after the secretion channel is open, which is induced by low calcium concentration or host cell contact. When the channel opens ExsE releases ExsC and is secreted. ExsC then binds ExsD which releases ExsA which then activates transcription (189). How, or even if, these two regulatory cascades interact is not known.

P. aeruginosa encodes four effector proteins, ExoS, ExoT, ExoU and ExoY. ExoS and ExoT share 76% amino acid identity with each other, and both are bifunctional cytotoxins. The N-terminal region of both encodes a Rho GTPase activating protein (RhoGAP) domain, and the C-terminus encodes an ADP-ribosylating domain (13). The RhoGAP domains of both proteins have the same substrate specificities. Both target Rho, Rac and Cdc42 in eukaryotic cells, which results in rearrangement of the actin cytoskeleton and inhibition of phagocytosis (13). The ADP-ribosylating domains, however, have different intracellular targets. ExoS has polysubstrate specificity targeting

several proteins including vimentin and several members of the H- and K-Ras family (59). On the other hand, ExoT targets CrkI and CrkII only (13). The ADP-ribosylating activity of both proteins requires a host protein, Factor Activating ExoS (FAS), which is a eukaryotic protein belonging to the 14-3-3 protein family (59). The result of the ADP-ribosylating activity of ExoS is cell death while the ADP-ribosylating activity of ExoT blocks focal adhesion development and phagocytosis (13). ExoU is a potent cytotoxin that possesses phospholipase A2 activity. Upon injection into host cells, it quickly acts to disintegrate host cell membranes. ExoU requires a eukaryotic host cell factor in order for it to be active which probably ensures that it only functions inside the host cell (165). The last effector protein, ExoY, is an adenylate cyclase. Injection of ExoY into eukaryotic cells results in the increase in cAMP levels and morphological changes. Interestingly, like the other three type III effector proteins, ExoY needs a host cell factor for the full activation of its activity (204).

The exact function of each of these effectors in the pathogenesis of *P. aeruginosa* is still largely unknown, although the ability to produce these effectors and secrete them is associated with the virulence of the organism. A study by Roy-Burman et al correlated the ability of clinical isolates to secrete type III proteins with a higher mortality rate and a higher relative risk of mortality in acute infections. Interestingly they found that clinical isolates from chronic infections such as seen in CF patients could not secrete type III proteins (159). In agreement with these results, a longitudinal study of CF clinical isolates showed that early in the infection the isolates produced and secreted type III proteins, but after the infection became chronic, the isolates lost the ability to secrete type III proteins (107). This suggests a role for type III secretion in early colonization events

that are no longer needed once the infection is established. Animal infection models have also correlated the ability of *P. aeruginosa* to secrete type III proteins with increased virulence (81, 107, 167, 192). A recent study by Lee et al attempted to elucidate the contribution of each secreted effector protein in infection. They found ExoS to be the major cytotoxin of this system in strain PAK, and deletion of it led to the recovery of fewer bacteria in the lungs, livers and spleens of infected mice. They also found that the introduction of ExoU into strain PAK enhanced its virulence by increasing the number of bacteria recovered from the lungs, livers and spleens (107). Thus, it appears that the effectors, specifically ExoS and ExoU, aid in dissemination. A competitive infection model suggested that the type III secretion system and effector proteins do this by enhancing the survival of *P. aeruginosa* in the blood stream of the host (192).

1.1.3 *Regulation of virulence:* The regulation of virulence in *P. aeruginosa* is a very complex process. Several virulence factors are under the control of quorum sensing. This is a mechanism by which the cells sense their numbers and coordinately express genes, as a group, once an appropriate density is reached instead of as individuals. *P. aeruginosa* possesses two distinct yet interconnected quorum sensing systems. The first system identified was the LasRI system. This system consists of the transcription regulator protein, LasR, the homoserine lactone autoinducer synthetase, LasI, and N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), the autoinducer synthesized by LasI. Once levels of 3-oxo-C12-HSL reach a certain threshold, it binds to and activates LasR. LasR can then bind to its own promoter and enhance the transcription of itself and LasI. Along with inducing its own synthesis, LasR directly controls the transcripton of several other virulence factors including LasB, LasA, alkaline protease, exotoxin A and

the *exp* genes encoding components of the type II secretion system (191). The Las system also positively regulates the second quorum sensing system, the RhlRI system. This system consists of the transcriptional regulator, RhlR, the autoinducer synthetase, RhlI, and the autoinducer synthetized by RhlI, N-butyryl-homoserine lactone (C4-HSL). Like LasR-HSL, once RhlR binds to C4-HSL it autoinduces its own transcription as well as RhlI. RhlR-HSL also directly controls the synthesis of rhamnolipids, cytotoxic lectins (PA-I and PA-II) and RpoS. It also enhances the synthesis of LasB, LasA and alkaline protease (41, 191). A third autoinducer that connects these two systems was recently identified. This molecule, 2-heptyl-3-hydroxy-4-quinolone (PQS, *Pseudomonas* quinolone signal) enhances the production of RhlRI and the genes required for its synthesis are controlled by LasR (41, 123). The exact function of this molecule in quorum sensing is still being elucidated. Also largely unknown are other factors, besides autoinduction, that control the transcription of the quorum sensing systems. RpoN controls the transcription of LasR and RhlR, probably through GacA which positively controls both of these genes (76). Vfr, the cAMP receptor protein of *P. aeruginosa*, binds to the promoter region of LasR and enhances its transcription (2). Conversely, AlgR2, a regulator of alginate production, binds to LasR and RhlR promoters and represses their transcription (104). Several other regulators control these systems as well. MvfR and RelA activate them while QscR, RsaL, DskA and RpoS repress them (188, 191). The complexity of control of the quorum sensing systems highlights their importance in the growth of this organism.

Along with the virulence factors already mentioned, quorum sensing also controls several other genes. Microarray analysis has found that 616 genes are controlled by the

two quorum sensing systems. These genes include those involved with attachment, motility, chemotaxis, cell wall and LPS synthesis, multi-drug efflux pumps, and biofilm development (194). Interestingly, 222 of these genes were repressed by the quorum sensing systems, such as the type III secretion (20). In addition 37 of the regulated genes are known or putative transcriptional regulators, or part of two-component systems (194). This may indicate that quorum sensing does not control all 616 genes directly but may work through other regulators, setting up a hierarchical network of gene expression. The majority of the genes identified, though, have unknown function, indicating that there is a lot more to learn about this organism (194).

Due to the ability of quorum sensing to direct the transcription of so many genes involved in virulence, this system is considered a major virulence factor of *P. aeruginosa*. Indeed mutants defective in either the Las and/or Rhl system are attenuated in several animal models (174). In a burn mouse model and two models of acute pulmonary pneumonia, adult and neonatal, defects in quorum sensing resulted in fewer deaths due to the inability of the organisms to spread locally and disseminate systematically (134, 161, 173). In humans, the quorum sensing autoinducers are detected in sputum samples from infected CF patients indicating that this system functions *in vivo* (171). Recently was discovered that the autoinducers themselves can trigger immune responses. A study by Smith et al demonstrated the ability of 3-oxo-C12-HSL to stimulate inflammation upon intradermal infection (173). This inflammation showed edema, polymorphonuclear and mononuclear cell infiltration and epidermal tissue destruction. These events co-incited with the production of inflammatory chemokines MIP-1β, MIP-2, MCP-1, IP-10 and TCA-3, and cytokines, IL-1α and IL-6. They also found that injection of 3-oxo-C12-

HSL induces the expression of Cox-2 (173). In a later study this same group showed that the induction of Cox-2 lead to increased production of PGE2 which can cause fever, induce pain, enhance vascular endothelial permeability and enhance mucus secretion which may contribute to the lung pathology seen in infected individuals (175). Both 3-oxo-C12-HSL and PQS modulate T cell responses. Both inhibited T cell proliferation usually triggered by T cell receptor and CD28 stimulation. Also 3-oxo-C12-HSL inhibited IL-2 secretion, but PQS did not (83).

1.2 Host Defenses

Even with all of its virulence factors, *P. aeruginosa* is unable to infect and cause disease in healthy, immunocompetent individuals. In order for it to establish an infection a predisposing event must occur such as severe trauma or immunosuppression due to chemotherapeutic agents or other underlying disease. It is only when the host defense mechanisms fail to function appropriately that an infection can establish itself and cause serious disease to the host.

The major defense against infection is the epithelial cell barrier (162). Without outside help, *P. aeruginosa* is unable to breach this defense mechanism. If this barrier is breached, several innate immune mechanisms work together to clear any invading organisms.

The major cell type responsible for the clearance of *P. aeruginosa* is the neutrophil (135, 162). This is demonstrated by the high susceptibility of neutropenic patients to *P. aeruginosa* infections and the use of neutropenic mouse models to study *P. aeruginosa* infections (135, 192). Macrophages also function in the clearance of the organism, but they are probably more critical for alerting adaptive immune cells (162).

Recently the role of dendritic cells (DC) in alerting the adaptive immune system to *P. aeruginosa* infection is starting to come to light (74, 91, 187, 201). In a study by Toliver-Kinsky et al enhancement of DC production by the administration of Fms-like tyrosine kinase-3 ligand (Flt-3) increased the resistance to *P. aeruginosa* infection after thermal injury (187). The authors did not know the mechanism of how this increased resistance occurred, but they suggested that this may compensate for the immunosuppression seen after burn injury. Others have shown that targeting *P. aeruginosa* to DC enhances immunity to infection (91, 201). These studies also suggest the importance of T cells in resistance to *P. aeruginosa* infections. Several studies using knock-out mice imply that $CD4^+$ cells, capable of secreting IL-4, are critical for immunity (74, 91, 201). Lastly, B cells and the ability to produce opsonic antibody are critical for anti-*P. aeruginosa* immunity (91, 135).

Along with these immune cells other factors contribute to the defense against *P. aeruginosa* infections. The Toll-like receptors (TLRs) are pattern recognition molecules that function to alert the immune system to invading pathogens. Immune cells, such as macrophages and DC, as well as epithelial cells express TLRs. Interaction with these molecules induces a signaling cascade that results in the activation of MAP kinases, NF-κB activation and transcription of genes important for immune activation. Several TLRs recognize various components of *P. aeruginosa*. TLR2 interacts with pili and other non-pilus adhesins (113, 162). TLR4 interacts with LPS; however this interaction may not stimulate signaling in epithelial cells, suggesting that this is not a significant mechanism in alerting the immune system (10, 110). Both TLR2 and TLR4 on monocytes interact with ExoS. Interestingly the C-terminal domain of ExoS activates TLR2 while the N-

terminal domain activates TLR4 (53). TLR2, along with TLR5, also participates in the recognition of *P. aeruginosa* flagella by epithelial cells (1, 206). Another molecule capable of initiating a signaling cascade in epithelial cells is asialo-GM1. This signaling is initiated by the binding of flagellin and results in the release of ATP that triggers G-protein activation, cleavage of PIP_2 to IP_3 and calcium mobilization. This leads to the activation of MEK1/2 and Erk1/2 and the transcription of the Muc-2 mucin gene (124). Since *P. aeruginosa* binds to mucin, increased mucin production may prevent colonization by increasing mucociliatory clearance.

Cytokines and chemokines are also important in the host defense against *P. aeruginosa* infections. IL-8 is a potent C-X-C chemokine that attracts and activates neutrophils but not monocytes (97). Several components of *P. aeruginosa* were identified that stimulate IL-8 production. These include ExoS, flagella, pili and the 3-oxo-C12-HSL autoinducer (42, 52). The binding of ExoS, flagella and pili to TLRs induces the IL-8 secretion seen for these components; however the host receptor for 3-oxo-C12-HSL is unknown. TNFα and IL-4 were shown to increase bacterial clearance, whereas IL-1 and IL-18 were shown to impair bacterial clearance (162). The role of other cytokines in the resolution of infection is less well defined. In the lung IFNγ seems to aid in bacterial clearance, but in the eye overproduction of if seems to contribute to the pathogenesis of the infection (73, 162).

Several other factors assist in the innate clearance of *P. aeruginosa*. Complement aids in the clearance of *P. aeruginosa*. This was demonstrated by the inability of C5 deficient mice to clear the infection after aerosolized exposure (135). At mucosal surfaces, mucus binds to *P. aeruginosa* (7, 111, 149, 153). This binding may prevent

bacterial adherence to the underlying epithelial cell layer and promote clearance of the organism (114). Surfactants found at the mucosal surface of the lungs also contribute to defense by agglutinating and enhancing the phagocytosis of *P. aeruginosa* cells (3, 117, 162). It was recently discovered that lung epithelial cells possess a membrane associated factor that can degrade 3-oxo-C12-HSL. The exact nature of this factor is unknown, but the authors of the study speculate that it may be an enzyme (30). The degradation of this autoinducer could prevent quorum sensing and the secretion of several virulence factors.

1.3 Previous Vaccination Attempts

Over the last 4 decades intense research has gone into the development of a vaccine against *P. aeruginosa* infections. Since it is highly resistant to antibiotic therapy, infections are often difficult to treat and are associated with high mortality rates. Thus alternative ways to treat the infections are needed. By studying naturally-acquired immunity to the organism after infection, it became obvious that vaccination to prevent the disease might be possible. Several types of vaccines were investigated ranging from whole cell immunizations to subunit and DNA immunizations. More recently dendritic cell vaccinations have been studied. Even with all of this effort no vaccine has been fully developed for widespread use, indicating that there is still more to learn about the pathogenesis of, and immunity to, this versatile pathogen.

The first widely studied vaccines were based on the immunization with LPS. The interest in this antigen was spurred by the fact that high titers of anti-LPS antibodies were observed in patients with bacteremic infections and this was associated with survival of these patients (135). However, conserved residues of the LPS core region are not protective and the highly immunogenic O-polysaccharide region shows considerable

diversity between strains leading to problems in achieving cross-protection between serogroups. Even strains within the same serogroup can have different chemical structures where the protective epitope may be part of the sub-type variant structure and not the serotype common structure (136). Despite all these issues several promising vaccine candidates were developed. Two of the first formulations to be tested, Pseudogen® and PEV, were purified LPS from 7 or 16 different serogroups, respectively (35). Both of these were tested extensively and showed promising results, especially in burn victims; however due to their toxicity they are no longer being pursued as viable vaccine candidates (77, 136). The next generation of LPS-based vaccines consisted of purified high molecular weight O-polysaccharide. These preparations were less toxic than the purified LPS vaccines, and they were immunogenic when injected into animals or humans. The antibodies elicited from monovalent preparations demonstrated opsonophagocytic killing of the homologous strain *in vitro* and passive protection from the homologous strain in several animal model systems. Unfortunately, when a heptavalent formulation was tested in rabbits and mice different cross-reactive patterns were seen. There was a high degree of variability in the ability of the generated antibodies to mediate opsonic killing of non-vaccine strains between these two species (68). Due to these inconsistencies, this vaccine is no longer under investigation. The most promising of the LPS-based vaccines is a conjugate vaccine consisting of purified O-polysaccharide, from 8 different serogroups, covalently linked to exotoxin A. This vaccine is non-toxic, non-pyrogenic and well tolerated upon injection into humans (36). Early studies demonstrated the ability of the vaccine to increase antibody titers to the 8 different serotype strains and extotoxin A. These antibodies could neutralize ETA and

were protective upon passive transfer to mice challenged with 7 of the 8 serotypes (36). This vaccine also induced high titers of IgG against all 8 serogroups and ETA in non-colonized CF patients. These responses were maintained up to 6 months after immunization (36). Currently this vaccine is being tested in CF patients in a phase III trial and is close to being marketed under the name Aerugen in Europe (136, www.bernabiotech.com).

Alginate is another vaccine candidate that has been studied. In CF patients the major virulence factor produced by *P. aeruginosa* in chronic infections is alginate. These patients are able to produce antibodies against alginate, but the antibodies tend to be non-opsonic, and they do not mediate opsonic killing of the bacteria (137). This was due to the difference in their ability to deposit the complement protein C3 onto alginate (137). Pier et al discovered that immunizing with high molecular weight polymers of alginate elicited opsonic antibodies that were capable of killing several heterogeneous strains that produce alginate with different ratios of mannuronic to guluronic acid; however the number of vaccinated individuals that responded was low (138). This was due to pre-existing, non-opsonic antibodies against alginate which inhibited the production of opsonic antibodies. To overcome this problem a conjugate vaccine, consisting of high molecular weight alginate polymers covalently linked to keyhole limpet hemocyanin (KLH), was constructed and evaluated in mice and rabbits. This conjugate construct elicited IgG capable of opsonizing and killing the vaccine strain as well as several other clinical mucoid CF isolates (185). More importantly, this conjugate induced opsonic antibodies in pre-exposed mice, partly overcoming the inhibitory effects of pre-existing antibodies. This study also showed that the opsonic antibody binds to O-acetyl groups of

native alginate and that antibodies against the backbone polymer are non-opsonizing (185). Work on this vaccination strategy is continuing.

Since the observation that non-flagellated and non-motile mutants of *P. aeruginosa* were avirulent in mouse burn models (48), flagella have been an attractive vaccination candidate. The fact that there are only 2 types of flagella makes it a more desirable candidate than LPS, which can be divided into over 20 serogroups. Early studies showed that immunization of mice with purified flagella from one serotype protected them from challenge with homologous strains in a burn mouse model (82). Incorporation of both flagellar types into the immunization protected mice from *P. aeruginosa* with type A or type B flagella (78). Passive transfer studies in burned mice showed that the protection afforded by vaccination was due to anti-flagellar antibodies. These antibodies have the ability to inhibit the motility of *P. aeruginosa* and to enhance opsonophagocytosis (5, 49). Anti-flagellar vaccines have also been tested in ocular infection models. Rudner et al showed that both active and passive immunization protected mice from corneal perforation (160). A flagellar vaccine was also formulated for use in humans. This vaccine was highly immunogenic and showed no adverse side effects after injection (34). The antibodies elicited enhanced the phagocytic killing of *P. aeruginosa* and were protective when passively transferred to challenged mice (34). Based on these results, a phase III clinical trial in CF patients was set-up in Europe (47). However the results of this trial have not been reported. Even though no other anti-flagellar clinical vaccine has been developed yet, this is still an active area of pseudomonal vaccine research.

Recently there has been a lot of interest in the use of outer membrane proteins (OMPs) as vaccines. This interest is due to the highly conserved nature of OMPs and their exposure on the cell surface. Several studies in different rodent models have shown that injection of OMPs protects the animals after challenge (77). Based on these results, OMPs vaccines have been tested in humans. A study by Lee et al tested the ability of sera from immunized burn patients to protect mice. This study showed that the antibodies elicited by immunization were capable of mediating opsonophagocytic killing *in vitro*, and they protected mice challenged with *P. aeruginosa* after passive transfer (106). Naturally occurring antibodies, probably induced by infection, did not have these protective capabilities (106). In a follow-up clinical trial this same group determined the optimal immunization schedule in burn patients to be 1.0 mg doses at 3 day intervals (93). This study also demonstrated the efficacy of vaccination by PCR, where the presence of *P. aeruginosa* was not detected in blood samples from immunized patients 21 days post-burn, whereas *P. aeruginosa* was detected in the placebo group at this time (93). A second group has also conducted a clinical study testing the ability of a recombinant OprF-OprI hybrid protein to induce antibodies in burn patients. This study showed that this recombinant protein caused seroconversion in 7 of the 8 immunized individuals making this another promising vaccine candidate (119). While neither of these vaccines are in widespread use the positive results seen so far are encouraging.

The newest antigen to be considered as a vaccine candidate is PcrV. This protein is part of the type III secretion system. Interest in this protein arose because it shares high homology with LcrV from *Yersinia*, which is a protective antigen (60). Also PcrV is required for the translocation of the type III effector proteins (167). In a murine model

of acute lung infection, mice vaccinated with PcrV had better survival, associated with fewer bacteria isolated from the lungs (167). In a burn mouse model, immunizing with PcrV, actively or passively, enhanced survival of mice challenged with different serogroups of *P. aeruginosa*, suggesting PcrV is conserved among different strains (80, 131). Protection in this model was associated with reduced bacterial numbers in the livers of the mice, indicating that *P. aeruginosa* was unable to spread from the burn site (80). With these positive preliminary results it seems PcrV is a very promising vaccine candidate.

1.4 Genetic Immunization

Genetic immunization against *P. aeruginosa* infection has also been attempted. In this type of vaccination, a gene of interest is cloned into a eukaryotic expression vector which is then injected into the host. This vector is taken up by the host cells where it migrates to the nucleus, and the target gene is expressed. The host detects this expressed protein as being foreign and mounts an immune response against it. Several different target genes were examined in these vaccination attempts. The gene encoding a non-enzymatically active exotoxin A was used in the first study. Upon intramuscular injection into mice, high titers of anti-ETA antibodies were detected that were capable of neutralizing ETA *in vitro* and protected mice upon toxin challenge (39). Immunizing with an *oprF* DNA vaccine and a *fliC* type B DNA vaccine has also been done (32, 145). In these studies mice produced antibodies that afforded protection upon bacterial challenge. The results seen in these studies reflected results seen with protein immunization, highlighting the ability of genetic immunization to elicit protective immunity against *P. aeruginosa*.

Genetic immunization has several advantages over protein or whole cell immunization making it the more desirable vaccination method. Some of these advantages include 1) DNA vaccines are relatively easy to make and purify free of contaminants, 2) they induce long lasting immune responses, 3) they induce humoral and cell mediated immune responses, 4) they can induce immune responses in neonates even in the presence of maternal antibodies, 5) they contain built in adjuvants in the form of CpG motifs, 6) DNA is more stable at higher temperatures than proteins which reduces the risk of denaturation of the vaccine upon shipment and storage (98, 118). DNA vaccination, however, has a couple of disadvantages. First DNA vaccination does not stimulate robust antibody responses, which are typically seen with protein immunization. Antibody titers can take 8-12 weeks to peak, and several boosts may be required (98). DNA immunization has been quite efficacious in small animals but scale-up to larger animals and humans has been problematic. The main reason for this is probably poor transfection efficiency and antigen expression by host cells (9, 118). Several approaches to improve the immune response in large animals have been examined, but so far not one method has been universally effective (118).

Multiple factors affect the ability of DNA vaccines to generate an effective immune response. Efficient protein expression is correlated with higher immune responses against the antigen. Several elements were engineered into the plasmid backbone to increase gene expression. The CMV-IE promoter is the strongest promoter based on *in vitro* protein expression where it is 40X stronger than the SV40 promoter (98, 118). Strong tissue specific promoters, such as the muscle-creatine kinase promoter, have also been used; however responses are usually not as good as when the CMV

promoter is used (118). The presence of intron A from CMV and a polyadenylation sequence increase protein expression as well (118). Modification of the target gene to reduce codon bias may also enhance protein expression by allowing efficient translation using the most abundant tRNA species of the host (118).

Along with efficient expression of the protein the final location, cytoplasmic, membrane bound or secreted, affects the immune response. A study using the hepatitis C virus nucleocapsid gene found that the DNA construct that encoded the secreted form of the antigen induced higher antibody titers and higher rates of seroconversion than the construct encoding a non-secreted antigen (86). Similar results were obtained from DNA constructs encoding cytoplasmic and secreted forms of ovalbumin. The secreted form induced up to 100-fold higher titers of antibodies (25). The method and route of DNA injection also influence the immune response. Needle injection and gene gun bombardment are the two most common methods used to inject DNA vaccines. Injection via a needle produces immune responses that show a Th1 bias whereas injection via the gene gun produces immune responses that show a Th2 bias (55, 196). These responses were independent of the route of injection which seems to influence the magnitude and longevity of the response. Using gene gun mediated injections, Ito et al found that intramuscular injection gave a slower, weaker but more sustained response in contrast to intradermal injection which gave a faster, stronger response initially, but it was not sustained (87). Systemic delivery of DNA vaccines does not elicit good mucosal responses. Mucosal delivery, however, will induce responses at all mucosal sites, and it may induce systemic responses as well (118).

1.5 Targeting Specific Arms of the Immune System

One of the advantages of genetic vaccines is they can induce humoral and cell mediated immunity. By manipulating the vaccine formulation it is possible to stimulate specific arms of the immune response. A Th1 type of response is usually needed when battling viral or other intracellular infections. This type of response stimulates strong cell mediated responses with the production of INFγ. Th1 cells also stimulate B cells to produce IgG2a. The Th2 type of response is needed to fight extracellular pathogens. This response stimulates strong humoral immunity with the production of IL-4 instead of INFγ. Here IgG1 is the major antibody isotype produced. The inclusion of cytokine genes, co-stimulatory molecule genes and chemokines genes along with the target antigen gene in the DNA vaccine has shown promise in targeting these two types of immune responses. The co-administration of IL-12, INFγ or GM-CSF in combination with IL-2 enhanced Th1 responses (29, 94, 96). To enhance Th2 responses, the co-administration of IL-4, IL-5 and IL-10 were effective (29, 96). Co-injection of plasmids encoding CD80 and CD86, two co-stimulatory molecules important for T cell activation, also modulates the immune response to the target antigen. However, these responses differed depending on the route of injection and the target antigen used (57, 95), warranting further investigation into their mode of action.

Another way of enhancing DNA vaccine potency is to target the expressed antigen to antigen presenting cells (APCs). To do this, a genetic fusion is created where the antigen gene is cloned in-frame with a gene encoding a ligand that binds to APCs. Fusion to the Fc region of human IgG enhanced $CD8^+$ T cell responses, $CD4^+$ T cell responses and B cell responses (205). Fusion to fms-like tyrosine kinase 3 ligand (Flt3L)

and heat shock protein 70 specifically enhanced $CD8^+$ T cell responses but not $CD4^+$ T cells responses (69, 85). In contrast to these fusions, the fusion of CTLA4 to a target antigen enhanced Th2 responses with increases in IgG1 production (24).

A similar method of using fusion proteins to target antigens has been studied using peptides. In this method the antigen is targeted to T cells via a T cell binding ligand. Several T cell binding peptides were tested, but only two were found that specifically stimulated either a Th1 or a Th2 response (208). The J peptide, which is amino acids 35-50 of β-2 microglobulin, stimulated Th1 responses. When fused to the 38.G epitope of the 38 KDa protein of *Mycobacterium tuberculosis*, high titers of IgG2a were obtained (208). When fused to the H1 epitope of the HSV-1 immediate-early protein ICP27, this peptide induced a delayed-type hypersensitivity response. This response was not observed after injection of the H1 peptide alone (157). On the other hand the G peptide, which is amino acids 135-149 of the β2 domain of MHCII, induced a Th2 response. This was demonstrated by the induction of high titers of IgG1 when the G peptide fused to the 38.G epitope was injected into mice (208). While these fusions were done with peptides they could easily be adapted to genetic immunization.

1.6 Dendritic Cells

In the last few years the importance of dendritic cells (DC) in initiating and regulating immune responses has come to light. These unique cells are present in almost every tissue of the body where they function to detect invading organisms and alert the immune system. While in the periphery, they exist in an immature state capable of phagocytosis. Upon stimulation, either by encountering a pathogen or inflammatory signals, DC will mature and migrate to local lymph nodes. The maturation process

causes a down regulation in phagocytosis by the cell and an up regulation of surface MHCII and co-stimulatory molecule expression. MHCII expression can increase 5-20 fold while CD86 expression can increase 100 fold (126). The up regulation of these molecules allows DC to be potent stimulators of naive and memory $CD4^+$ T helper cells (103, 109). DC also are potent stimulators of $CD8^+$ T cell responses. Most antigens presented to $CD8^+$ T cells are endogenously produced, either originating from an intracellular pathogen or a self antigen. DC have the ability to cross-present exogenously acquired antigen by a process where the engulfed antigens are transported out of the endocytic compartment and into the cytosol to undergo processing and presentation on MHCI (103, 125). In addition to activating T cell responses, DC influence B cell responses in several ways. First, DC retain unprocessed antigen that is transferred to B cells upon contact with them. Contact with DC also sends a signal to the B cells to class switch from IgM to IgG (202). Lastly, the interaction of DC with T and B cells brings these antigen specific cells together where B cells receive the proper signals from T cells to differentiate and start to secrete antibody (50). Along with being potent stimulators of adaptive immune responses, DC function to tolerize the immune system to self antigens. This function is attributed to immature DC that have captured antigen in the absence of danger signals so they do not express the co-stimulatory molecules required to fully activate T cells. Since these T cells do not receive the proper stimuli they are subsequently deleted (21, 70, 125).

Discovering the pathway of DC development from hemopoietic precursors has been elusive. It seems that DC can develop from both myeloid and lymphoid precursors (37). Several subsets of DC have been described; however it is unknown whether these

subsets are a result of different lineages or if they are the same lineage at different stages of differentiation. For example, the presence of CD8α was thought to denote a DC of lymphoid lineage, but it is now thought to be a marker of differentiation (197). Several studies have tried to assign specific functions to these subsets but there is little consensus among different studies (197). One point of consensus has been the identification of plasmacytoid DC (pDC). These are a unique population of DC that have a plasma-cell like appearance and secrete large amounts of type I interferons upon exposure to viruses (19). These cells require IL-3 and Flt3L for their development unlike classic DC which require GM-CSF (19, 31). pDC also differ from classic DC in their expression of TLRs and chemokine receptors, giving pDC different functions in immunity (31). Like classic DC, the exact function of pDC is still being unraveled.

With the potent immunoregulatory activity of DC, recent research has focused on targeting DC to induce immunity against microbial pathogens, to stimulate anti-tumor immunity, and to control allergies and autoimmune disorders. Both *ex vivo* and *in vivo* targeting methods have been developed. *Ex vivo* targeting consists of the isolation and culture of DC *in vitro*. These cells are then pulsed with antigen before injection back into the host. DC have been pulsed with whole cells, cell lysates, whole proteins or peptides, DNA and RNA (56, 91, 92, 176, 180, 201, 210). While this method has stimulated potent anti-microbial and anti-tumor responses, DC culture is expensive and time consuming. To skip the *ex vivo* culturing step, *in vivo* methods have also been studied. DNA vaccination, as mentioned above, has used plasmids encoding fusion proteins that target APCs, including DC. These vaccines enhanced B and T cell responses above that seen with the untargeted vaccine (69, 85, 205). However these responses take time to

develop and will only be useful for prophylactic treatment of diseases. Protein immunizations have also targeted DC *in vivo*. These methods have shown faster induction of immune responses. In one system a fusion between *Bordetella pertussis* adenylate cyclase toxin, CyaA, and amino acids 257-264 of ovalbumin targeted DC through the specific binding of CD11b by CyaA. One injection of this fusion protein induced strong cytotoxic T lymphocyte responses by day 7 (65). A second system involves the use of a hamster monoclonal antibody (mAb), N418, which binds to CD11c on DC. Conjugation of a target antigen to this mAb resulted in rapid antibody titers against the target antigen. This was first demonstrated by Wang et al who non-covalently bound a model antigen, goat anti-hamster IgG to N418 and injected this into mice. Anti-goat antibody titers were seen 7 days after injection (195). Subsequently Conwell covalently cross-linked purified recombinant FliC protein from *P. aeruginosa* to N418 and saw high anti-flagellar titers 7 days after injection (32). The rapid induction of both T and B cell responses by these systems has enormous potential for the development of therapeutic vaccines for the treatment of diseases where traditional remedies fail.

CHAPTER 2

GENETIC IMMUNIZATION WITH THE TYPE A *fliC* GENE OF *Pseudomonas aeruginosa*

2.1 Introduction

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen. It is a major cause of nosocomial infections, infecting immunosuppressed patients, severely burned patients and cystic fibrosis (CF) patients. The infection process involves the use of multiple virulence factors including pili, flagella, alginate, secreted toxins and quorum sensing. Treatment of *P. aeruginosa* infections is quite difficult due to its inherent resistance to several antibiotics. The outer membrane provides a permeability barrier. *P. aeruginosa* also produces several multi-drug efflux pumps that export several types of antibiotics along with biocides, antiseptics and disinfectants (142, 169). Lastly, *P. aeruginosa* maintains antibiotic resistance plasmids as part of its genome. Only fluoroquinolones, gentamicin and imipenam are effective against *P. aeruginosa*, but not all strains (186). Vaccination to prevent infections is much more desirable than trying to treat such an aggressive infection.

Several potential vaccines against *P. aeruginosa* have been tried with varying success (77). One potential vaccine target is flagella. *P. aeruginosa* possesses a single polar flagellum that functions in motility and chemotaxis as well as adherence to host cells (54, 111). This external structure is highly immunogenic and conserved (4, 177). In

animal models it has been found that flagella minus mutants are less virulent than their flagellated wild-type parents (48). Active and passive immunization against flagella also provide protection to animals challenged with *P. aeruginosa* (49, 78, 82). There are only two antigenically distinct types of flagella in *P. aeruginosa*, type A and type B, with very little cross-reactivity between them. Type B flagella are a homologous group with a molecular weight of 53 kDa and no variation between strains (4). Type A flagella are a heterogeneous group with molecular weights between 45 kDa and 52 kDa, showing some variability between strains; however all type A strains share a common a_o epitope (4).

These potential flagellar vaccines have been made using purified flagella from *P. aeruginosa*. However, there is a lot of contamination with lipopolysaccahride (LPS) that must be removed before injection. One way to avoid this purification nightmare is to utilize DNA vaccination. In DNA vaccination, the gene of the antigen of interest is cloned into a eukaryotic expression vector and then injected into the host. Once in the host, the DNA is taken up by host cells and the gene is expressed then an immune response is mounted against the expressed protein (98). The advantages of this type of vaccination over protein based vaccination are 1) DNA is easier to purify with very little endotoxin contamination, 2) DNA is more stable and easier to store than protein, 3) both humoral and cell mediated immune responses are stimulated (98).

Previous studies using a DNA vaccine encoding the type B *fliC* gene from *P. aeruginosa*, PAO1, showed good protection in mice when challenged in a burn mouse model (32). In these studies the *fliC* gene from the type B strain PAO1 was cloned into the eukaryotic expression vector, pVR1020. Mice injected with this construct were protected upon challenge with a homologous type B strain but not when challenged with

a type A strain (32). In this study, DNA vaccination against type A flagella was examined. Two separate DNA vaccines were constructed but protection was not afforded by either. A possible reason may be the post-translational glycosylation of type A flagella.

2.2 Materials and Methods

Cloning and large-scale preparation of pVR1020-fliC(A-pak) and pVR1020-fliC(A-sbin)

To create the plasmids pVR1020-*fliC*(A-pak) and pVR1020-*fliC*(A-sbin), the *fliC* genes from *Pseudomonas aeruginosa* strains PAK and SBI-N were cloned into pVR1020 (Vical, Inc, San Diego, CA). The genes were amplified from genomic DNA using the following primers: forward 5'-GGACGGATCCATGGGCCTGCAGATCTCCAACCG-3' which incorporates a BamHI restriction site into the product and reverse 5'-GGACAGATCTCGGCAGCTGGTTGGCCTGG-3' which incorporates a BglII restriction site into the product. Template genomic DNA was isolated from *P. aeruginosa* strains PAK and SBI-N using Invitrogen's (Carlsbad, CA) Easy-DNA kit. The PCR reaction was carried out as follows: Template DNA was allowed to denature at 94°C for 1 minute prior to the start of cycling, which consisted of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, extension at 72°C for 1 minute, repeating for 30 cycles followed by a final extension at 72°C for 10 minutes. The PCR products were then ligated to the TA vector (Invitrogen, Carlsbad, CA) according to protocol and then transformed in TOP10 cells. The cells were plated on LB agar plus kanamycin and X-gal for blue/white screening and incubated at 37°C overnight. The next day white colonies were picked to screen for presence of the PCR insert.

An EcoRI fragment was removed from the TA vectors that contained the PCR insert. This fragment was gel purified using Qiagen's (Valencia, CA) Qiaex II kit according to protocol. The gel purified EcoRI fragment was then digested with BamHI and BglII overnight at 37°C. The next day the BamHI-BglII fragment was gel purified and ligated, using T4 ligase, to pVR1020 that had been digested overnight at 37°C with

BglII, CIAP treated for 30 minutes at 37°C and gel purified. The ligation was incubated for 2 hours at room temperature. The ligation mixture was then transformed into SubCloning Efficiency DH5α *E. coli* cells (Gibco, Grand Island, NY) using the supplied protocol. After the outgrowth the cells were plated onto LB agar with kanamycin and incubated overnight at 37°C. Colonies were screened for plasmids that contained the *fliC* insert by restriction digests.

Plasmids that contained the *fliC* gene were then screened for expression using a transient transfection assay. Plasmids that were able to express the *fliC* gene in culture were then purified using Qiagen's (Valencia, CA) Mega Prep Kit. The purified DNA was resuspended to 1 mg/ml in sterile PBS.

Transient Transfection Assay

UM449 cells growing in normal growth medium (RPMI 1640, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum) were plated at a concentration of $1x10^5$ cell/well in 6 well tissue culture plate. These cells were allowed to grow until 40-60% confluent (approximately 18 hr) at 37°C with 5% CO_2. Then Lipofectin® Reagent (Gibco, Grand Island, NY) was diluted 1:10 in 100 µl of serum free medium per well and incubated for 30 minutes at room temperature. The appropriate DNA samples were diluted in serum free medium to a concentration of 2.5 µg/100 µl per well. Next, equal volumes of the Lipofectin and appropriate DNA solutions were mixed and allowed to incubate for 15 minutes at room temperature to allow to Lipofectin-DNA complexes to form. After the 15 minute incubation 0.8 ml of serum free medium was added to the complexes to give a final volume of 1 ml per well which was then added to the UM449 cells that had been washed once with serum free medium. The cells were

then incubated for 20 hr at 37°C, 5% CO_2 before replacing the liposome-DNA medium with normal growth medium. After 48 hr of incubation, cell supernatants were collected and cell lysates were harvested by adding 250 µl of 2x-SDS-PAGE loading buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% Bromphenol Blue, pH 6.8) to the each well for analysis of gene expression by immunoblot.

Purification of flagella

P. aeruginosa strains PAK and SBI-N were grown overnight in TSB with shaking at 37°C. The next day the culture was centrifuged at 7,000xg for 30 minutes and the supernatant was decanted. The cells were resuspended in 0.01 M Tris-Cl, pH 8.0. The flagella were sheared off using an Omni mixer at low speed for 30 seconds. The suspension was then centrifuged at 10,000xg for 30 minutes to pellet the bacteria. The supernatant was collected and centrifuged for 60 minutes at 100,000xg. The pellet was then resuspended in 0.01 M Tris-Cl, pH 8.0 and microcentrifuged for 2 minutes to remove large particles. The concentration and purity of the flagella was determined by using the Bradford Assay and SDS-PAGE, respectively.

Deglycosylation of flagellin

Purified flagellin was chemically deglycosylated using the GlycoFree kit (Oxford Glycosystems, Bedford, MA) according to the manufacturer's protocol.

SDS-PAGE and Immunoblot

SDS-PAGE. The proteins were electrophoresed in a 12% polyacrylamide mini-gel containing SDS for 1 hour at 200 V. To stain, the gel was placed in Coomassie dye for 1 hr and then destained for 1 hour with acetic acid:methanol.

Immunoblot. The antigens were electrophoresed as above and then transferred to a PDVF membrane for 30 minutes in Towbin transfer buffer (0.025 M Tris-Cl, 0.192 M glycine, 0.1% SDS, 20% methanol). After transfer, the membrane was blocked for 30 minutes in Tris buffered saline plus 0.05% Tween (TTBS) at room temperature with shaking. After blocking, the primary antibody, polyclonal rabbit anti-FliC type A, diluted 1:1000 in TTBS was added and allowed to incubate for 1 hour at room temperature with shaking. The membrane was then washed 3 times in TTBS for 5 minutes each. Then the secondary antibody, goat anti- rabbit- alkaline phosphatase conjugate (Jackson Immunoresearch, West Grove, PA), diluted 1:5000 in TTBS was added and allowed to incubated for 1 hour at room temperature with shaking. The membrane was then washed again as before. Finally, the membrane was developed by adding a solution of NBT (0.3 mg/ml, final concentration) and BCIP (0.15 mg/ml, final concentration) in carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) and allowing it to incubate until bands were seen. The reaction was stopped by rising with water.

Immunization protocol

Balb/c or Cr1:Cf1 BR non-Swiss mice, 6-8 weeks old, were injected with 1 µg of DNA using the BioRad Helios Gene Gun (Hercules, CA). The DNA was coated onto 1 micron gold beads according to protocol (BioRad, Hercules, CA). The bullets were then made by coating plastic tubing with the DNA coated gold with each bullet contained 1 µg of DNA. Mice received a single shot from the gene gun at 400 psi in the shaved abdominal dermis. Mice were injected on days 0, 14 and 28 and bled by retro-orbitally on days 28 and 42.

Analysis of antibody responses

Total IgG ELISA. Immulon II (Dynatch Laboratories Inc, Chantilly, VA) microtiter plates were coated with 100 µl of 1 µg/ml purified flagella diluted in 0.1 M carbonate buffer, pH 9.6. Mouse sera were diluted 1:100 in 0.05% Tween- Tris buffered saline (TTBS). The plate was washed three times with TTBS, and 100 µl of the diluted mouse sera was added and allowed to incubate at room temperature for 1 hour. The plate was washed again three times with TTBS, and then 100 µl of 1:5000 diluted goat anti-mouse alkaline phosphatase conjugate (Jackson Immunoresearch, West Grove, PA) was added. After incubating 1 hour at room temperature, the plate was washed three times with TTBS followed by three washes with Tris buffered saline (TBS) and then 100 µl of substrate, p-nitrophenyl phosphate (Sigma, St. Louis, MO), diluted to 1 mg/ml in diethanolamine buffer, pH 9.8, was added. After 30 min. of color development, the absorbance was measured at 405 nm in a BioRad Model 550 plate reader. To determine the antibody titer the mouse sera were 2-fold serially diluted in TTBS before being added to the plate. Antibody titer was defined as the highest dilution that produced an optical density reading equal or above 0.200.

IgG Isotyping ELISA. Immulon II microtiter plates were coated with 100 µl of 1 µg/ml purified flagella diluted in 0.1 M carbonate buffer, pH 9.6. Mouse sera were diluted 1:100 in TTBS and added to the plate after it had been washed three times with TTBS. After 1 hour at room temperature, the plate was washed as before and 100 µl of anti-mouse IgG1 alkaline phosphatase conjugate, diluted 1:1500 or anti-mouse IgG2a alkaline phosphatase conjugate, diluted 1:1000 (Zymed Laboratories, San Francisco, CA) was added. The secondary antibodies were diluted in such a way so that their standard

curves were identical and the absorbance readings could be compared directly. The secondary antibody was detected with p-nitrophenyl phosphate substrate diluted to 1 mg/ml in 10% diethanolamine buffer, pH 9.8. The absorbance was read using the BioRad Model 550 plate reader.

The concentration of IgG1 or IgG2a was determined by comparing absorbance readings to standard curves. The standard curve was established by coating Immulon II plates with 100 µl of 1 µg/ml purified rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a (Zymed Laboratories, San Francisco, CA). The primary antibody used was either purified mouse myeloma IgG1 or purified mouse myeloma IgG2a (Zymed Laboratories) at 10, 20, 40, 60, 80 and 100 ng. The same secondary antibodies were used as above to detect the mouse IgG1 or IgG2a. The standard curves were graphed in Excel and linear regression was used to determine the concentrations of IgG1 and IgG2a.

Burn mouse model

The burn mouse model was performed at Shriner's Hospital for Children in Cincinnati, OH and was followed according to the protocol established by Holder (80). Female Cr1:Cf1 BR non-Swiss, 6-8 week old, mice were immunized with gene gun injections of pVR1020 or pVR1020-*fliC*(A-pak)on days 0, 14 and 28. On day 42 mice were anesthetized and given a partial thickness alcohol burn over 15% of their body surface. Saline was injected intraperitoneally to replace fluids lost during the burn. Next, a lethal dose, 2 x 10^3 cfu, of *P. aeruginosa* strain SBI-N was injected subcutaneously at the burn site. Survival was monitored over the next 10 days.

Motility inhibition assay

Motility plates were prepared as follows: 1 ml of motility media (tryptic soy broth plus 0.4% agar) was mixed with 50 µl of the appropriate serum or phosphate buffered saline (PBS) for control in a sterile test tube before being placed in a well of a sterile 24 well plate. Overnight cultures of *P. aeruginosa* strains PAK, SBI-N and PAO were then stabbed into the center of the appropriate wells. The plates were incubated at 37°C for 12 hours and then the diameter of the zone of motility was measured. The assay was performed in duplicate.

2.3 Results

Immunization with pVR1020-fliC(A-pak). The *fliC* gene from *P. aeruginosa* was cloned into the eukaryotic expression vector pVR1020 (Vical, Inc, San Diego, CA). This vector contains the cytomegalovirus promoter/enhancer and the bovine growth hormone poly A terminator. It also has the tissue plasminogen activator secretion signal that allows for the secretions of any expressed proteins (Fig. 2.1). Expression of the PAK *fliC* gene was observed after transient transfection of UM449 cells in tissue culture. Figure 2.2 shows the immunoblot of both the cell lysate and supernatant of the transfected culture demonstrating the presence of the cloned *fliC* gene product in both.

After demonstrating expression in culture Cr1:Cf1 BR non-Swiss mice were injected with pVR1020 and pVR1020-*fliC*(A-pak) on days 0, 14 and 28. On days 28 and 42 the mice were bled retro-orbitally and the sera were analyzed by ELISA. Anti-flagellar antibodies were present and a boost in titer was seen from day 28 to day 42 (data not shown). The sera from day 42 were analyzed for total IgG, IgG1 and IgG2a. As shown in figure 2.3 there was a significant amount of anti-flagellar IgG in the pVR1020-*fliC*(A-pak) group as compared to the vector control group. The major isotype produced was IgG1 (fig. 2.3). On day 42 the mice were challenged with 2×10^3 cfu of *P. aeruginosa* strain SBI-N in the burn mouse model. Survival 10 days post-burn in the vector control group was 47% and in the pVR1020-*fliC*(A-pak) group was 37% (Table 2.1). No protection was seen.

To investigate the lack of protection the antibody titer for the sera from the pVR1020-*fliC*(A-pak) group on day 42 was determined for both PAK flagella and SBI-N flagella. As seen in Table 2.2 the sera had a higher titer against PAK flagella then to

SBI-N flagella. Sera collected from the mice that survived the challenge were tested by ELISA for their ability to bind to PAK and SBI-N flagella. Fig 2.4 shows that the sera from the pVR1020-*fliC*(A-pak) group have significantly greater binding to PAK flagella than to SBI-N flagella.

Immunization with pVR1020-fliC(A-sbin). The *fliC* gene from *P. aeruginosa* strain SBI-N was also cloned into pVR1020. Expression of the gene was observed in the transient transfection assay (data not shown) before the construct was injected into mice. Balb/c mice were injected on days 0 and 14 and then bled on day 28. The sera were tested for their ability to bind to both PAK and SBI-N flagella. As figure 2.5 shows, the sera from the pVR1020-*fliC*(A-sbin) group binds better to the PAK flagella than to the SBI-N flagella. Next, the pVR1020-*fliC*(A-sbin) sera along with pVR1020 vector control, rabbit anti-flagellar type A and rabbit anti-flagellar type B sera were tested for their ability to inhibit the motility of *P. aeruginosa* strains PAK, SBI-N and PAO in the motility inhibition assay. The rabbit sera functioned as positive controls. Negative control wells received no sera or vector control sera. As seen in Table 2.3 all 3 strains were motile when no serum was present. They were also motile when vector control serum was present. The rabbit anti-flagellar type A serum was able to inhibit the motility of the type A strains, PAK and SBI-N, and the rabbit anti-flagellar type B serum was able to inhibit the motility of the type B strain, PAO. The pVR1020-*fliC*(A-sbin) serum was unable to inhibit the motility of any of the strains, indicating that this serum would not be protective.

Deglycosylation of purified flagella. Purified flagella preparations from *P. aeruginosa* strains PAK and SBI-N were deglycosylated using the GlycoFree kit. These

deglycosylated flagella preparations along with the purified flagella preparations were used to coat ELISA plates to test for differences in antibody titer from various immunized groups. Sera were collected from mice that had been injected with pVR1020-*fliC*(A-pak), pVR1020-*fliC*(A-sbin), purified PAK flagella or purified SBI-N flagella. Tables 2.4 and 2.5 show that the serum titers from the DNA immunized mice were lower than the titers from the protein immunized mice, which is typical of DNA immunization. Table 2.4 also shows that the sera from the pVR1020-*fliC*(A-sbin) mice have a higher titer to the deglycosylated flagella preparations. The sera from pVR1020-*fliC*(A-pak) mice have the lowest titer with the purified SBI-N flagella preparation. Table 2.5 shows sera from the SBI-N flagella immunized mice have a higher titer to the purified flagella preparation and lower titers with the deglycosylated flagella preparations. The sera from PAK flagella immunized mice have a higher titer to PAK flagella, both untreated and deglycosylated, and lower titers to SBI-N flagella, both untreated and deglycosylated.

2.4 Discussion

Infections with *P. aeruginosa* are particularly difficult to treat. The organism is inherently resistant to many antibiotics. Thus, vaccination to prevent infection is much more desirable. Several antigens from *P. aeruginosa* have been proposed as possible vaccine targets. One particularly intriguing candidate is flagella because they are highly conserved and only have two major serogroups. Active immunization with purified flagella protected mice in the burn mouse model seemingly by preventing the spread of the organism to the liver (82). Passive immunization showed that this protection was due to antibodies against flagella (49). Protein immunization has several disadvantages such as the difficulty of purification and problematic long term storage. DNA vaccination avoids these issues. Previous work in this lab showed the protective capacity of a DNA vaccine encoding the type B *fliC* gene of *P. aeruginosa* (32). Upon gene gun immunization, mice produced good titers of antibodies of the IgG1 isotype. Mice were also significantly protected when challenged with a lethal dose of a homologous strain of *P. aeruginosa*. As with protein vaccination, there was no protection when the mice were challenged with the heterologous type A strain. Further analysis suggested that this protection was due to the inhibition of motility and possibly enhanced phagocytosis of the organisms due to opsonizing antibodies. This study showed that DNA vaccination was possible against an extracellular pathogen, giving similar results as protein vaccination (32).

The goal of this study was to expand on the possibility of DNA vaccination against *P. aeruginosa* by investigating immunization with the type A flagellin gene. To accomplish this, the *fliC* gene from *P. aeruginosa* strain PAK was cloned into the

eukaryotic expression vector, pVR1020. After injection into mice, via the gene gun, good titers of anti-flagellar antibodies were seen. These antibodies were mainly of the IgG1 isotype, as expected for gene gun immunizations (55, 196). These antibodies were also expected to be protective since they mirrored the response seen with the type B gene gun studies; however this was not the case. After challenge with strain SBI-N, no protection in the immunized group was seen (37.5% survival vs. 47.5% survival in vector control). PAK was not used as the challenge strain because it is not virulent in the burn mouse model however SBI-N is virulent and a type A flagellar strain. In studies by Holder and Naglich, immunization with flagella from a type A strain cross-protected when challenged with other type A strains but not when challenged with type B strains (78).

To investigate the lack of protection pre- and post-challenge sera were tested for their ability to bind to PAK or SBI-N flagella. Both pre- and post-challenge sera bound to PAK flagella with higher affinity. Type A flagellar genes show some sequence variability but they are 96-98% identical at the amino acid level (177). However, it is possible that this slight variation occurs at protective epitopes so that the antibodies produced are not cross-protective. So the *fliC* gene from strain SBI-N was cloned into pVR1020 and used to immunized mice. These sera were also tested for their ability to bind to PAK and SBI-N flagella. Once again the antibodies bound to PAK flagella with greater affinity. This suggests something other than amino acid sequence was contributing to the lack of binding of the antibodies induced by pVR1020-*fliC*(A-pak) to SBI-N flagella. The sera also were tested for their ability to inhibit the motility of *P. aeruginosa* since the lack of motility or the inhibition of motility is associated with

protection (48, 49, 132). The sera were unable to inhibit the motility of both type A strains tested, suggesting these sera would not be protective.

Recently it has been found that several prokaryotic organisms post-translationally modify their proteins (143). *P. aeruginosa* flagella, both types A and B are phosphorylated while type A flagella are glycosylated (26, 90). Brimer and Montie showed that much of the size variability in type A flagella was due to the presence of glycosyl groups and when these were removed there was a downshift in the size of the flagellin bringing it closer to its predicted molecular weight (26). Then Arora et al discovered the presence of a glycosylation island in all type A strains tested but not in type B strains. This island contains 14 predicted genes and is inserted between the *flgL* and *fliC* genes (6). When mutants of two of the genes, orfA and orfN, were constructed, the ability to glycosylate flagellin was lost, but it was recovered when complimented with the wild-type genes (6). Later it was shown that there is sequence polymorphism among the glycosylation genes and that this leads to variation in the amount of glycosylation seen on the flagellin proteins (8, 168).

It is possible that the lack of protection seen with the type A *fliC* DNA vaccines is due to the lack of glycosylation of the expressed proteins. Evidence to support this include, the fact that protein immunization with type A flagella is protective, and the preliminary data comparing DNA immunized sera and protein immunized sera titers to untreated and deglycosylated PAK and SBI-N flagella. The pVR1020-*fliC*(A-sbin) sera seems to bind to deglycosylated flagellin better than to untreated flagellin, while SBI-N protein sera seems to bind to untreated flagellin better. This may be due to the lack of antibodies against glycosyl groups in the DNA immunized group and presence of

antibodies against glycosyl groups in the protein immunized sera. Also SBI-N flagella may be more extensively glycosylated, as seen by the sera from pVR1020-*fliC*(A-pak) and PAK flagella immunized groups binding with higher affinity to untreated or deglycosylated flagella.

At this time the function of the glycosyl groups on *P. aeruginosa* flagellin is unknown (168). They are not required for flagellar assembly or function (168). Their role in virulence and immunity has not been examined thus far. In *Pseudomonas syringae* pv. glycinea, flagellar glycosylation contributes to host specificity (182). So it is possible that in *P. aeruginosa* flagellar glycosylation may aid in recognition and binding of host cells. In *Campylobacter coli* the glycosyl groups are responsible for LAH serotype (43). Thus it is possible for the glycosyl groups to contribute to protective epitopes. Flagella are known to be potent stimulators of the innate immune response through TLR5 (1, 71, 206). The effect of *P. aeruginosa* flagellar glycosylation on TLR5 activation of innate immunity is not known. Data presented in here suggests that the presence of the glycosyl groups are needed for protective immunity to *P. aeruginosa* type A strain infections and DNA vaccination with this antigen will not work.

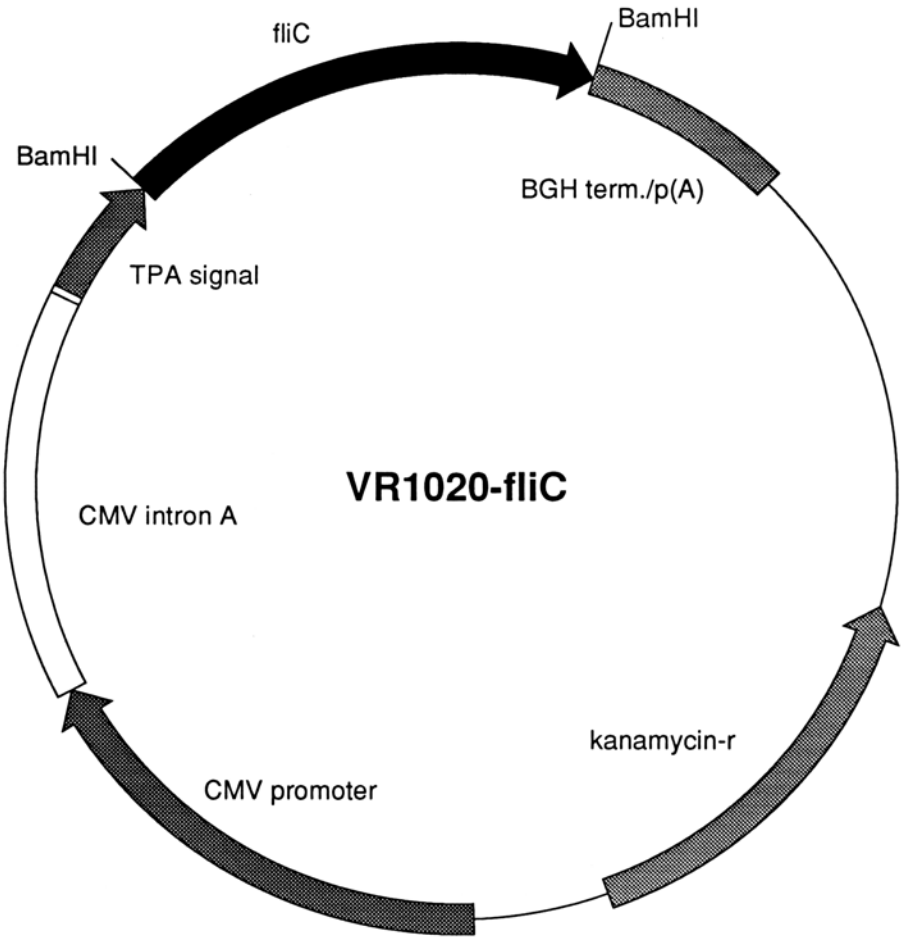


Figure 2.1. Map of pVR1020 showing the position of the cloned *fliC*(A) gene. This vector contains the cytomegalovirus promoter and enhancer and bovine growth hormone poly A terminator for expression in eukaryotic cells. It also contains the tissue plasminogen activator secretions signal for secretion of the expressed protein.

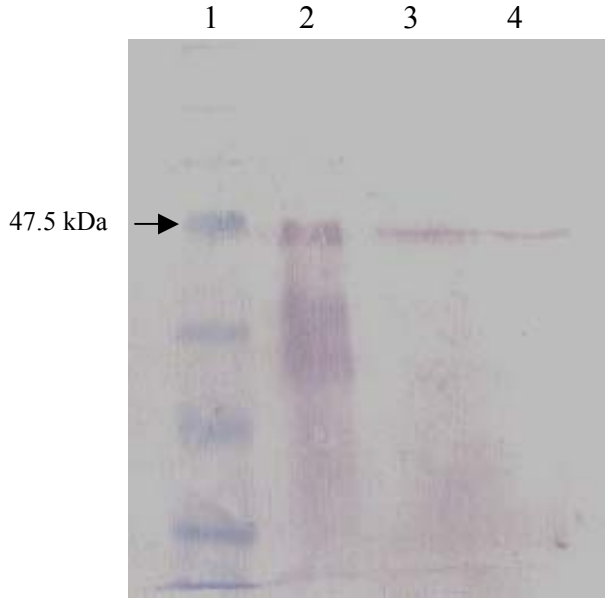


Figure 2.2. Immunoblot of transient transfection of UM449 cells with pVR1020-*fliC*(A-pak). UM449 cells were transfected as described in Material and Methods. After 48 hr of incubation the culture supernatants and cell lysates were harvested and analyzed for expression. As seen in lanes 3 and 4 the gene was expressed and the product is recognized by anti-flagellar type A antibodies. Lane 1: Molecular weight markers, Lane2: purified PAK flagella, Lane 3: cell lysates, Lane 4: culture supernatants.

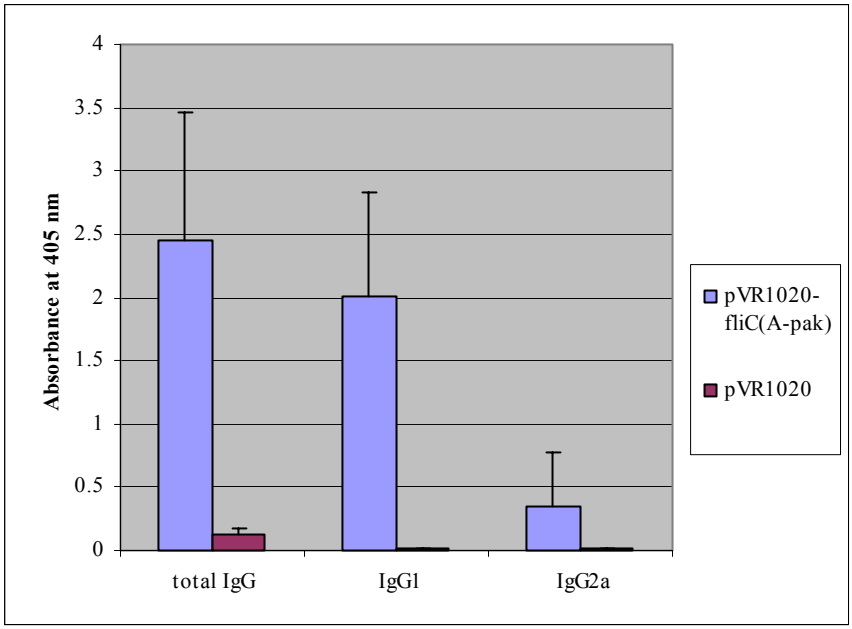


Figure 2.3. Serum IgG, IgG1 and IgG2a on day 42 from mice injected with pVR1020-*fliC*(A-pak) and pVR1020. Mice received 1 µg of DNA via the Helios Gene Gun on days 0, 14 and 28. Serum was analyzed in an ELISA for total IgG, IgG1 and IgG2a prior to challenge.

Immunization group[a]	Survivors
pVR1020	7/15 (47%)
pVR1020-*fliC*(A-pak)	6/16 (37%)

[a]Mice were immunized on days 0, 14 and 28 with 1 μg of DNA via the Helios Gene Gun

Table 2.1. Survival 10 days post-burn. Cr1:Cf1 BR non-Swiss mice were challenged with $2x10^3$ cfu of *P. aeruginosa* strain SBI-N on day 42.

Flagella type	Titer
PAK	1:6400
SBI-N	1:400

Table 2.2. Serum titers of pVR1020-*fliC*(A-pak) immunized mice to PAK and SBI-N flagella. Serum from day 42 before challenge was pooled and titered in an ELISA.

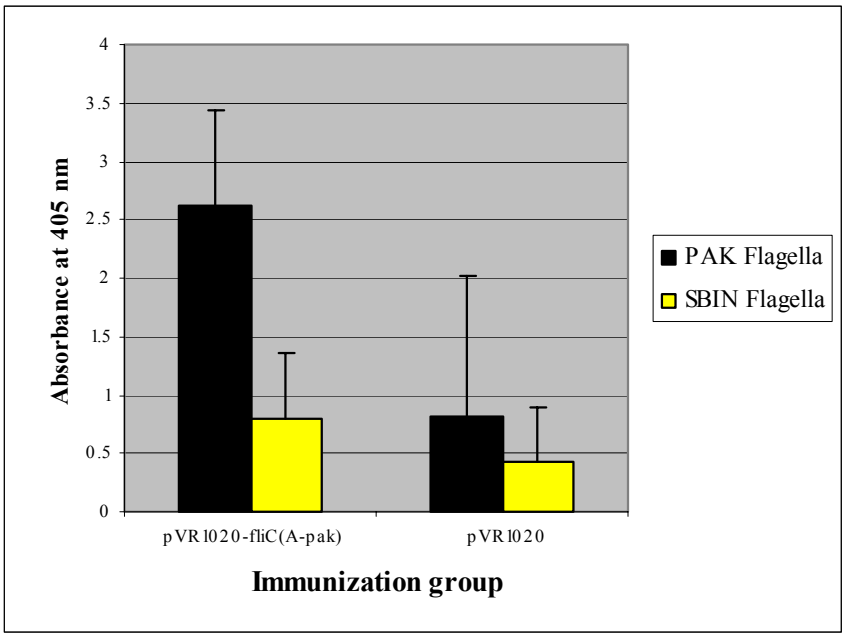


Figure 2.4. Total IgG of post-challenge survivor sera. Mice that had survived 10 days post challenge were bled and the sera were tested for total IgG to PAK and SBI-N flagella. Sera were diluted 1:100 before being added to plates coated with either PAK flagella or SBI-N flagella.

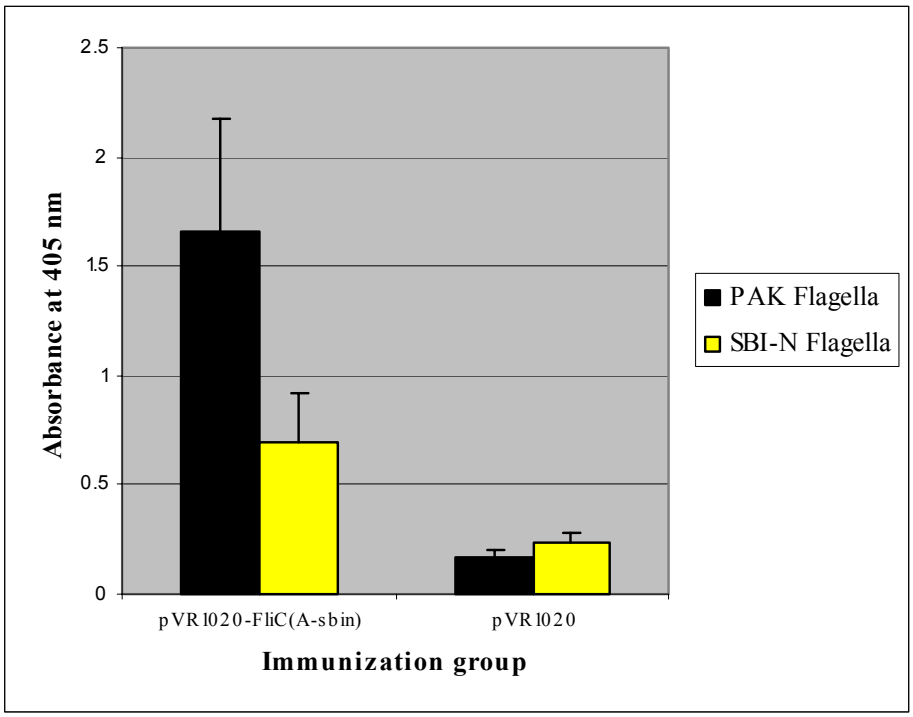


Figure 2.5. Total IgG from pVR1020-*fliC*(A-sbin) immunized mice to PAK and SBI-N flagella. Mice were injected with pVR1020-*fliC*(A-sbin) or pVR1020 on days 0 and 14. Mice were bled on day 28 for analysis of the IgG response. Sera were diluted 1:100 before being added to plates coated with PAK or SBI-N flagella.

	Serum tested				
Strain tested	None	pVR1020	pVR1020-*fliC*(A-sbin)	Rabbit anti-flagella type A	Rabbit anti-flagella type B
PAK (A)[a]	8	9.5	9.5	4.25	13.25
SBI-N (A)[a]	16	16	16	9.5	16
PAO (B)[b]	16	16	16	7	4

[a]Type A strain
[b]Type B strain

Table 2.3. Motility inhibition assay. Motility plates prepared with the appropriate sera before plating in sterile 24-well plates. Overnight cultures were stabbed into the center of each well and the plates were incubated at 37°C for 12 hours.

Flagella type	pVR1020-*fliC*(A-pak)	pVR1020-*fliC*(A-sbin)
Untreated PAK	1600	1600
Deglycosylated PAK[a]	1600	3200
Untreated SBIN	800	1600
Deglycosylated SBIN[a]	1600	3200

[a]Flagella were chemically deglycosylated and used to coat ELISA plates to test for antibody titer from DNA immunized mice

Table 2.4. Titers from DNA immunized mice sera against untreated and deglycosylated PAK and SBI-N flagella.

Flagella type	PAK Flagella	SBIN Flagella
Untreated PAK	102400	51200
Deglycosylated PAK[a]	102400	25600
Untreated SBIN	51200	51200
Deglycosylated SBIN[a]	51200	25600

[a]Flagella were chemically deglycosylated and used to coat ELISA plates to test for antibody titer from protein immunized mice

Table 2.5. Titers from protein immunized mice sera against untreated and deglycosylated PAK and SBI-N flagella.

CHAPTER 3

GENETIC IMMUNIZATION TO PROMOTE A Th2 RESPONSE AGAINST THE TARGET ANTIGEN

3.1 Introduction

Genetic immunization has been called the third revolution in immunization technology. In genetic immunization, the gene of the target antigen is cloned into a eukaryotic expression vector. This plasmid construct is then injected into the host where it is taken up by the host cells and translocated to the nucleus. Once in the nucleus, the gene is transcribed and then translated into an immunogenic protein. The immune system mounts both cell mediated and humoral responses against this expressed protein. Activation of both arms of the immune system is just one advantage of DNA vaccines. Other advantages include longevity of the response, easier purification and storage, and the ability to modulate the immune response.

Upon the detection of a foreign antigen the immune system can respond in several different ways. Different subsets of T-helper (Th) cells help to determine what type of response will occur. Th1 cells encourage the development of cell mediated responses. This type of response is characterized by activation of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells and macrophages, the production of interleukin-12 (IL-12) and interferon gamma (IFN-γ) and the production of antibodies of the IgG2a isotype. Th2 cells encourage the development of humoral immune responses. Here interleukin-4 (IL-

4) and interleukin-10 (IL-10) are produced and B cells are activated to produce mainly IgG1 antibodies. Th1 responses are necessary to eradicate viral and intracellular pathogens while Th2 responses are needed to defend against extracellular pathogens. In the case of *P. aeruginosa* burn wound infections a Th2 type of response seems to be necessary for protection (32, 91). Animals that were vaccinated with a DNA construct containing the type B *fliC* gene were protected after gene gun injection of the plasmid. This type of immunization tends to produce a Th2 type of response (55, 196). This was seen by the production of mainly IgG1 antibodies in these mice (32). However mice injected with the plasmid intramuscularly by a hypodermic needle were not protected (32). This type of injection tends to produce a Th1 type of immune response (55, 196). These mice produced mainly IgG2a antibodies (32). Scale-up of gene gun immunizations is impractical, due to the cost of gold and the equipment required to perform the injections (118), so a method to direct the immune response to a Th2 type of response upon intramuscular immunization is much more desirable.

A novel way of directing the immune response to peptides has been reported in several papers where the antigenic peptide is joined to a peptide of a T cell binding ligand (157, 208, 209). It was found that the G peptide, which consists of a non-polymorphic region, amino acids 135-149, of major histocompatibility complex class II (MHCII) β2 domain, was able to direct a Th2 type of response against a mycobacterial peptide and HSV-1 peptide (157, 208). In contrast, the J peptide, which corresponds to amino acids 35-50 of β-2 microglobulin, was able to direct a Th1 response to both the mycobacterial and HSV-1 peptides (157, 208). This study examines the use of the G peptide fused to a whole protein to direct the immune response with a DNA vaccine. To accomplish this,

the DNA corresponding to the G peptide was cloned 5' of the type B *fliC* gene of *P. aeruginosa* strain PAO1. This construct was then injected into mice and the antibody responses were quantitated. It was found that immunization with this fusion does not direct the immune system towards a Th2 response.

3.2 Materials and Methods

Cloning and large scale preparation of pcDNA-fliC(B) and pcDNA-fliC(B)-G

To create pcDNA-*fliC*(B), a 1.3 kb *fliC* gene fragment from *P. aeruginosa* strain PAO1 was amplified from genomic DNA. The genomic DNA was purified from PAO1 using the Easy-DNA kit (Invitrogen, Carlsbad, CA), following the supplied protocol. The PCR reaction was carried out using the following primers: forward, 5'-CAAGGAATTCGCCATGGGCCTGCAGATCTCCAACCG-3', which incorporated an EcoRI restriction site into the amplified product and reverse, 5'-TCAGCTCGAGCTACGGCAGCTGGCAGCTGGTTGGCCTGG-3', which incorporates an XhoI restriction site into the amplified product. The program for amplification was the same as previously described (chapter 2). After amplification, the product was ligated to the TOPO-TA cloning vector (Invitrogen) as per protocol. This was then transformed into *E. coli* TOP10 cells and plated onto LB supplemented with ampicillin and X-gal for blue/white screening. The plates were incubated overnight at 37°C and the next day white colonies were picked. Purified plasmids were digested overnight with EcoRI at 37°C to screen for incorporation of the PCR insert. The EcoRI fragment was then gel purified using Qiagen's (Valencia, CA) QIAquick Gel extraction kit. This fragment was then digested overnight with XhoI at 37°C and then purified using QIAquick PCR purification kit (Qiagen). The EcoRI-XhoI fragment was then ligated to pcDNA3.1 vector DNA that had also been digested with EcoRI and XhoI before purification. The ligation was transformed into Subcloning Efficiency *E. coli* DH5α cells (Gibco, Grand Island, NY) and plated onto LB plus ampicillin. After overnight incubation at 37°C, colonies were picked and miniprepped, to screen for the presence of

the insert by restriction digests. Plasmids with the *fliC* insert were also screened for expression of *fliC* in the transient transfection assay.

To create pcDNA-*fliC*(B)-G the DNA for the G peptide was cloned 5' of the *fliC*(B) sequence of pcDNA-*fliC*(B). The G peptide DNA was amplified from the pCEXV-A_{β}^{d} plasmid, which was a gift from Ronald Germain (129). The primers used for amplification were as follows: forward, 5'-ATGTGGATCCGCCATGAATGGCCAGGAGGAGACAG-3', which incorporates a BamHI restriction site into the product and reverse, 5'-CGCCGAATTCAATAAGCTGTGTGGATGAGAC-3', which incorporated an EcoRI restriction site into the product. The PCR was run using 30 cycles of 1 min at 94°C, 1 minute at 55°C, 55 seconds at 72°C and finished with 10 minutes at 72°C as a final extension. The presence of a 50 bp product was confirmed by agarose gel electrophoresis before the G-DNA was ligated into the TOPO-TA vector following the provided protocol (Invitrogen). This was then transformed into TOP10 cells for blue/white screening. White colonies were picked and miniprepped so that the G-DNA could be digested out of them. A double digest was done overnight using BamHI and EcoRI at 37°C. The G-DNA was then gel purified using a phenol freeze fracture method (18) as follows; the gel slices were cut into 1-2 millimeter squares and an equal volume of phenol was added to them. This was then frozen at -70°C for 10 minutes and then thawed at 37°C. A second volume of phenol was added to the tube and then it was frozen again. After the sample thawed at 37°C, 50 µl of water was added and the tube was vortexed for 30 seconds. Then the sample was microcentrifuged for 20 minutes and the aqueous layer was collected. The DNA was cleaned up using a phenol:chloroform extraction and one

chloroform extraction. Finally the DNA was precipitated using sodium acetate and isopropanol and then incubated for 30 minutes at -70°C. The DNA pellet was washed once with 70% ethanol and allowed to dry before being resuspended in 10 mM Tris-Cl, pH 8.5. The G-DNA fragment was then ligated to pcDNA-*fliC*(B) that had been digested with BamHI and EcoRI and gel purified using the QIAquick Gel Extraction kit (Qiagen). The ligation was then transformed into Subcloning Efficiency *E. coli* DH5α cells (Gibco) and plated onto LB supplemented with ampicillin. After the overnight at 37°C incubation, colonies were picked for minipreps and screened for the G-DNA insert by PCR and restriction digests. Plasmids that contained the G insert were then screened for expression in a transient transfection assay.

The plasmids, pcDNA3.1, pcDNA-*fliC*(B) and pcDNA-*fliC*(B)-G, were purified using Qiagen's MegaPrep Kit. The concentration of each plasmid was adjusted to 1 mg/ml in PBS.

Transient Transfection Assay and Immunoblot

The transient transfection assay and immunoblot procedures were performed as previously described (chapter 2) with the following changes. The membrane was incubated with the primary antibody, rabbit anti-flagellar type B diluted 1:1000 in TTBS for 1 hour before washing. Then the membrane was incubated with the secondary antibody, alkaline phosphatase conjugated anti-rabbit IgG (BioRad) diluted 1:1000 in TTBS before washing and color development.

Immunization schedule

Six to eight week old female Balb/c mice were injected intramuscularly with 100 µg of pcDNA3.1, pcDNA-*fliC*(B) or pcDNA-*fliC*(B)-G in the rear quadriceps on days 0 and 14. On day 28, mice were bled retro-orbitally and the sera were analyzed for antibody responses to flagella.

Analysis of antibody responses

Total IgG ELISA. The total IgG ELISA was performed as described previously (chapter 2). In brief, immulon II plates were coated with 1 µg/ml of PAO1 flagella. The plate was blocked for and washed with TTBS. The mouse sera were diluted 1:100 in TTBS added to the plate and incubated for 1 hour at room temperature. After washing the secondary antibody, alkaline phosphatase conjugated anti-mouse IgG (BioRad) diluted 1:1000 in TTBS was added and incubated for 1 hour at room temperature. Finally the plate was washed with TTBS and TBS before the addition of the substrate. Color was allowed to develop for 30 minutes before the absorbances were read at 405 nm in a BioRad Model 550 plate reader.

Isotyping ELISA. The IgG1 and IgG2a ELISAs were performed as previously described (chapter 2). In brief, immulon II plates were coated with 1 µg/ml of purified PAO1 flagella. After blocking the mouse sera were diluted 1:100 in TTBS and added to the plate and incubated for 1 hour at room temperature. After washing, the appropriate secondary antibody was added and incubated for 1 hour at room temperature. The secondary antibody used was either alkaline phosphatase conjugated anti-mouse IgG1 diluted 1:1500 in TTBS or alkaline phosphatase conjugated anti-mouse IgG2a diluted 1:1000 in TTBS (both Zymed Laboratories). Finally the plates were washed and the

substrate was added. The absorbances were read at 405 nm in the BioRad Model 550 plate reader. The standard curves for both IgG1 and IgG2a were standardized to each other so that direct comparisons of the absorbances could be made.

3.3 Results

Cloning and expression of pcDNA-fliC(B) and pcDNA-fliC(B)-G. A 1.3 kb fragment of the *fliC* gene from *P. aeruginosa* strain PAO1 was amplified from purified genomic DNA. This fragment is missing the first 138 bp and last 27 bp. These regions encode conserved portions of flagellin that are believed to be involved in subunit-subunit interactions and are not naturally accessible to the immune system (139, 198). The *fliC* fragment was then cloned into pcDNA3.1, a eukaryotic expression vector which contains the CMV promoter and enhancer, and the bovine growth hormone polyadenylation sequence needed for the expression of the cloned gene. Expression from this plasmid was confirmed using a transient transfection assay. Lane 2 from Figure 3.1 shows the cell lysate from UM449 cells transfected with pcDNA-*fliC*(B). The G-DNA sequence was then cloned into pcDNA-*fliC*(B) upstream of the *fliC* gene. The G-DNA was amplified from pCEXV-A_{β}^{d}. Expression of the G-FliC protein was also confirmed by transfection of UM449 cells. Lane 1 of Figure 3.1 shows the immunoblot of cells transfected with pcDNA-*fliC*(B)-G. As can be seen, G-FliC runs higher in the gel than FliC (lane 2) and both FliC and G-FliC proteins are smaller than purified PAO1 flagellin (lane 4) due to the missing165 bp. Lastly cells that were transfected with pcDNA3.1 (lane 3) show no product capable of reacting with anti-flagellar type B antibodies.

Antibody responses. Three separate trials were conducted where groups of 3-4 mice were injected with pcDNA3.1, pcDNA-*fliC*(B) or pcDNA-*fliC*(B)-G. Each mouse received 100 µg of DNA suspended in phosphate buffered saline (PBS) via intramuscular needle injection, on days 0 and 14. On day 28, the mice were bled and the sera were analyzed for anti-flagellar antibody responses. Each trial showed the same overall results. Figure

3.2 shows the results from one trial but it is representative of all three trials. By day 28 both the pcDNA-*fliC*(B) and pcDNA-*fliC*(B)-G plasmids stimulated good levels of anti-flagellar type B (anti-FlaB) antibodies, whereas the pcDNA3.1 vector plasmid did not stimulate any anti-FlaB antibodies. In all three trials more anti-FlaB antibodies were produced after injection of pcDNA-*fliC*(B) than pcDNA-*fliC*(B)-G but this was not a significant difference. Isotype analysis showed more IgG1 production than IgG2a in both pcDNA-*fliC*(B) and pcDNA-*fliC*(B)-G groups (Fig. 3.2). Comparison of the IgG1:IgG2a ratios from all 3 trials show no difference between the pcDNA-*fliC*(B) and pcDNA-*fliC*(B)-G groups (Table 3.1).

3.4 Discussion

Genetic immunization has several advantages over whole cell and protein subunit vaccines. One advantage is the ability of genetic vaccines to induce both humoral and cellular immunity. This is not seen with most protein subunit vaccines or killed whole cell vaccines, which usually only induce humoral immunity (98). Another advantage is the ability to modulate the immune response initiated by DNA vaccines to more of a Th1 type response of a Th2 type of response. Several ways have been investigated including the use of traditional adjuvants and genetic adjuvants, such as cytokine genes, chemokine genes and co-stimulatory molecule genes (164). For this study the use of the G peptide fused to FliC(B) in a genetic vaccine to direct a Th2 response upon intramuscular immunization was examined.

Two vaccine constructs were created. The pcDNA-*fliC*(B) construct contained the *fliC* gene from *P. aeruginosa* strain PAO1 cloned into pcDNA3.1. Upon transfection in cell culture, the cells produced a gene product that was recognized by anti-FlaB antibodies. The pcDNA-*fliC*(B)-G construct consists of the pcDNA-*fliC*(B) plasmid with the G peptide DNA cloned 5' of the *fliC* gene. Upon transfection in cell culture, a larger gene product was seen that also was recognized by anti-FlaB antibodies. So the presence of the G peptide fused to the N-terminus of FliC had little, if any interference with the ability of polyclonal anti-flagellar antibodies to bind to the FliC portion of the G-FliC protein. The presence of the G peptide also did not interfere with the ability of the G-FliC protein to induce anti-FliC antibodies that were able to recognize native purified flagella in an ELISA. Both the transfection and immunization results suggest that the G

peptide did not interfere with the proper folding or antigenicity of the FliC portion of the gene product.

On day 28, large amounts of anti-flagellar antibodies were detected in both pcDNA-*fliC*(B) injected mice and pcDNA-*fliC*(B)-G injected mice, while no anti-flagellar antibodies were detected in the vector control mice. The pcDNA-*fliC*(B)-G group was expected to produce higher amounts of IgG as in the previous works by Zimmerman et al (208) and Rosenthal et al (157), where the presence of the G peptide induced higher antibody responses than control peptides or other test peptides. In this case, the pcDNA-*fliC*(B) group always gave slightly higher antibody responses, although there was not a significant difference between the two groups. Examination of the IgG isotypes showed more IgG1 than IgG2a in both pcDNA-*fliC*(B) and pcDNA-*fliC*(B)-G groups; however, comparison of the IgG1:IgG2a ratios showed no difference between these two groups. This was unexpected due to the previous reports of the G peptide being able to induce an overwhelming IgG1 antibody response (208). These results indicated that the G peptide was unable to direct the immune response to an overwhelming Th2 response in this system.

This study was the first to attempt to use the G peptide fused to a whole protein in a DNA vaccine to direct the immune response. Unfortunately no modification of the immune response was seen. This is probably due to the fact that a whole protein antigen was used instead of a peptide antigen. In all previous studies using the G peptide it was fused to peptide containing B and/or T cell epitopes (157, 208). In these studies the presence of the G peptide induces a Th2 response. In this study a whole protein was used. It is possible that when the protein folded the G peptide was buried in the protein

in such a way that it was inaccessible to the immune system. Thus it would have been unable to modify the immune response. The exact mechanism of how these modulatory peptides work is unknown (209). It may be that they only function to modulate the immune response against peptides and not whole proteins. Thorough investigation of their mechanism of action is needed to determine this.

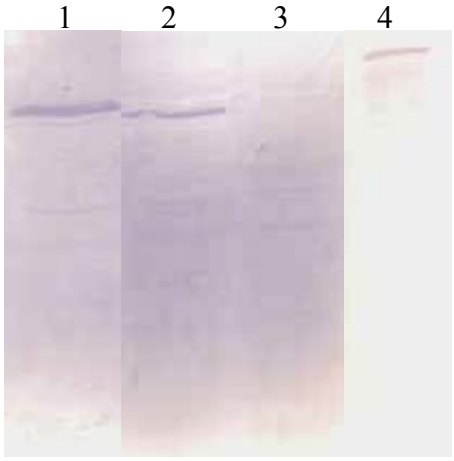


Figure 3.1. Immunoblot of transient transfection of UM449 cells. UM449 cells were transiently transfected as described in Materials and Methods. After 48 hours of incubation the cell lysates were and analyzed for gene expression. Immunoblots were exposed to anti-flagellar type B antibodies. Lane 1: pcDNA-*fliC*(B)-G, lane 2: pcDNA-*fliC*(B), lane 3: pcDNA3.1, lane 4: PAO1 purified flagella.

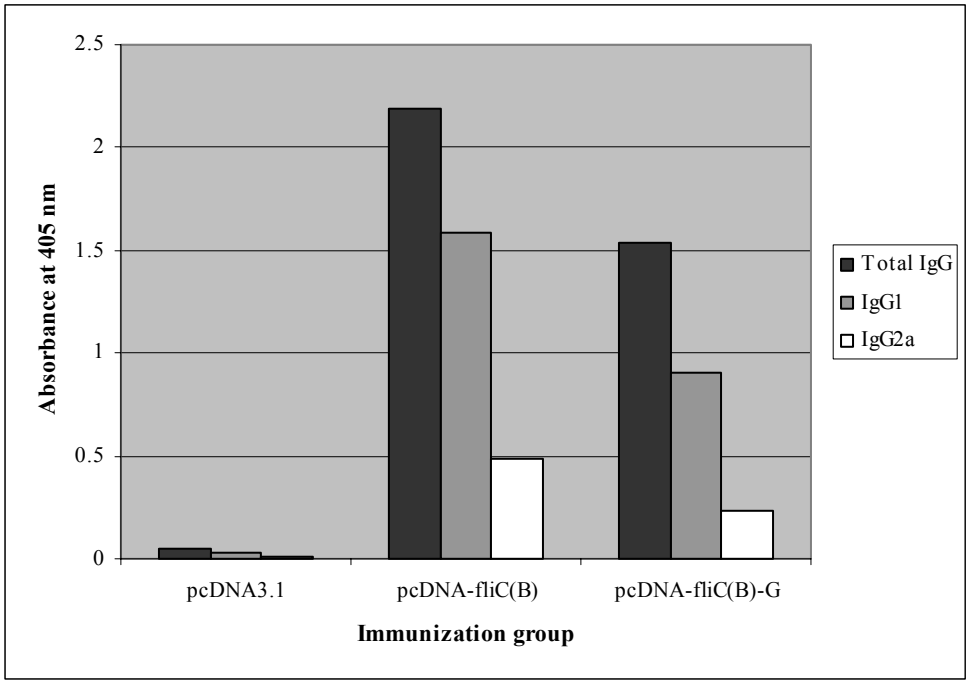


Figure 3.2. Immune responses detected after genetic immunization with the G peptide. Mice were injected with intramuscularly with 100 µg of DNA on days 0 and 14. On day 28 the mice were retro-orbitally bled and the sera were analyzed for anti-flagellar antibodies. Graph is from one representative trial. Data represents the means of 3 or 4 mice per group.

Immunization Group	Trial I	Trial II	Trial III
pcDNA-*fliC*(B)	4.64	2.32	3.28
pcDNA-*fliC*(B)-G	4.37	1.68	3.83

Table 3.1. IgG1:IgG2a ratios from 3 separate immunization trials.

CHAPTER 4

IN VIVO DENDRITIC CELL TARGETING

4.1 Introduction

P. aeruginosa is capable of infecting almost any site in the human body after a predisposing event, such as catherization, intubation, severe trauma or burn. Thus *P. aeruginosa* is one of the leading causes of nosocomial infections (115). Treatment of these infections is difficult due to *P. aeruginosa*'s inherent resistance to many antibiotics. Only fluoroquinilones, gentamicin and imipenam are effective at treating *P. aeruginosa* infections (186). Vaccination to prevent infection is more desirable, however, most vaccine strategies require several weeks and several booster shots before an effective immune response is mounted (195). Unfortunately there is usually not enough time to administer all the shots required for effective immunization before the onset of an infection after hospitalization. Prophylactic vaccination of the whole population is not cost effective, but neither is extensive antibiotic treatment; however, a vaccine that could induce rapid and protective immune responses in at risk individuals could provide a needed solution to this problem.

A new trend in vaccine development is dendritic cell (DC) targeting. DC have been termed 'nature's adjuvant' (58). These cells are potent stimulators of innate and adaptive immune responses (58). Immature DC reside in almost every tissue of the body where they actively sample their surroundings, in order to detect pathogens (126). Upon

encountering an infectious agent, DC rapidly up regulate their expression of MHC (class I and II) molecules along with co-stimulatory molecules, such as CD80 and CD86, and migrate to secondary lymphoid organs in order to activate T cells (58, 126).

Several methods have been used to target DC. One method involves the *ex vivo* culture of DC and then pulsing them with bacterial cells, whole tumor lysates, tumor peptides, RNA or DNA (56, 176, 180, 201). While this method has shown promise, the *ex vivo* culture of DC is difficult and requires expensive reagents, such as cytokines. Another method of targeting DC involves genetic fusions in DNA vaccines. In this case a genetic fusion is created between the target antigen and a molecule that binds to DC, such as Flt3-ligand, the Fc region of IgG or heat shock protein (69, 85, 205). This method has been shown to enhance both cytotoxic T cell responses and T helper cell responses but humoral responses still took 4 weeks (69, 205).

A third method to target DC has been described by Wang et al that rapidly induces antibody responses (195). In this study antigen was targeted to DC *in vivo* by the use of a hamster monoclonal antibody (mAb), N418, which binds CD11c on DC. Seven days after injection high titers of antibodies against the target antigen were seen. However the target antigen, goat anti-hamster IgG, used in the study is not relevant in a pathogenic model, so Conwell (32) tested the ability of N418 to induce rapid antibody responses against an antigen that is relevant in a pathogenic model. Flagellin was chosen as the target antigen because it is the major component of *P. aeruginosa* flagella, which are expressed during the acute phases of infection (54, 62), and active and passive immunization against *P. aeruginosa* flagella is protective (49, 78, 160). In her studies, Conwell chemically cross-linked flagellin to N418 and injected this conjugate into mice.

A rapid anti-flagellar antibody response was seen by day 7 in the conjugate injected group but not in the flagellin injected group; however the protective efficacy of the conjugate vaccine was inconclusive (32).

This study extends on the work done by Conwell. First the question of whether the mAb, N418, is necessary to induce the rapid antibody responses seen or whether these responses were due to conjugation to flagellin was examined. To do this two conjugates, one consisting of flagellin conjugated to random hamster IgG and one consisting of N418 conjugated to BSA, were constructed and antibody responses analyzed. Next the ability of the N418-FliC conjugate to provide protection in the burn mouse model was shown. Lastly the ability of the conjugate to activate dendritic cells and then stimulate T cells was examined.

4.2 Materials and Methods

Monoclonal antibody purification

Both the N418 hybridoma cell line and DEC-205 hybridoma cell line were cultured according to the supplier's instructions (American type culture collection, ATCC, Manassas, VA). The growth medium for the N418 hybridoma was RPMI-1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 uM 2-mercaptoethanol and 7.5% fetal bovine serum (FBS). The growth medium for the DEC-205 hybridoma was RPMI-1640 supplemented with 2.5g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS. Both cell lines were incubated at 37C plus 5% CO_2 in a humidified environment.

In order to concentrate the antibodies for purification, they were precipitated out of the culture supernatant as follows. First the pH of the culture supernatant was adjusted to 5.5 with concentrated hydrochloric acid. Then 575 g/L of ammonium sulfate was slowly added and mixed for 1 hour at room temperature (84). The precipitant was collected by centrifuging for 15 minutes at 8500 rpm and discarding the supernatant. Finally the precipitant was dissolved in binding buffer (0.5 M Tris, pH 7.0) for application to the affinity chromatography column.

The N418 antibody was purified using Protein A affinity chromatography (Amersham Bioscience, Piscataway, NJ) and the DEC-205 antibody was purified using Protein G affinity chromatography (Amersham Biosciences). The purification was done as follows. First the precipitated antibodies in binding buffer were applied to the equilibrated column. Then the column was washed with 10 bed volumes of binding

buffer. Finally the antibodies were eluted using 5 bed volumes of 0.1 M glycine, pH 3.0 and collecting 1 ml fractions. Absorbance of the fractions was read at 280 nm. Fractions with an optical density at or above 0.1 were saved and pooled. The purity of the antibodies was checked by SDS-PAGE before dialysis in 1 mM Tris, pH 7.0. The dialyzed antibodies were then lyophilized and stored at -20°C.

Recombinant flagellin expression and purification

The *fliC* gene from *P. aeruginosa* strain PAO1 was previously cloned into the bacterial expression vector pET16b (32). This plasmid was then transformed into *E. coli* strain BL21(DE3) for expression. The expression was followed according to the Novagen protocol for expression from a T7 promoter (Novagen pET system manual, 2000 edition) with some modifications. A 16 ml overnight culture of E. coli BL21(DE3) containing pET-fliC-B in LB plus 75 μg/ml ampicillin was used to inoculate 400 ml of LB plus ampicillin. The culture was allowed to grow at 37°C with shaking until an absorbance at 600 nm of approximately 0.6 was reached. It was then induced with 1 mM IPTG and incubated for another 6 hours. At the end of the expression period the cells were harvested by centrifugation and resuspended in denaturing binding buffer, pH 7.8 (20 mM sodium phosphate, 500 mM NaCl, 8 M urea). Then the cells were lysed using a French Pressure Cell at 15,000 psi and passing them through the press twice. The lysed cells were then centrifuged at 19,000 rpm for 30 minutes to remove debris.

To purify the His-tagged recombinant flagellin protein, nickel affinity chromatography was used. The protein was batch bound to the nickel charged resin overnight at 4°C with rotation in a serum wheel. The resin with bound protein was then washed once with denaturing binding buffer before the resin was transferred to the

column. Next the resin was washed with denaturing wash buffer, pH 6.0 (20 mM sodium phosphate, 500 mM NaCl, 8 M urea) until the absorbance at 280 nm of the flow through was below 0.05. Then the resin was washed with denaturing wash buffer, pH 5.3 until the absorbance at 280 nm of the flow through was below 0.05. Finally the recombinant flagellin was eluted from the resin using elution buffer, pH 4.0 (20 mM sodium phosphate, 500 mM NaCl, 8 M urea) in 1 ml fractions. The absorbance of each fraction was measured at 280 nm and fractions with an absorbance at or above 0.1 were pooled together. The purity of the eluted protein was checked by SDS-PAGE. Then the pooled fractions were dialyzed against 10 mM Tris, pH 8.0 with 1% Triton X-100, then 1 mM Tris, pH 8.0 and finally water. After dialysis the protein was lyophilized and stored at -20°C.

Cross-linking of antibodies and target antigens

The crosslinker used to conjugate the purified N418 mAb, purified DEC-205 mAb or purified hamster IgG (Jackson Immunoresearch, West Grove, PA) to purified recombinant flagellin or bovine serum albumin (BSA) was Sulfo-SMCC (Pierce, Rockford, IL) following the supplied protocol. In brief, the antibody, dissolved in PBS and 5 mM EDTA, was reduced with 0.5 M 2-mercaptoethylamine-HCl (Pierce) for 90 minutes at 37°C. After the reduction period, the reducing agent was removed using D-Salt Excellulose Plastic desalting column (Pierce, MW cut-off 5,000) and PBS with 5 mM EDTA as the solvent. The target protein, dissolved in PBS, was reacted with Sulfo-SMCC for 60 minutes at room temperature. The excess cross-linker was then removed using the desalting column (MW cut-off 5,000) and PBS as the solvent. Fractions with the highest concentration of antibody and target protein, as determined by Bradford

assay, were mixed and incubated overnight at 4°C. The concentration of the conjugate was determined using the Bradford assay before dilution to 0.4 mg/ml in PBS for injection.

Immunization

Six to eight week old female Balb/c mice were injected subcutaneously with 30 µg/75 µl of conjugate, control proteins or saline on the days indicated. The mice were bled retro-orbitally on the indicated days in order to analyze the sera for anti-flagellar or anti-BSA antibodies.

Serum Analysis

Total IgG ELISA. The ELISAs were performed as previously described with some modifications (chapter 2). Immulon II plates were coated with 2 µg/ml of purified flagella or BSA overnight at 4°C. The plates were washed and blocked with 0.05% Tween-Tris buffered saline (TTBS). The sera were serially 2-fold diluted in the plate and allowed to incubate for 1 hour at room temperature. After washing the secondary antibody, alkaline phosphatase conjugated anti-mouse IgG diluted 1:1000 was added to each well. The plates were incubated for 1 hour at room temperature before washing and addition of the substrate. After 30 minutes of color development, the absorbance was read at 405 nm. Antibody titer was defined as the highest dilution that gave an absorbance reading equal to or above 0.2.

Isotyping ELISA. The isotyping ELISAs were performed as previously described (chapter 2). A standard curve was constructed in order to determine concentration of IgG1 and IgG2a present in the serum samples.

Challenge

The burn mouse model challenge was performed at Shriner's Hospital for Children in Cincinnati, Ohio and was followed according to Holder (80). Female Cr1:Cf1 BR non-Swiss, 6-8 week old mice were immunized subcutaneously with 30 µg/75 µl of N418 alone, recombinant FliC alone, N418-FliC conjugate or saline on days 0 and 7. On day 14 the mice were anesthetized and given a partial thickness alcohol burn over 15% of their body surface. Saline was injected intraperitoneally to replace fluids lost during the burn. Next, a lethal dose, $2x10^3$ cfu, of *P. aeruginosa* strain M-2 was injected subcutaneously at the burn site. Survival was assessed over the next 5 days.

Isolation and culture of dendritic cells

Balb/c mice were dissected to remove their femurs and tibias. The muscles were removed from the bones and then the bones were sterilized in 70% ethanol for 1 minute. After sterilization, the bones were placed in fresh dendritic cell (DC) growth medium (RPMI 1640, 2 uM glutamine, 50 mM 2-mercaptoethanol, 0.01 M HEPES and 5% FCS). To remove the marrow, both ends of the bones were snipped off and the marrow was flushed out using a syringe, 25-gauge needle and DC growth medium. After all the marrow was collected, the red blood cells were lysed using ACK lysis buffer (0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4). The cells were then filtered through a 0.45 um cell strainer and counted. Finally, the cell concentration was adjusted to approximately $8x10^5$ cells/ml in fresh DC growth medium supplemented with 100 U/ml of murine GM-CSF and 100 U/ml murine IL-4. The cells were plated in 24 well tissue culture plates and incubated at 37°C and 5% CO_2 for 8-10 days. On days 3, 6 and 8 the media was changed by removing 50% of the old medium and centrifuging it at 1100

rpm for 10 minutes to save and non-adherent cells. Then fresh DC growth medium supplemented with 200 U/ml murine GM-CSF and 200 U/ml murine IL-4 was added to the collected cells and they were added back to the plate.

Activation of immature DC

After 8 days of culture, the bone marrow derived DC were stimulated with TNFα (500 U/ml), 1 μg/ml of the indicated antigen or an equal volume of PBS. After 24 hours of incubation with the stimulus, the cells were harvested and analyzed for the up-regulation of MHCII and CD86. The harvested cells were washed twice in FACS buffer (PBS, 1% FCS, 1% BSA) before blocking with Fc Block (BD Pharmigen) for 30 minutes at room temperature. After washing, the appropriately labeled antibodies were added to the cells and allowed to incubate for 1 hour at room temperature, in the dark. The antibodies used were FITC-anti-CD11c, PE- anti-I-A^d and PE- anti-CD86 and the appropriate isotype matched controls (all BD Pharmigen). After the incubation, the cells were washed and then fixed with 2% paraformaldehyde. Fluorescent signals were analyzed using BD BioSciences FACS Caliber and CellQuest Pro software.

Antigen presentation

Ninety-six well tissue culture plates were seeded with 100 DC per well. The DC were then stimulated with 1μg/ml of the indicated antigen or an equal volume of PBS for 4 hours before $2x10^5$ or $1x10^5$ isolated axillary lymph node cells were added to each well. The lymph nodes were harvested from mice that had been immunized on day 0 and then harvested on day 7 or from mice that had been immunized on days 0 and 7 and harvested on day 14. The plates were incubated for 72 hours at 37°C with 5% CO_2. After 72 hours, the supernatants were analyzed for IL-2 production by ELISA.

IL-2 ELISA

Immulon II plates were coated with 2 μg/ml of rat anti-mouse IL-2 (BD Pharmigen). The plate was then blocked with 1% BSA in PBS for 1 hour at room temperature. After washing 3 times with 0.05% Tween-PBS (PBS-T), the culture supernatants from the antigen presentation assay as well as IL-2 standards were added to the plate. For the IL-2 standards, recombinant murine IL-2 (BD Pharmigen) was diluted serially from 1000 pg/ml to 0 pg/ml in assay buffer (0.05% Tween- PBS, 1% BSA). The plate was then incubated for 2 hours at room temperature. After washing 4 times in PBS-T, 1 μg/ml of biotinylated rat anti-mouse IL-2 (BD Pharmigen) diluted in assay buffer was added to each well and incubated for 1 hour at room temperature. Once again the plate was washed 4 times and then alkaline phosphatase-strepavidin (BD Pharmigen) diluted 1:2000 in assay buffer was added to each well. After incubation for 30 minutes at room temperature the plate was washed 5 times in PBS-T and the substrate solution, p-nitrophenyl phosphate (Sigma) dissolved in glycine buffer (7.51 g glycine, 0.203 g $MgCl_2$, 0.136 g $ZnCl_2$ per 1 liter, pH 10.4), was added. The absorbance was read at 405 nm after 60 minutes of color development in the dark.

4.3 Results

Serum Analysis. First, to repeat the results seen by Conwell, the N418 mAb was cross-linked to purified recombinant flagellin (FliC). Mice were then injected subcutaneously on days 0 and 7 with 30 μg of N418-FliC conjugate, FliC alone or PBS. On days 7 and 14 the mice were bled and the serum was analyzed for anti-flagellar type B (anti-FlaB) antibodies. Injection of PBS did not induce any anti-FlaB titers on day 7 or 14 (data not shown) and no anti-FlaB antibodies were seen after the injection of FliC alone on day 7. A second with FliC alone injection on day 7 produced slight anti-FlaB titers by day 14 (Fig. 4.1), whereas injection of the N418-FliC conjugate produced anti-FlaB antibodies by day 7 and a second injection significantly boosted the titers by day 14 (Fig. 4.1).

To test if the rapid induction of antibodies was due to the presence of the N418 mAb or if conjugation to any antibody would induce a rapid antibody response a control conjugate consisting of hamster IgG (HamIgG) cross-linked to FliC was made. Mice were then injected with this or with the N418-FliC conjugate as before. Analysis of the sera from day 7 showed that only the N418-FliC conjugate was able to induce anti-flagellar antibodies (Fig. 4.2). The HamIgG-FliC control conjugate was only able to induce slight amounts of antibodies on day 14, after receiving 2 injections. The antibody titer on day 14 of the HamIgG-FliC group was very similar to the titer induced by injection with FliC alone (Figs. 4.1 and 4.2). Once again, after 2 injections of the N418-FliC conjugate a significant increase in anti-flagellar antibody titer was seen (Fig. 4.2).

Next, to test if conjugation of N418 to any antigen would induce rapid antibody responses or if this phenomenon was due to N418 being cross-linked to FliC, BSA was conjugated to N418. Mice were then injected with N418-BSA, BSA alone or PBS on

days 0 and 7. Serum was analyzed on days 7 and 14 for anti-BSA antibody responses. As seen in Figure. 4.3, PBS injection induced no anti-BSA antibodies at day 7 or 14. Injection of BSA only induced slight anti-BSA titers by day 14. The N418-BSA conjugate, however, was able to induce anti-BSA titers at day 7 with a significant increase in titer after boosting by day 14 (Fig. 4.3).

A second mAb that also binds DC was tested for its ability to induce rapid antibody titers. The NLDC-145 (DEC-205) mAb was conjugated to FliC and injected into mice on days 0 and 7. On day 7 the DEC-205-FliC conjugate induced an anti-flagellar titer of 700 which increased to 3200 on day 14. Since the N418-FliC conjugate gave higher titers, the DEC-205-FliC conjugate was not investigated further.

Boost Responses. To examine the optimal boost schedule, mice were injected with 30 μg of N418-FliC on day 0 and then boosted on day 3, 5, 7 or days 3 and 5, or they were not boosted. One group of mice was injected with FliC alone on day 0 and boosted on day 7 for comparison. The mice were then bled on days 7, 10, and 14 to analyze the anti-FlaB responses. The results are shown in Figure. 4.4. By day 7 all groups had some anti-FlaB antibodies titers with the group boosted on day 3 having the highest. However, this response decreased by day 7 and had only slightly improved by day 14. Boosting on day 5 and 7 produced very similar results with boosting on day 7 giving a higher titer. No increase in titer was seen in the group that was not boosted. Once again injecting FliC alone gave very slight titer by day 14 similar to the no boost group.

Next, the ability of the N418-FliC conjugate to prime the response to a FliC only boost was tested. Mice were primed with either N418-FliC or FliC alone and then boosted on day 7 with either N418-FliC or FliC. The group that received 2 injections of

FliC alone produced low titers of anti-FlaB antibodies on day 14. The groups that received the N418-FliC conjugated had similar anti-FlaB titers on day 7. The antibody titer increased similarly in both of these groups whether they were boosted with N418-FliC conjugate or with FliC alone (Fig. 4.5).

Challenge. To test the effectiveness of the rapid antibody response to protect against challenge of *P. aeruginosa*, mice were injected with 30 μg of N418-FliC, N418 alone, FliC alone or PBS on days 0 and 7. On day 14 the mice were burned as described in materials and methods and challenged with $2X10^3$ cfu of *P. aeruginosa* strain M-2, which is virulent in the burn mouse model and has type B flagellin. Mortality was assessed for 5 days. Table 4.1 shows the results from this study. Immunization with N418 alone provided no protection to the mice with 85% of them having died. Immunization with FliC alone provided some protection when compared to the group immunized with saline ($p<0.05$). The N418-FliC immunized group showed significant protection relative to the other groups ($p<0.05$ vs. N418 only and FliC only groups). The antibody titers in this group were much higher than in the FliC only group (Table 4.2). The major IgG isotype produced was IgG1 (Table 4.2)

Dendritic cell activation. To test if the conjugate could up regulate the expression of MHCII and CD86, which is associated with DC activation, bone marrow derived dendritic cells (BMDC) were incubated with 1 μg/ml of N418-BSA, BSA alone or HamIgG alone for 24 hours before staining for FACS analysis. BMDC were incubated with PBS, as a negative control, and as a positive control they were incubated with 500 U/ml TNFα. As expected, TNFα was able to increase the percentage of MHCII and CD86 expressing cells as compared to incubation with PBS (Table 4.3). N418-BSA was

also able to increase the percentage MHCII and CD86 expressing cells. This increase was greater than that induced by TNFα. BSA alone was unable to activate the BMDC as seen by the lower percentage of $MHCII^+$ cells and $CD86^+$ cells as compared to the BMDC incubated with PBS. HamIgG slightly activated the BMDC; however this increase was not nearly as high as the BMDC exposed to TNFα.

Antigen presentation. After showing that the N418 conjugate can activate DC, the ability of the activated DC to present antigen to T cells was tested. T cells were harvested from the draining lymph nodes of mice that had been injected with N418-BSA or BSA alone. The T cells were mixed with BMDC that had been incubated with N418-BSA, BSA alone or PBS for 24 hours. T cells were incubated without BMDC, as a negative control, to get background IL-2 production levels. IL-2 production was assessed by ELISA 72 hours after the start of the co-culture to detect the level of T cell proliferation stimulated by DC. At day 7, the T cells from N418-BSA primed mice produced large amounts of IL-2 when incubated with N418-BSA pulsed DC, while much less IL-2 was produced when these T cells were exposed to BSA pulsed DC (Fig. 4.6A). There was no difference in the IL-2 production between BSA pulsed DC and PBS pulsed DC and very little IL-2 was produced by T cells cultured without DC. The T cells from BSA primed mice showed no difference in the amount of IL-2 produced after co-culture with PBS pulsed DC, N418-BSA pulsed DC or T cells cultured without DC. No IL-2 was produced after co-culture of BSA primed T cells with BSA pulsed DC (Fig. 4.6B).

Similar results were seen using T cells harvested on day 14 from mice that were injected on days 0 and 7 with N418-BSA or BSA alone. The N418-BSA T cells produced more IL-2 when co-cultured with N418-BSA pulsed DC than when co-cultured

with PBS or BSA pulsed DC or than T cells cultured without DC (Fig. 4.7A). T cells from mice injected with BSA alone produced more IL-2 when co-cultured with N418-BSA pulsed DC than when co-cultured with PBS or BSA pulsed DC or T cells cultured without DC (Fig. 4.7B). Overall, more IL-2 production is seen from the T cells harvested from N418-BSA injected mice than from BSA injected mice.

4.4 Discussion

Vaccination to induce humoral immune responses is important to prevent *P. aeruginosa* infections, however, the induction of antibodies titers high enough to prevent infection can take anywhere from 2-6 weeks (115). For acute infections with *P. aeruginosa*, this is too long to establish protective antibody titers. A unique way to induce rapid antibody responses was described by Wang et al (195) and this work was applied to *P. aeruginosa* vaccination by Conwell (32). The work presented in this study explores the underlying mechanism by which the N418-FliC conjugate is able to induce rapid, protective anti-flagellar antibody responses.

Since both N418 and FliC can bind to receptors on DC, the first question to address was whether N418 or FliC or the combination of them working together lead to the responses seen. The results indicate that the N418 antibody is the critical component necessary for this response. This is supported by the fact that N418 cross-linked to BSA induced rapid anti-BSA responses as was seen with the N418-FliC conjugate. In contrast, FliC cross-linked to HamIgG did not induce rapid antibody responses. This indicates that the conjugate binds to DC via a N418-CD11c interaction and not a FliC-TLR5 interaction (71, 206). It is unlikely that the conjugate binds to DC by Fc receptors because the target antigen is chemically linked to the Fc portion of the antibody.

The next question was whether DC or another cell type was being targeted since CD11c can also be found on monocytes/macrophages, neutrophils and NK cells (97). The original specificity studies of N418 found that it did not bind significantly to peritoneal macrophages, blood monocytes, tissue macrophages, or bone marrow granulocytes but it was able to bind to DC (127) suggesting that N418 may recognize an

epitope on CD11c that is only present when it is expressed on DC or that the expression of CD11c on these other cells is below the limit of detection. This would imply that the majority of the conjugate binds to DC *in vivo*. To test this further DEC-205, a second mAb that binds to CD205 on DC and thymic epithelial cells (100), was cross-linked to FliC and injected into mice. Once again anti-flagellar antibodies were seen within 7 days. Since the only cell type that both N418 and DEC-205 bind to is the DC, then the rapid induction of antibodies seen is most likely due to the targeting of the antigen to DC.

Since antibodies were seen in 7 days and the titer could be increased by boosting with conjugate on day 7, the next question was what boosting protocol would give the best titers by day 14. By boosting on different days it was found that the absence of a boost yielded the lowest titers and boosting on day 7 yielded the highest. Boosting on day 3, 5, and both yielded intermediate titers. The results seem to indicate that giving a boost on day 3 has some inhibitory effect to the induction of high titers of antibodies. This is seen in both the day 3 only boost group and the days 3 and 5 boost group. The nature of this inhibition is unknown at this time. Since boosting on day 5 or 7 gave very similar results, either of these days could be used to induce high titers. The ability of the conjugate to prime an immune response to an injection of flagellin was also examined. The results showed that one injection of conjugate was enough to prime the immune system when one injection of FliC was not. This indicates that injection of conjugate leads to faster priming of T cells which are needed for antibody production (97).

To clarify the protection results seen by Conwell (32), larger groups of mice were challenged in the burn mouse model. Here it was shown that the N418-FliC conjugate significantly protected the mice above all the other groups from *P. aeruginosa* challenge

($p<0.05$). The N418 mAb alone does not protect the mice ruling out non-specific activation of the immune system, and showing that N418 must be cross-linked to the target antigen in order to achieve protection. It should be noted, as previously seen (32), protection was associated with a high concentration of IgG1 indicating a Th2 response.

The last question this work examined was whether the conjugate could activate DC which would then activate T cells. After incubation with the N418-BSA conjugate, BMDC up-regulated their expression of MHCII and CD86. This up-regulation translated into these DC then being able to activate T cells in culture. BMDC exposed to BSA alone were unable to up-regulate MHCII and CD86 and they were unable to activate T cells. This shows that the N418-CD11c interaction leads to the internalization of the N418-BSA conjugate and activation of DC that are then able to stimulate T cells to produce IL-2, which is a marker of T cell activation and proliferation (97). The ability of the conjugate to activate DC to activate T cells *in vivo* is also suggested by the fact that T cells that recognize N418-BSA bearing DC could be isolated 7 days after injection of N418-BSA conjugate. In contrast, no T cells that recognized N418-BSA or BSA loaded DC were isolated from mice 7 days after BSA injection.

The information reported here along with the work done by Wykes et al and Skok et al lead to a possible *in vivo* mechanism of how the N418-FliC conjugate is able to produce rapid, protective antibody responses within 14 days. Wykes et al showed that DC are capable of retaining unprocessed antigen, which can be transferred directly to B cells. The authors also report that direct contact of DC with B cells leads to class switching of the antibody from IgM only, to IgM and IgG (202). Skok et al found that the interaction of DC, B cells and T cells drives a Th2 response (172). Thus, after

subcutaneous injection, the N418-FliC conjugate binds to CD11c bearing DC. The conjugate is taken up by the DC, which then starts to mature as it migrates to the draining lymph node. Once in the lymph node, the DC interacts with antigen-specific T cells and activates them. The DC also interacts with antigen-specific B cells, which acquire unprocessed antigen and a signal to class switch directly from the DC. Since the DC is interacting with both B cells and T cells, the T cell signals the B cell to differentiate and produce antibodies. Meanwhile, the B cell influences the differentiation of the T cell into a Th2 cell. The result of this is high titers of IgG antibodies detectable in the serum at day 7. The major isotype of this antibody is IgG1, which indicates a Th2 response. A boost on day 7 allows this cycle to repeat allowing for the activation of both naive B cells, T cells and possibly memory cells causing a significant increase in IgG titer, still of the IgG1 isotype, by day 14. At this time there is a sufficient antibody response to protect from a lethal challenge with *P. aeruginosa*. While this mechanism seems probable, more data needs to be gathered regarding the interactions of conjugate exposed DC with B cells, T cells and the cytokines produced during this interaction.

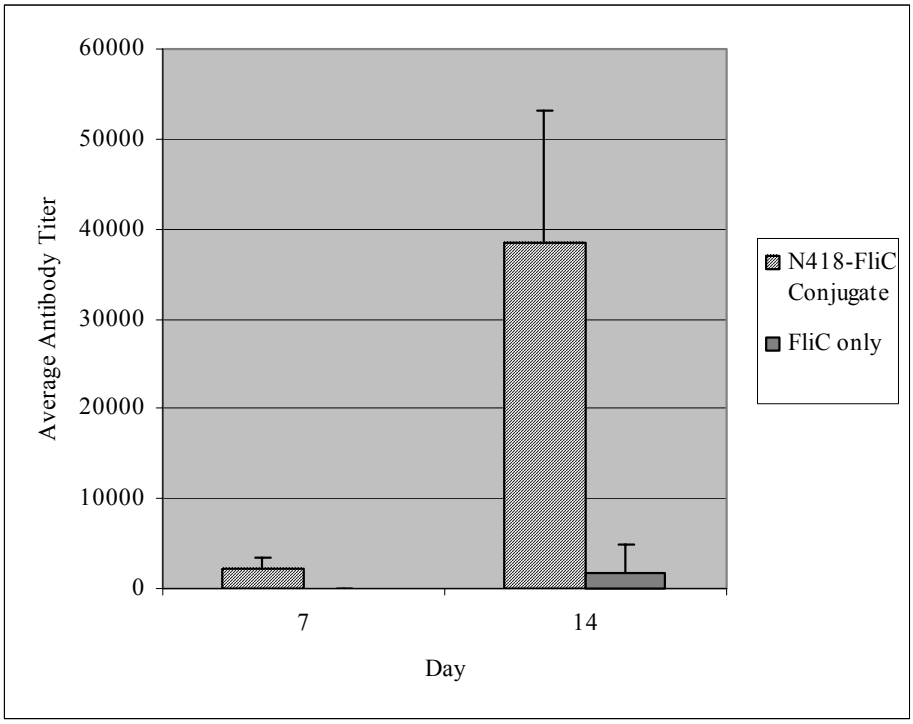


Figure 4.1. Antibody responses to the N418-FliC conjugate and recombinant FliC protein. Mice were injected on days 0 and 7 with 30 μg of either N418-FliC conjugate or FliC alone subcutaneously. On day 14 the mice were bled and the sera were tested for anti-flagellar antibodies by ELISA.

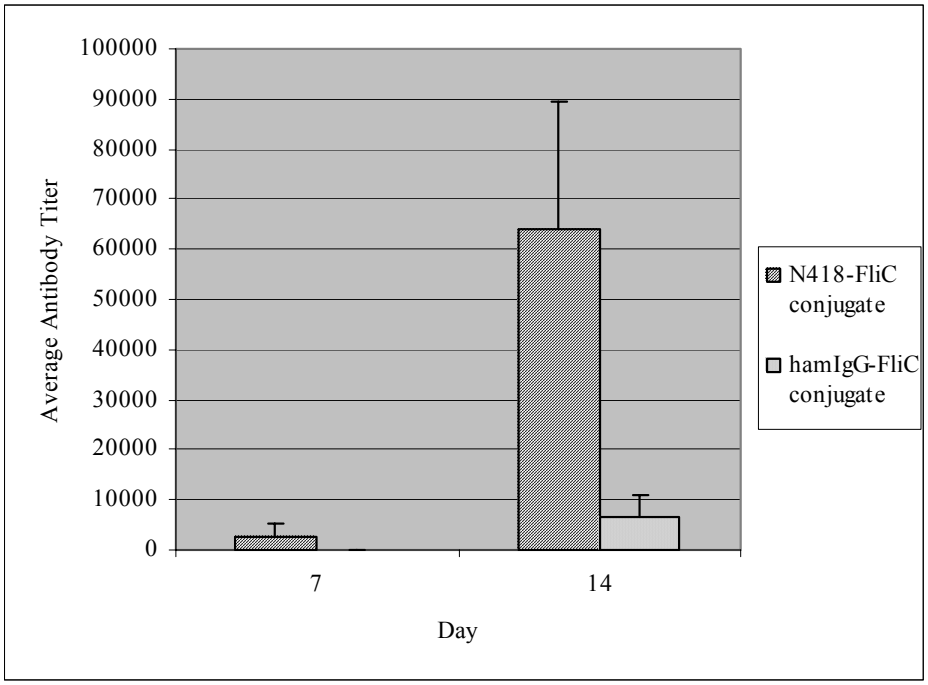


Figure 4.2 Antibody responses to the control (hamster IgG-FliC) conjugate. Mice were injected on days 0 and 7 with 30 µg of hamster IgG-FliC conjugate or N418-FliC conjugate subcutaneously. On day 14 the mice were bled and the sera were tested for anti-flagellar antibodies by ELISA.

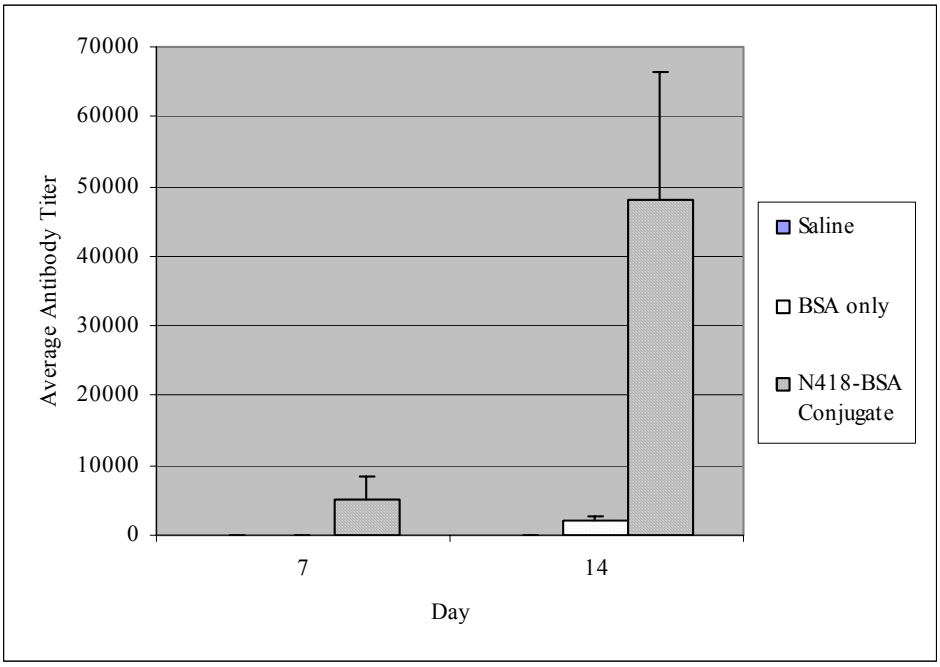


Figure 4.3. Antibody responses to the N418-BSA conjugate. Mice were injected on days 0 and 7 with 30 µg of N418-BSA conjugate, BSA alone or saline subcutaneously. On day 14 the mice were bled and the sera were tested for anti-BSA antibodies by ELISA.

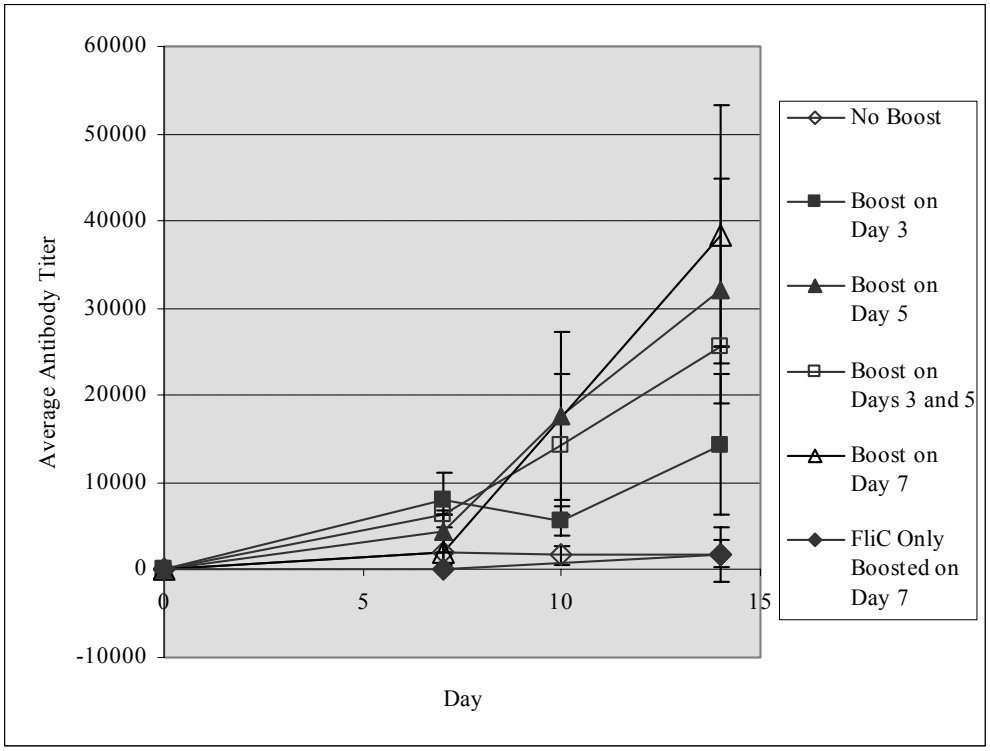


Figure 4.4. Boost response of Balb/c mice. Mice were injected with 30 μg of N418-FliC conjugate or FliC alone subcutaneously on day 0 and boosted on the indicated day. Mice were bled on days 7, 10 and 14 and the sera were analyzed for anti-flagellar antibodies by ELISA.

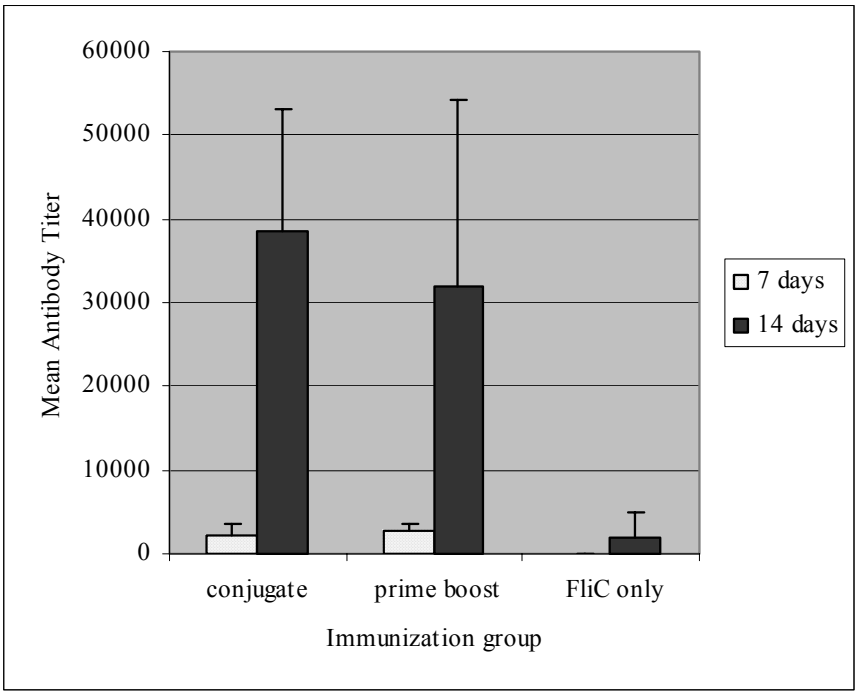


Figure 4.5. Prime-boost response in Balb/c mice. Mice were injected with 30 µg of N418-FliC conjugate or FliC on day 0 subcutaneously. On day 7 conjugate primed mice were boosted with 30 µg of N418-FliC conjugate (conjugate) or FliC (prime boost), FliC primed mice were boosted with FliC (FliC only). On day 14 the mice were bled and the sera were analyzed for anti-flagellar antibodies by ELISA.

	Mortality [dead/total (%)]				
Treatment[c]	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
Saline	0/37 (0)	24/37 (65)	37/37 (100)	37/37 (100)	37/37 (100)
N418	0/39 (0)	16/39 (41)	33/39 (85)	33/39 (85)	33/39 (85)
FliC	0/25 (0)	18/25 (72)	18/25 (72)[a]	18/25 (72)[a]	18/25 (72)[a]
N418-FliC	0/25 (0)	6/25 (24)[b]	9/25 (36)[b]	10/25 (40)[b]	10/25 (40)[b]

[a]Significantly more protective than saline, $p<0.05$

[b]Significantly more protective than either N418 alone or FliC alone, $p<0.05$

[c]CF-1 mice were immunized on days 0 and 7 with 30 μg of N418-FliC conjugate, N418 alone, FliC alone or saline. On day 14, animals were challenged with a lethal dose of *P. aeruginosa* in the burn mouse model. The data represent combined results from 3 experiments.

Table 4.1. Mortality of mice after challenge with *P. aeruginosa* strain M-2.

Treatment	Average Titer	IgG1 (µg/ml)	IgG2a (µg/ml)
FliC only[a]	47,360	256	10.7
N418-FliC[a]	160,000	587	27.2

[a]Separate groups of CF-1 mice were injected, as for the challenge, to assess anti-flagellar antibody titer and isotype

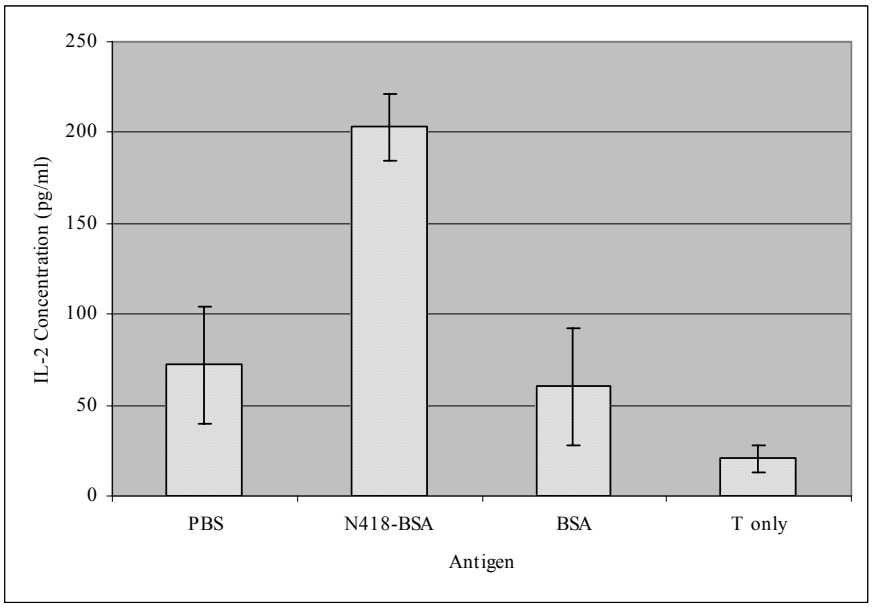
Table 4.2. Titer and isotype of sera on day 14 from challenge mice.

Treatment[a]	MHCII positive (%)[b]	CD86 positive (%)[b]
PBS	20.58	12.43
TNFα	33.59	32.32
N418-BSA	53.02	54.21
BSA	17.13	8.82
HamIgG	24.14	18.15

[a]BMDC were cultured as in Materials and Methods. Cells were incubated with 500 U/ml TNFα or 1 μg/ml of N418-BSA conjugate, BSA or hamster IgG for 24 hr. After incubation the cells were washed and stained for FACS analysis.
[b]Cells were gated on $CD11c^+$ cells

Table 4.3. *In vitro* activation of BMDC.

A



B

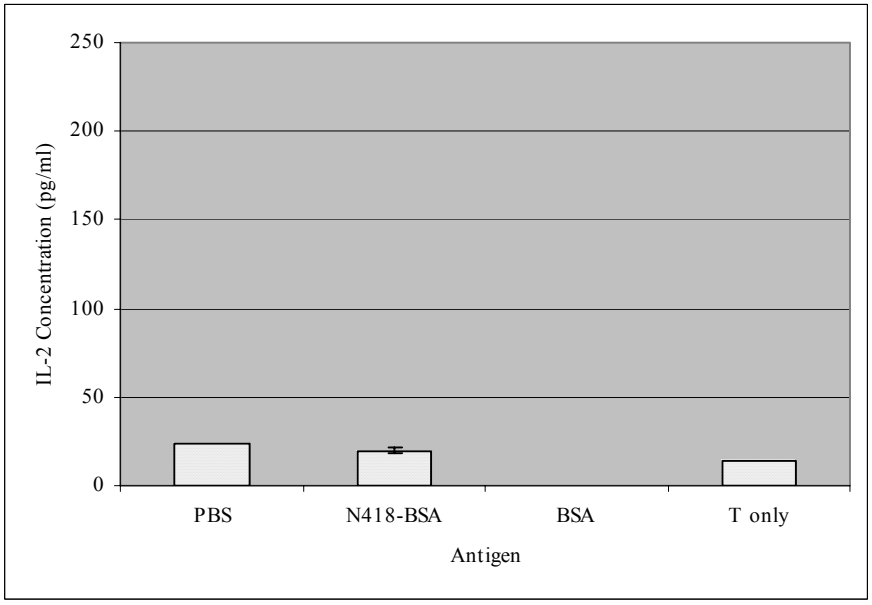
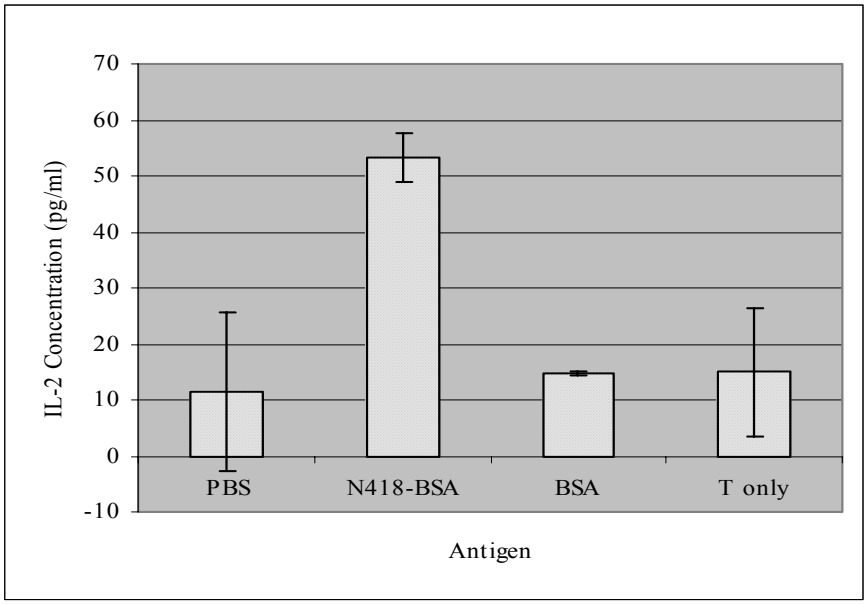


Figure 4.6. Antigen presentation assay. Mice were injected with 30 µg of N418-BSA (A) or BSA (B) on day 0 subcutaneously. On day 7 the draining lymph nodes were harvested. BMDC were cultured for 8 days and then incubated with PBS, N418-BSA or BSA for 24 hr. DC and T cells were mixed at a ratio of 1:1000 and incubated for 72 hr. After incubation the supernatants were tested for IL-2 concentration by ELISA. As a control T cells were incubated in medium alone.

A.



B.

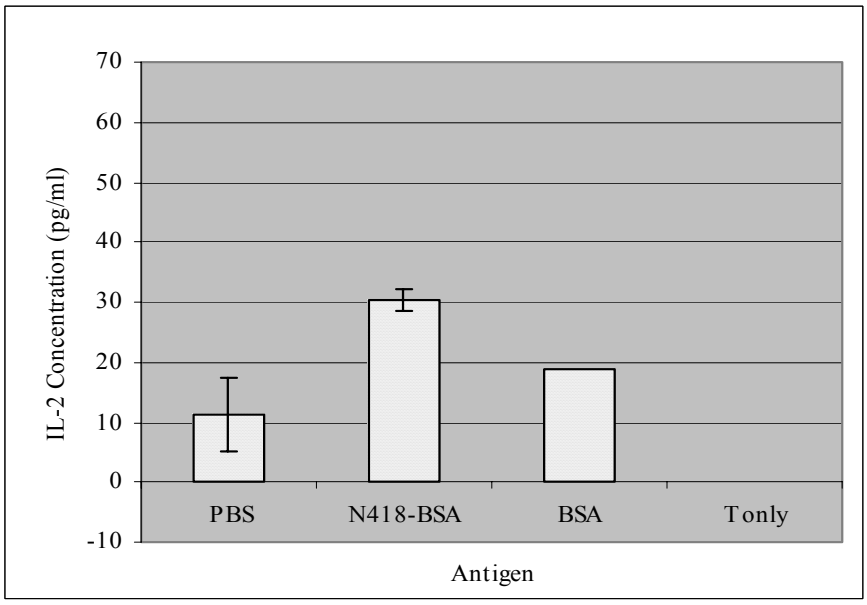


Figure 4.7. Antigen presentation assay. Mice were injected with 30 µg of N418-BSA (A) or BSA (B) on days 0 and 7 subcutaneously. On day 14 the draining lymph nodes were harvested. BMDC were cultured for 8 days and then incubated with PBS, N418-BSA or BSA for 24 hr. DC and T cells were mixed at a ratio of 1:2000 and incubated for 72 hr. After incubation the supernatants were tested for IL-2 concentration by ELISA. As a control T cells were incubated in medium alone.

SUMMARY

Pseudomonas aeruginosa is a versatile opportunistic pathogen that can infect every tissue in the body. Treatment of these infections is quite difficult due to the inherent resistance of this organism to most antibiotics. Since antibiotic therapy is so difficult, vaccination to stimulate the immune system to fight off this organism is a desirable alternative. Typically *P. aeruginosa* only infects immunosuppressed patients so vaccination of the whole population is unnecessary and impractical. A vaccine that can be administered to at risk populations and that can stimulate protective immunity quickly with few injections would be ideal. Traditional protein based vaccines usually require several injections and protective immunity is not obtained until weeks or even months after the first vaccination is given. Traditional protein based vaccines also typically induce strong humoral responses but do not stimulate strong cell-mediated immune responses which may also play a role in the eradication of the infection so different vaccination strategies need to be explored.

The choice of antigen employed for vaccination is quite important. *P. aeruginosa* produces several virulence factors that could be possible vaccine candidates. A good vaccine candidate will elicit protection against all strains of *P. aeruginosa*, will be expressed when the organism is first invading the host, and will be non-toxic to the host. The flagellum of *P. aeruginosa* meets most of these criteria. There are only 2 types of flagella in this organism, type A and B, and a vaccine incorporating both provides

protection against all strains. Flagella are expressed when *P. aeruginosa* is invading the host and are accessible to the immune system. Lastly, injection of purified flagella is non toxic to the host.

The studies presented in this work examined different strategies to elicit protective humoral immune responses against *P. aeruginosa* using flagella as the target antigen. In the first study the efficacy of using a DNA vaccine encoding the type A *fliC* gene was explored. Here two different DNA vaccine constructs were examined. The first construct contained the *fliC* gene from strain PAK, a type A strain, cloned into the eukaryotic expression vector pVR1020. Injection of the construct into mice elicited significantly higher anti-flagellar antibody titers than vector immunized mice; however this was not protective upon challenge in a burn mouse model. The second construct contained the *fliC* gene from strain SBI-N, a type A strain, cloned into pVR1020. Sera from mice injected with this construct were unable to inhibit the motility of type A *P. aeruginosa* which is associated with protection, suggesting that these sera would not be protective to *P. aeruginosa* challenge. Closer analysis of the sera from mice immunized with these two showed that the antibodies elicited by both groups had higher affinity to PAK flagella over SBI-N flagella. Type A flagella are glycosylated by *P. aeruginosa*. Polyacrylamide gel analysis shows that SBI-N flagella have a larger molecular weight than PAK flagella suggesting that SBI-N flagella are more heavily glycosylated. The flagellins expressed by DNA vaccines are not glycosylated as suggested by their smaller molecular weight as seen by immunoblot analysis. This lack of glycosylation may be the reason protective immunity was not obtained with these two constructs. Preliminary studies showed that antibodies from DNA-immunized mice preferentially bound to

deglycosylated flagella preparations where SBI-N flagella-immunized mice produced antibodies that bound to untreated flagella better. While this preliminary result is far from being conclusive, it warrants further investigation into the function of glycosylation of flagella in pathogenesis and immunity. Collectively the results from this study suggest that target antigens that are post-translationally modified by the organism may not be good candidates for genetic immunization especially if the modification is part of the protective epitope.

The next vaccination strategy examined the ability of a T cell binding ligand to promote a Th2 response in a DNA vaccine. Previous studies suggested that a Th2 response and the production of IgG1 antibodies were necessary for protection against *P. aeruginosa*. This type of response is typically seen after gene gun injection of a DNA vaccine but not after intramuscular injection. Gene gun injections however are impractical for widespread use due to the cost of gold and the equipment required. A better strategy would be to achieve a Th2 response after intramuscular immunization. A novel method of directing immune responses, using peptides, is the fusion of an antigenic peptide to a T cell binding ligand peptide. This was adapted to a DNA vaccine by cloning the DNA sequence for amino acids 135-149 from the β2 region of MHCII in front of the type B *fliC* gene from strain PAO1 into the eukaryotic expression plasmid pcDNA3.1. This construct was injected intramuscularly into mice to test its ability to promote a Th2 response. Separate groups of mice were injected intramuscularly with a construct containing the type B *fliC* gene cloned into pcDNA3.1 or with the pcDNA3.1 alone in order to compare the humoral immune responses elicited. Sera were analyzed by ELISA for total IgG, IgG1 and IgG2a to indicate the type of immune response induced.

While the vector immunized mice did not produce significant titers of antibodies, comparison of the other two groups showed no difference in their antibody titers of isotypes indicating that this T cell binding ligand was unable to promote a Th2 response when fused to the whole FliC protein. The most probable reason for this was that a whole protein was fused to the ligand instead of a peptide. After expression the protein could have folded in such a way to bury the peptide or it is possible that this system will only work with peptide antigens. While this study was the first attempt to use this method of immune response modulation in a DNA vaccine further studies of this method using peptide DNA sequences in genetic immunizations are justifiable.

The last study examined *in vivo* dendritic cell (DC) targeting. *P. aeruginosa* infections do not occur in healthy individuals, so vaccination of the entire population is not necessary. *P. aeruginosa* is a major nosocomial pathogen that can quickly establish an infection in immunosuppressed individuals. A vaccination strategy that induces rapid immune responses could greatly reduce the incidence of *P. aeruginosa* related disease in this at risk population. Previous work showed that N418, a hamster monoclonal antibody (mAb) specific for CD11c on DC, chemically conjugated to purified recombinant type B flagellin (FliC) from strain PAO1, could enhance the humoral immune response to this antigen resulting in the production of antibodies 7 days after it was administered. This study expanded on this previous work. In order to determine which component of the N418-FliC conjugate caused the rapid induction of antibodies, two different conjugates that replaced either N418 or FliC were constructed. The first conjugate consisted of purified hamster IgG cross-linked to FliC. This conjugate was unable to stimulated high titers of anti-flagellar antibodies like the N418-FliC conjugate 7 days after injection. The

second conjugate consisted of N418 cross-linked to bovine serum albumin (BSA). This conjugate was able to induce high anti-BSA titers 7 days after being injected into mice. Together these results indicate that the presence of N418 in the conjugate is necessary for the rapid antibody responses observed. This is probably due to the ability of N418 to bind to DC, thus targeting the antigen to them. This was confirmed by the used of a third conjugate consisting of DEC-205 cross-linked to FliC. DEC-205 is a rat mAb that binds to CD205 on DC. Injection of this conjugate also induced high titers of antibody by day 7. The results obtained from the injection of the N418-BSA conjugate also suggest that the rapid induction of antibody is not unique to the N418-FliC conjugate but that conjugation of any antigen to N418 could enhance the humoral response to that antigen. Different immunization protocols were also examined. In the first study groups of mice were injected with the N418-FliC conjugate on day 0 and then boosted with conjugate at different times. Boosting on day 3 resulted in the lowest antibody titers of all the groups that were boosted, perhaps indicating that boosting on this day may inhibit the enhancement of the humoral response. Giving a second injection on day 7 resulted in the highest anti-flagellar titers but these titers were not significantly higher than titers seen after boosting on day 5. These results suggest that in order to achieve maximal production of antibodies a booster injection should not be given until day 5 or 7. The next study looked at the ability of the N418-FliC conjugate to prime a response that could be boosted with FliC alone. Here mice were injected initially with conjugate and then boosted on day 7 with either conjugate or FliC alone. The antibody titers, on day 14, were similar in both groups. Another group of mice primed and boosted with FliC alone produced significantly less anti-flagellar antibody than the groups primed with N418-

FliC. This indicates that targeting the antigen to DC primes the immune system more efficiently than untargeted antigen. Next, to assess the protective efficacy of the antibody response observed in conjugate immunized mice, a challenge study was conducted. Mice that were immunized with N418-FliC on days 0 and 7 before being challenged on day 14 in a burn mouse model of lethal *P. aeruginosa* infection were significantly protected. This protection was associated with high anti-flagellar antibody titers mainly of the IgG1 isotype. Finally, the mechanism of how the N418 containing conjugates were able to induce such rapid humoral responses was studied. *In vitro* the N418 conjugate was able to stimulate DC to up regulate their expression of both MHCII and CD86, indicating that it could activate these cells which would then be able to stimulate T cells. This was confirmed by co-culturing DC, exposed to the N418 conjugate, with primed T cells isolated from immunized mice. The activated DC stimulated the T cells to produce higher amounts of IL-2 than DC exposed to PBS or unconjugated antigen. These co-culture experiments also indicate that injection of the N418 conjugate can rapidly prime T cell *in vivo*. At 7 days after injection, T cells isolated from conjugate primed mice produced large amounts of IL-2 upon restimulation in culture. T cells isolated from unconjugated antigen primed mice did not produce IL-2 after restimulation in culture. These results suggest that targeting antigen to DC, via the N418-CD11c interaction, stimulate DC to mature and migrate to local lymph nodes where they interact with and stimulate B and T cells. This results in the rapid production of high titers of protective antibodies.

The overall goal of the work presented here was to investigate different vaccination strategies that would enhance the humoral immune response in order to

provide protection against acute *P. aeruginosa* infections. While the genetic immunization studies were not efficacious, significant protection was observed with the N418 DC targeting method. Since this method was able to induce such a rapid response it would be interesting to test the therapeutic efficacy of this vaccine in infected hosts. I would also be interesting to see if the N418 conjugate can induce mucosal immunity by either systemic injection or mucosal delivery. Due to the ability of different N418 conjugates to induce rapid titers of antibodies against different target antigens, this vaccination strategy may have enormous potential in the prevention or treatment of several nosocomial infections.

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