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TLR4 adjuvanted vaccine against Pseudomonas aeruginosa virulence factor, Pf bacteriophage, induces increased immunoglobulin response and highly mutated antigen-specific B-cell receptors

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Abstract

Pseudomonas aeruginosa is listed as a priority 1 pathogen by the World Health Organization. Although this Gram-negative bacterium is ubiquitous and can naturally be found in the environment, it is opportunistic. *P. aeruginosa* has a wide range of host immune evasion strategies; one such strategy is the establishment of biofilms. Pf bacteriophage, a *P. aeruginosa* virulence factor, can strengthen the integrity of biofilms and can also misdirect the host immune response from an appropriate anti-bacterial response to an anti-viral response. Previous studies have established that antibodies against Pf bacteriophage can enhance clearance of *P. aeruginosa* infections. Next generation adjuvants such as TLR adjuvants have the ability to increase the magnitude of antigen-specific antibodies and generate a lasting immune response against pathogens. In this dissertation, we hypothesized incorporation of a TLR4 agonist will result in an enhanced immune response against the Pf bacteriophage. Herein, we demonstrate antibodies generated from the optimized vaccine are functional and able to recognize Pf virions and disrupt phage replication cycle. Additionally, we have optimized a Pf-bacteriophage-adjuvanted vaccine which incorporates antigen from two distinct clades and optimized a Pf-bacteriophage-adjuvanted vaccine for intranasal administration. Vaccination lead to mucosal immune response and broadened antibody response. Furthermore, after isolating antigen-specific B-cells we used single-cell sequencing techniques to explore the adjuvant effects on the humoral response. We discovered adjuvants affect clonality and heavy chain mutation accumulation in antigen specific B-cell population.

Chapter 1: Introduction

1.1 Brief history of vaccines

Vaccination is regularly featured as one of the Ten Greatest Public Health Achievements. It is one of the major reasons why the global life expectancy age has improved from just 49 years in 1955 to 66 years by 2000[1],[2]. More recently, the public health tool of vaccination was indispensable during the first couple of years of the SARS-CoV-2 pandemic [3]. As early as 200 BCE, peoples from early civilizations were exposing healthy individuals to smallpox in the hopes to prevent succumbing to the disease[4]. This process, known as variolation, was a continued practice globally in places like Turkey, China and India and then later was introduced to Europe[5][4]. The process included taking pustules from an individual with smallpox and rubbing the contents in an open wound in a healthy individual; the individual exposed to smallpox would then later have a mild case of smallpox when re-exposed [5]. In 1796 a young scientist by the name of Edward Jenner heard about the well-known phenomenon in which dairy-maids exposed to cowpox were naturally protected against smallpox. He later took a pustule from a dairy-maid named Sarah Nelms and inoculated a boy named James Phipps. James Phipps became mildly sick after the inoculation but made a full recovery. To prove that the James Phipps was protected from smallpox, Edward Jenner then took a pustule from a person infected with smallpox and inoculated James Phipps once more. This time, James Phipps did not become sick, and was fully protected against smallpox; thus, the first vaccine was born [6]. By the 1900's other scientists created the first successful vaccines against rabies and typhoid, and by the end of that century vaccines against pertussis, yellow fever, and polio were created [4]. Through a global public health campaign, smallpox was finally eradicated in 1977, and remains the only eradicated human disease in history [6].

1.2 Brief history of adjuvants

The first developed vaccines were primarily from live-attenuated or whole-inactivated pathogens [7]. The live-attenuated based vaccine contains viable pathogens which are manipulated in a way that renders them less virulent [7]. Whole-inactivated pathogen vaccines, on the other hand, contain pathogens which

are inactivated using methods such exposing the pathogen to high temperatures [7]. Although whole-inactivated vaccines and live-attenuated vaccines can create a robust immune response they also can pose a risk of incomplete attenuation or incomplete inactivation of the pathogen and cause disease [7]. Purified pathogen-derived proteins are an attractive alternative to whole-inactivated or attenuated pathogen vaccines [7]. Although protein subunit-based vaccines can be a safer alternative, the downside is that immunogenicity can be substantially decreased resulting in reduced immune protection [7]. This reduced immunogenicity can be in part because subunit vaccines lack pathogen-associated molecular patterns or PAMPS [7]. Vaccine additives known as "adjuvants" can trigger the innate immune response to the sub-unit protein and result in the mounting of an effective and lasting immune response [7]. Adjuvants were first discovered by a veterinarian named Gaston Ramon. Dr. Ramon made the observation that animals vaccinated against tetanus or diphtheria and had abscess at the site of injection also had higher titers of tetanus and diphtheria-specific antibodies compared to vaccinated animals without abscess formation. Having made this realization, he would vaccinate animals against diphtheria or tetanus inactivated-toxin and include tapioca or breadcrumbs to encourage the formation of sterile abscesses at the site of injection. This local inflammation resulted in increased titers of diphtheria toxin or tetanus toxin specific-antibodies in the sera [7]. During the same time period, Alexander Thomas Glenny was attempting to purify tetanus toxoid using potassium aluminum sulfate and made the discovery that vaccination using the potassium aluminum sulfate purified tetanus toxoid created higher tetanus antibody titers than soluble tetanus [8]. Since Alexander Thomas Glenny's discovery, alum has been extensively used as the adjuvant for vaccines such as tetanus, pertussis and Hepatitis B. The newest generation of adjuvants developed and approved for human use, leverage the innate immune system by targeting specific pattern recognition receptors (PRRs) such as toll-like receptors or TLRs [8].

1.2.1 TLR4 and TLR4 agonists

The immune system can be largely divided into the innate and the adaptive response[9]. While the adaptive response functions by generating memory against specific pathogens, the innate response functions

to respond quickly and non-specifically to invading pathogens [9]. Another distinct difference between the innate and the adaptive immune response is antigen receptors from the innate arm of the immune system are encoded in the germline and the antigen receptors from the adaptive response are generated somatically [9]. PRRs are receptors from the innate immune response which recognize a broad array of different proteins or molecules such as PAMPs, and initiate the innate immune response [9]. One such family of PRRs is the Toll-like receptors or TLRs, which were first identified in *Drosophila* [9]. TLRs have a signaling domain and an external domain which recognizes specific microbial ligands [9]. TLRs and other PRRs not only recognize specific microbial ligands and trigger downstream innate immune responses but also serve as a nexus to the adaptive response by facilitating the appropriate immune response to specific pathogens [9]. One such well-studied and well characterized TLR is TLR4 which naturally recognizes lipopolysaccharides or LPS from Gram-negative bacteria [10]. The structure of LPS can be divided into three components: lipid A, the core sugars (e.g., 3-deoxy-D-manno-oct-2-ulosonic acid) and O-antigen [10]. The complex of surface protein Myeloid Differentiation factor 2 (MD-2) along with TLR4 and LPS triggers the downstream intracellular signaling leading to the production of cytokines and other inflammatory mediators [10]. Lipid A has been discovered as the bioactive component of LPS. However, Lipid A as an adjuvant is extremely toxic. Further modification of lipid A in the form of 3'-deacylated monophosphoryl lipid A (MPL), demonstrated to in the triggering of T helper responses and increasing other immune responses while also limiting inflammatory toxicity [11]. Because of these adjuvant immunomodulatory properties, MPL is a component in multiple vaccines approved for human use [12]. Other lipid A mimetics such aminoalkyl glucosaminide-4-phosphates (AGPs), which contain a monosaccharide instead of a disaccharide, have shown promise as TLR4 agonists [11]. Animals treated with AGPs prior to influenza challenge have reduced viral loads compared to untreated animals, and AGP's protective qualities have been demonstrated in bacterial challenges as well [11]. INI-2002, another synthetic TLR4 agonist is a next-generation adjuvant, which has the ability to augment antigen--specific antibodies and generate a cell-mediated response (Chapter 2) while also maintaining robust structural integrity compared to AGPs.

1.3 Conjugate vaccines and linked recognition

1.3.1 Conjugate vaccines

It was established that adults had the ability to generate an immune response against purified *Haemophilus influenza* type b (Hib) capsular polysaccharides, but unfortunately high-risk groups such as children were unable to generate immunity against the polysaccharide antigen [13]. For this reason, researchers developed the first glycoconjugate vaccine [13]. The first conjugate vaccine approved for human use was in 1987 and was a vaccine created to target Hib bacterial infections which can result in meningitis and lead to death [14]. This vaccine is comprised of capsular polysaccharides conjugated to the protein carrier tetanus toxoid [14]. The conjugation of purified polysaccharides to a larger carrier protein results in highly immunogenic antigen, and this technology has been applied to the development of vaccines against other bacteria [13]–[15].

1.3.2 Linked recognition

There are two major ways to induce activation of B-cells, T-cell independent and T-cell dependent. During the T-independent process, an antigen such as a polysaccharide will crosslink the surface B-cell receptors [16]. Although T-cell independent activation results in a rapid response against the invading pathogen, B-cells activated through this response will not typically result in a robust immune response against the antigen because activated B-cells will not undergo immunological processes such as somatic hypermutation [16]. The T-cell dependent activation of B-cells relies on linked recognition, one of the central immunology dogmas [16]. Linked recognition is the process in which a B-cell and a T-cell respond to the same antigen [16]. In this process, the B-cell presents a peptide from the antigen onto its major histocompatibility complex class II (MHC II), and the T-cell receptor (TCR) recognizes and binds to the peptide-loaded MHC II [16]. The linking of the MHC II with the TCR and other accessory surface proteins such as CD40 to CD40 ligand, leads to a downstream effect in which the T-cell releases cytokines which

initiates B-cell proliferation and isotype switching [16]. Although B-cell and T-cell response to the same epitope is required for linked recognition to occur, the B-cell and T-cell do not necessarily need to recognize the same epitope [16]. The B-cell receptor can bind to an epitope on the outer portion of the antigen, endocytose the pathogen, and load a peptide from the inner portion of the antigen onto its MHC II. A T-cell specific for the peptide from the inner portion of the antigen will then interact with the B-cell and release cytokines and other activation signals which then will lead to the proliferation of the B-cell and release of antibodies specific for the outer-membrane (**Figure 1-1 A,B**) [16].

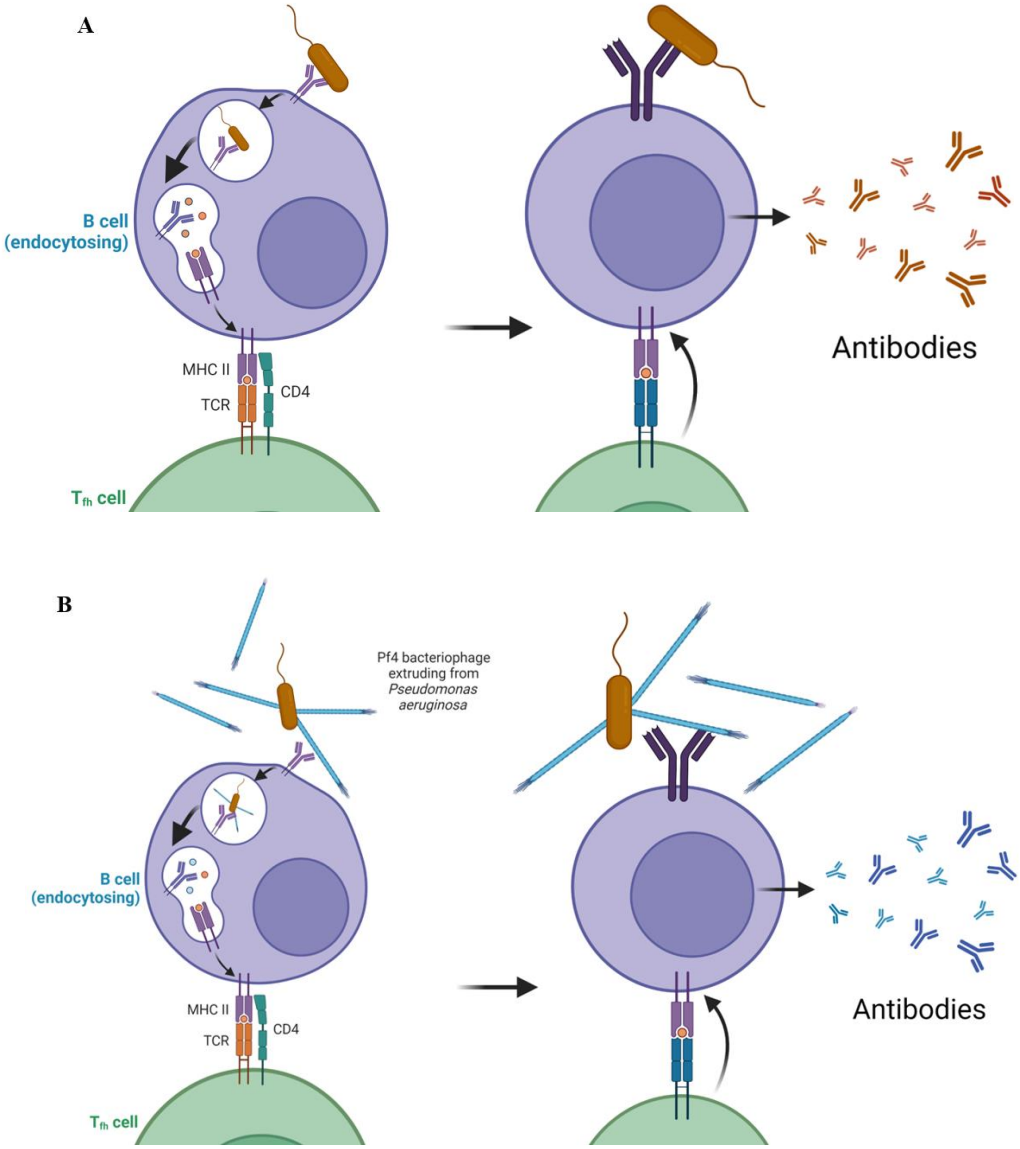

Figure 1-1 Schematic of two examples of linked recognition. In panel A, the B-cell receptor binds to an outer epitope on the *Pseudomonas aeruginosa* bacterium. The B-cell presents its MHC II loaded with a peptide from the digested *P. aeruginosa* bacterium. The T-cell receptor also recognizes the same epitope, and activates the B-cell resulting in the production and release of *P. aeruginosa*-specific antibodies. In panel B, the B-cell receptor binds to an outer epitope on the Pf bacteriophage extruding out of the *P. aeruginosa* bacterium. The B-cell endocytoses both the bacterium and the extruding bacteriophage, but presents its MHC II loaded with a peptide from the digested *P. aeruginosa* bacterium. The T-cell receptor recognizes the same epitope, and activates the B-cell resulting in the production and release of Pf bacteriophage-specific antibodies.

Studies involving haptens conjugated to a carrier protein demonstrated linked recognition and how conjugate vaccines can recruit T-cell help [13], [16].

1.4 B-cells

While the innate immune response is largely non-specific and fast acting, this branch of the immune response does not have memory capabilities [17]. The adaptive immune response on the other hand, is specific to pathogens and has the ability to generate memory against pathogens [17]. Within the arm of the adaptive immune response, there exists the cell-mediated adaptive immune response and the humoral response [17]. The cell-mediated response is primarily comprised of CD8 T lymphocytes and CD4 T lymphocytes, while the humoral response is primarily comprised of B lymphocytes [17]. Interactions between T lymphocytes or T-cells and B lymphocytes or B-cells results in the activation of B-cells, and this activation can lead to the generation of memory B-cells or antibody producing B-cells [17]. Antibodies are soluble proteins excreted from B-cells and have multiple effector functions against invading pathogens [17].

1.4.1 Development and maturation of B-cells

B-cells first derive from a pluripotent hematopoietic stem cell and can develop into B1 cells or B2 cells [17]. B1 cells are generated in the fetal liver and, in mice, largely reside in the peritoneum [18]. B1 cells are typically activated in a T-cell independent fashion and largely produce polyreactive IgM and IgA antibodies [19]. B1 cells are thought to assist with immune homeostasis, and antibodies generated from B1 cells play an important role in protecting against autoimmunity [18]. B2 cells on the other hand can be

further divided into marginal zone B-cells and follicular B-cells. Development of these B-cells starts in the bone marrow where they first differentiate into pro B-cells followed by the transition into pre B-cells and finally exit as immature B-cells. In the bone marrow the B-cells go through the first of several selection processes known as central tolerance [20]. It is estimated that up to 75% of developing B-cell are auto-reactive, and it is during the process of central tolerance that these auto-reactive B-cells further modify their B-cell receptor or are deleted [19], [20]. Once the immature B-cell leaves the bone marrow it travels to the spleen where it undergoes positive selection and differentiates into marginal zone B-cells or into follicular B-cells. B-cells which differentiate into marginal zone B-cells receive a weak signal through their B-cell receptor which leads to expression of neurogenic locus notch homolog protein 2 resulting in the differentiation of the B-cell [19]. Marginal zone B-cells are very similar to B1 cells except they also produce T-cell-dependent antibodies in addition to T-cell-independent antibodies. If the immature B-cell receives a strong signal through its BCR, Bruton tyrosine kinase becomes induced and results in differentiation into follicular B-cells [19]. While marginal zone B-cells remain in the spleen, follicular B-cells may exit the spleen and travel to other tissues such as Peyer's patches in the gut and lymph nodes [19]. After differentiation, marginal zone and follicular B-cells can become activated. Marginal zone B-cells will primarily become activated via antigen crosslinking of B-cell receptors and toll-like receptor signaling [19]. This engagement leads to differentiation into memory cells or short-lived IgM-producing plasmablasts [19]. Follicular B-cells become activated by binding antigen followed by presenting antigen peptides on MHC II molecules and engaging with T-cells. Engagement of the B-cell receptor in combination with receiving T-cell help leads to the differentiation of B-cells and proliferation [19].

1.4.2 VDJ Recombination

An aspect critical to the development of a B-cell is the process known as variable (V), diversity (D), joining (J) recombination or V(D)J recombination of the B-cell receptor genes. The B-cell receptor is comprised of a heavy chain which includes VDJ segments, and the light chain which includes V and a J segment. It is through the process of VDJ recombination that results in the highly diverse B-cell repertoire

of the adaptive humoral response [20]. One reason why VDJ recombination results in the diversification of immunoglobulins is because each gene segment has multiple copies [21]. In humans for example there are approximately 45 gene copies of the variable heavy gene, 23 of the diversity heavy chain gene, 6 of the joining heavy chain gene and 9 of the heavy chain gene [21]. At random, one of each gene segments combines with each other to form rearranged V(D)J joined gene segments. The gene segments are then spliced and translated into polypeptide chains and arranged to form either the heavy or light chain [21]. Each gene segment is flanked with a recombination signal sequence which contains a heptamer non-coding nucleotide acid sequence followed by a spacer of either 12 or 23 base pair nucleotides followed by a nonamer non-coding nucleotide sequence [21]. When the gene segments are rearranged and joined together, they are done so with the 12 base pair spacer joining the 23 base pair spacer [21]. This recognition and joining of spacer 12 and 23 are done by proteins RAG1 and RAG2 [21]. Other proteins then cut the DNA segment and ligate the two gene segments together [21]. V(D)J recombination occurs in the bone marrow during the pro B-cell phase of the cell. In the developing B-cell the heavy chain genes recombine first; the D and J segments join together followed by the (D)J combining with the V segment [21]. Once these segments successfully combine and produce a heavy chain, the newly formed receptor is checked for autoreactivity [21]. The B-cell then enters the preB-cell phase where the V segment combines with J segment and forms the light chain [21].

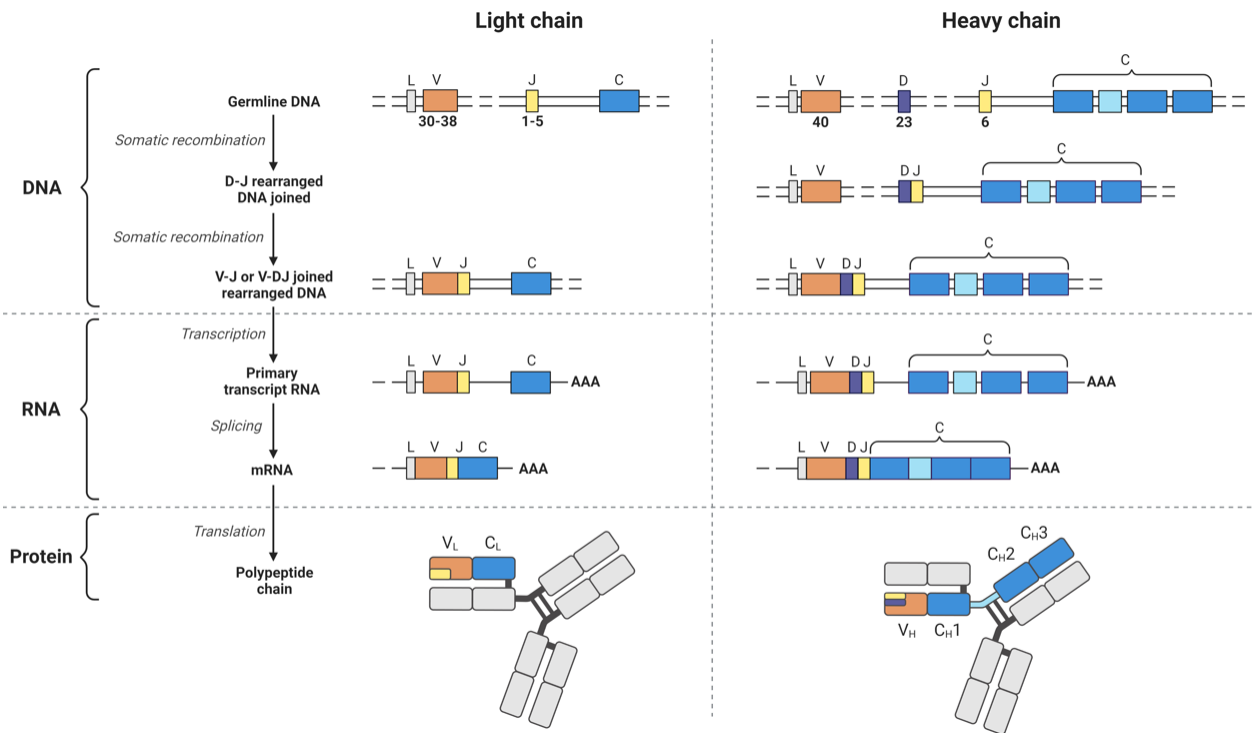

Figure 1-2: Variable region rearranged in the heavy and light chain of immunoglobulin. Schematic of V(D)J recombination of both variable regions in the heavy and light chain. In the heavy chain on of the 23 D-gene segments is combined with one of the six J-gene segments. The combined DJ-gene segments are then joined with one of the 40 V-gene segments. The combined V(D)J segments are then combined with a constant-gene segment and translated into a heavy chain peptide. This same process is repeated in the light chain, although the light chain does not contain a D-gene segment [22].

1.4.3 Germinal Centers

Once immature B-cells leave the bone marrow, they have 24 hours to enter a follicle in a secondary lymphoid tissue and encounter a T-cell, or they will undergo apoptosis [23]. It is in the secondary lymphoid tissue where the immature cell transitions into a mature naïve B-cell and upregulates the cell surface protein CD21 [24]. The mature B-cell will also express CXCR5 which is the receptor for the chemokine CXC13 (expressed by follicular dendritic cells) [25]. The follicular dendritic cells present antigen on their surface, and when the B-cell migrates to the follicular dendritic cells, it will then "sample" the antigens presented on the follicular dendritic cells [25]. The B-cell will then endocytose the antigen and migrate towards the

area in the secondary lymphoid organ where the T-follicular helper cells reside [25]. Through the phenomenon of linked recognition, the T-cell (which responds to the same antigen as the B-cell) will then release cytokines that result in the activation of the B-cell [23]. The T-cell and B-cell then migrate to a primary follicle where the B-cell starts to proliferate [26]. The proliferating B-cells will then push out resting cells into an area known as the mantle zone [26]. These proliferating B-cells ultimately form a germinal center which is comprised of the light zone and the dark zone [26]. The light zone contains follicular dendritic cells, which enhance survival of the B-cell, as well as T-follicular cells [27]. In addition to T-follicular cells in the light zone of germinal center, there are also a few CD8-positive T-cells which clear cells infected with virus as well as B-cell lymphomas in the germinal center [27]. The dark zone, on the other hand, is more homogenous in regards to distinct cell populations, for it is mainly comprised of tightly packed proliferating B-cells and few reticular cells [27]. Surrounding the light zone and the dark zone of the germinal center are tingible body macrophages which phagocytose B-cells which have undergone apoptosis [27]. Although there is an increase in proliferating B-cells, the germinal center size, once developed, is fairly stable; this is because there is an approximate equal rate of B-cells undergoing apoptosis [27]. The proliferating centroblasts (germinal center B-cells in the dark zone) eventually enter the growth phase becoming centrocytes (germinal center B-cells in the light zone) and make their way to the light zone[25]. If the B-cell has low affinity to antigen it will most likely undergo apoptosis, but if they receive T-cell help they may exit as memory cells. If the B-cell has a higher affinity for antigen it will make its way back to the dark zone where it can proliferate and undergo the process of somatic hypermutation [28]. The process of somatic hypermutation results in the accumulation of point mutations in the variable region of the B-cell receptor [21]. Most B-cells that undergo somatic hypermutation will undergo apoptosis as the mutations will result in a non-functional B-cell receptor [27]. However, the accumulation of mutations can also lead to higher affinity for antigen. Once the B-cell undergoes somatic hypermutation it will then travel back to the light zone and compete with other B-cells for antigen and T-cell help. If the mutations result in a higher affinity for antigen, then the B-cell can migrate back into the dark zone and either proliferate and undergo more somatic hypermutation or exit as a plasma cell [27]. The accumulation

of mutations resulting in increased affinity for antigen is the processes of affinity maturation, and on average a single B-cell in the germinal center will cycle through the light zone and the dark zone of the germinal center three times [27].

The differentiation of B-cells in into plasma cells starts in the light zone of the germinal center. The B-cells with higher affinity to antigen will receive help from follicular T-cells and express genes such as XBP1 and IRF4 which are important for plasma cell differentiation [27]. The B-cell then migrates to the dark zone and eventually leaves the germinal center and enters the periphery [27]. Memory B-cell precursors, on the other hand, have lower affinity for antigen [27]. This weaker affinity to antigen is thought not to induce a strong enough signal to re-enter the dark zone and thus contributes to the differentiation into memory B-cells [27].

1.4.4 T-cell help and class switching

Another process that further diversifies B-cells is the process of class-switching recombination [29]. Class-switching recombination is the process in which the DNA recombines in a way that replaces the expressed constant region [29]. Class-switching recombination was thought to happen in the germinal center along with somatic hypermutation, but recent studies have found that class-switching frequently occurs outside of the germinal center and stops once somatic hypermutation-related genes are turned on [29]. The mature naïve B-cell expresses both IgD and IgM surface immunoglobulins and initially can secrete IgM antibodies [30]. Class-switching recombination is guided by regions known as switch regions [23]. Switch regions are in the introns upstream of constant region exons and when class-switching recombination is initiated this eventually leads to the switch regions joining together and the removal of the intervening constant regions [23]. The follicular T-cell help is indispensable to the class-switching process. Ligation of B-cell surface protein CD40 results in the recruitment of proteins downstream which then eventually leads to the expression of activation-induced deaminase or AID [30]. AID is an enzyme essential for processes such as V(D)J recombination, somatic hypermutation, as well as class-switching [30]. In addition to follicular T-cells inducing the activation of AID expression through CD40 ligation, the T-cell

also provides signaling in the form of cytokines which influence polarization of immunoglobulin constant regions (**Figure 1-3**) [23].

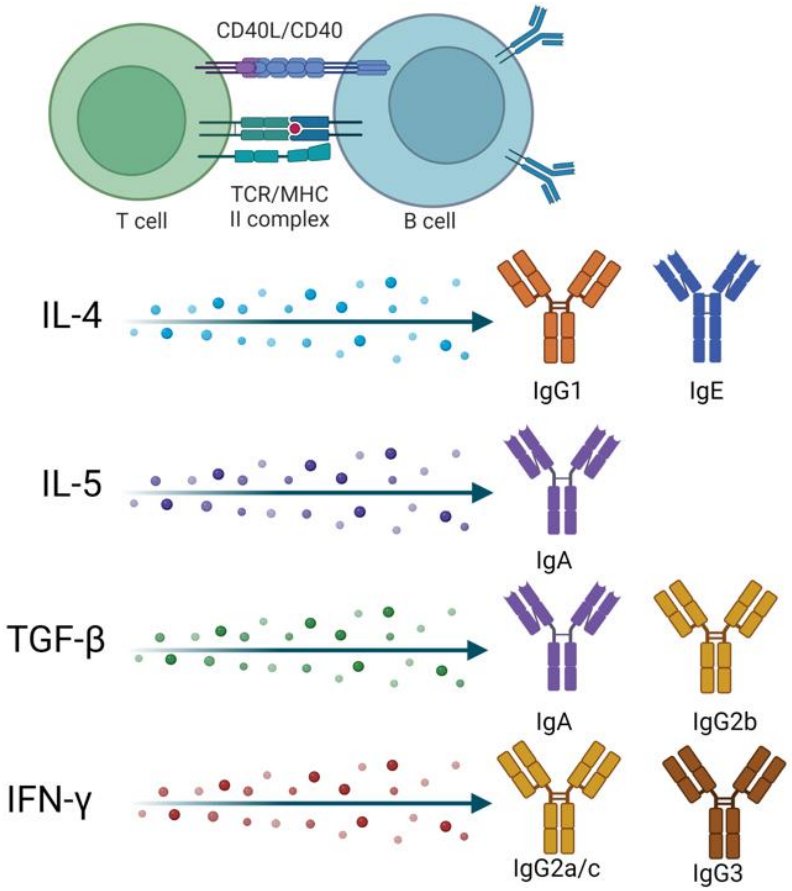

Figure 1-3: T-cell help via CD40 ligation and cytokine expression induces class-switching of activated B-cell. In the above schematic IL-4 expressed by follicular T-cells induces expression of IgG1 or IgE antibody secretion. IL-5 cytokine release induces class-switching of activated B-cells to IgA secretion. Expression of TGF-β by follicular T-cells induces class-switching of activated B-cells to secrete either IgA or IgG2b. IFN-γ cytokine release by follicular T-cells induces class-switching of activated B-cells to IgG2a/c or IgG3 secreting antibody B-cells [23].

1.4.5 Antibody mechanisms of action

Immunoglobulins have two main components, the Fab region and the Fc portion[31]. There are two Fab regions, which are the components of the immunoglobulins that recognize the antigen [31]. The Fab portions can be further divided into the variable region and the constant region; one from the light chain and the other corresponding to the heavy chain [31]. The Fc portion of the immunoglobulin is comprised

of the constant region and is from the heavy chain [31]. Where the Fab region is the antigen recognition component of the antibody, the Fc portion is the effector portion of the antibody [31]. Soluble antibodies play a huge role in clearance and neutralization of pathogens as well as their toxins [31]. There are four main ways antibodies can protect against invading pathogens: neutralization of the pathogen along with any produced toxins, opsonization of the pathogen leading to enhanced phagocytic uptake by a phagocyte, antibody-dependent cellular cytotoxicity, and through activation of complement via the classical pathway (**Figure 1-4**) [31].

Neutralization occurs when the Fab portion of the antibody binds the pathogen in a way that prevents entry of the pathogen or its toxins into the host-cell [31]. Neutralization can also form immune complexes and trigger the activation of complement [31]. Complement proteins such as C4b and C3b can bind receptors on erythrocytes, and the erythrocyte can transport the immune complex to either the spleen or liver [31]. Macrophages in these tissues can then phagocytose the immune complex while also avoiding consuming the erythrocyte [31]. Antibodies also have the ability to attach to epitopes on the pathogen. The accumulation of attached antibodies results in phagocyte cell receptor binding to the Fc portion of the antibodies, triggering phagocytic uptake [31]. Antibody-dependent cellular cytotoxicity is similar to the process of opsonization and phagocytosis of the pathogen except the Fc portions of the antibodies will crosslink Fc receptors on granulocytes such as eosinophils leading to the induction of release of granules [31]. These granules contain reactive oxygen species which can lyse the pathogen [23]. Finally, the other method antibodies can trigger the destruction of invading pathogens is through activation of complement through the classical pathway [31]. In this process, the antibody binds to the surface of the pathogen, and the protein C1q binds to the Fc portion of the antibody [31]. The activation of C1q then leads to a cascade event in which other complement proteins are cleaved and become activated and/or attach to the surface of the pathogen where eventually a membrane-attack complex is formed leading to cell lysis [31].

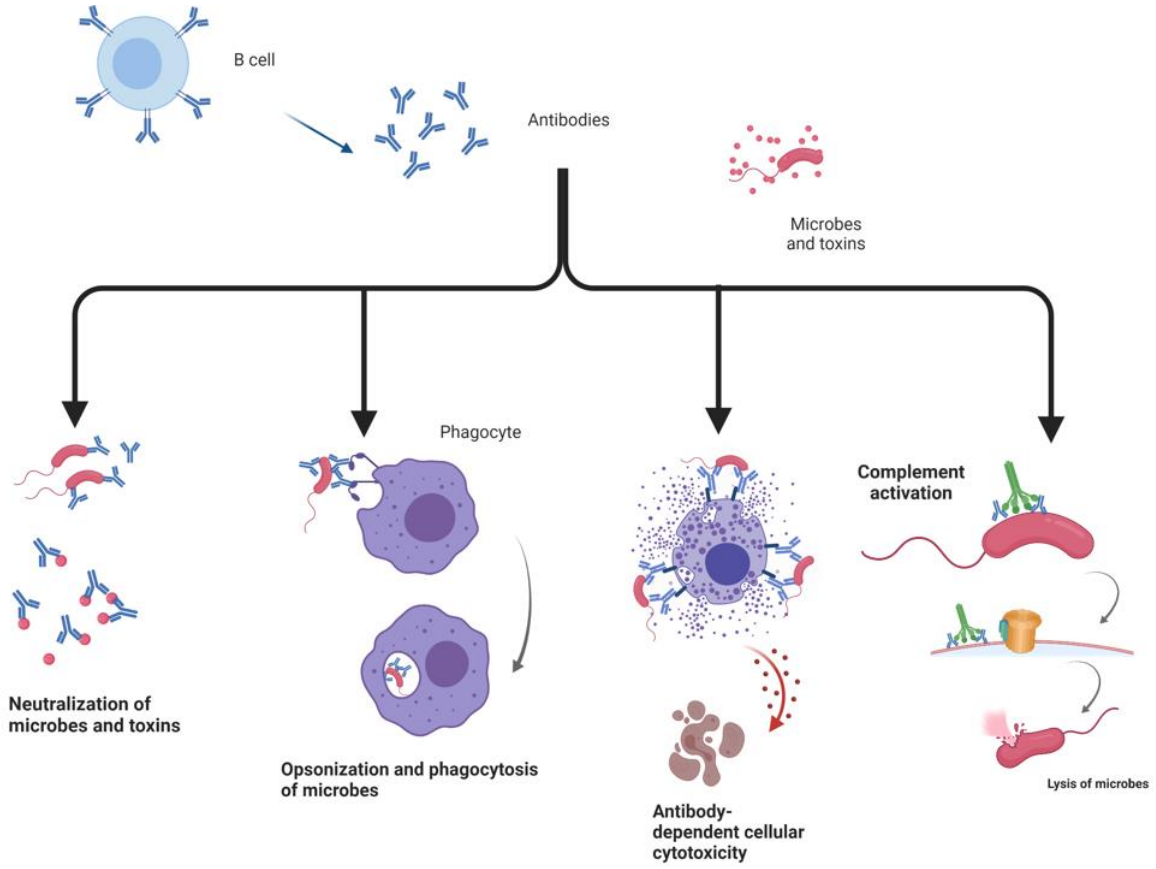

Figure 1-4: Mechanisms in which antibodies may confer protection against invading pathogens Schematic illustrating the four main ways in which antibodies can respond to pathogens. Antigen-specific antibodies have the ability to neutralize pathogens and their toxins. Crosslinking of Fc receptors bound to pathogens can trigger either phagocytosis or antibody-mediated cellular cytotoxicity. The process of complement can also be triggered by antibodies binding to the surface of pathogens and lead to the lysis of pathogens [32].

There are five main classes of human antibodies, including IgM, IgD, IgE, IgA, and IgG. IgG can be divided further into five main classes: IgG1, IgG2a/c, IgG2b, IgG3 and IgG4 [31]. These classes of antibodies can have different shapes, frequency levels and primary effector functions [31]. IgM is the primary antibody that is secreted during initial infection and does not require class-switching to develop [31]. Typically, IgM is in a pentameric form in serum and has low affinity, but due to its pentameric shape it can increase its avidity [31]. IgM is primarily found in the bloodstream and is primarily involved in activation of complement. IgA can form dimers and is most commonly found in secretions such as the epithelial lining of the mucosa [31]. IgA is the most abundant class of antibody found in the body and

primarily involved in neutralizing pathogens and their toxins [33]. IgE is monomeric and is found at the lowest frequency in serum compared to the other classes of antibodies [34]. IgE can trigger degranulation of granulocytes which results in antibody-dependent cytotoxicity [34]. All IgG antibodies are monomeric and are most commonly found in extracellular spaces and blood [23]. IgG1 is the most abundant of the IgG subclass and is associated with neutralization of pathogens as well as opsonization and phagocytosis; IgG2 is also associated with neutralization of pathogens [35] [36]. IgG3 although has the ability to trigger phagocytosis and neutralization. It is primarily involved in activating the complement cascade [35]. IgG4 is the least abundant IgG antibody found in the sera and its primary function is to neutralize pathogens [35]. IgD's function has historically been an enigma [37]. Studies in the last 10 years have found that secreted IgD is found in the mucosa and is involved in gut-homeostasis and can interact with basophils in an Fc independent fashion, resulting in the release of IL-4 [37].

1.5 Human TLRs influence on B-cells

Toll-like receptors or TLRs, are transmembrane innate cell receptors that belong to the family of pattern recognition receptors or PRRs [38]. TLRs recognize a variety of conserved microbial molecules known as pathogen-associated molecular patterns or PAMPS [38]. TLRs are expressed at different frequencies on a multitude of different cell types at various cellular stages [38]. In humans there are 10 different TLRs that are expressed; TLR3, TLR7, TLR8 and TLR9 are expressed on endosomes while TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the surface of the cell [39] [40]. Once the TLR ligand has been engaged, it triggers one of two signaling pathways, the MyD88-dependent pathway or the TRIF-dependent pathway [41]. Downstream of the signaling pathway for TRIF-dependent pathway leads to the production of type-1 interferons while the MyD88-dependent pathway leads to the production of inflammatory cytokines [41].

TLR1 will typically form a heterodimer with TLR2, and its endogenous ligands are triacyl lipopeptides [41]. Dendritic cells express TLR1, and engagement of TRL1 leads to enhanced maturation and antigen presentation which indirectly affects the humoral adaptive response [38]. TLR1 signaling has also been

found to be important in class-switching recombination in B-cells [38]. Studies have found that there seems to be an even frequency of TLR1 expression on naïve B-cells, and memory B-cells but TLR1 expression is found in higher frequencies on B-cells present in the germinal center [40]. In addition to forming a heterodimer with TLR1, TLR2 can form a heterodimer with TLR6 or form a homodimer[41]. Depending on the dimer arrangement, TLR2s natural ligands include triacyl and diacyl lipopeptides, zymosan, peptidoglycan, lipoteichoic acid, lipoarabinomannan, or hyaluronic acid [41]. TLR2s are expressed more on plasma cells than naïve or memory B-cells; when stimulated with TLR4 and TLR9 on follicular B-cells it triggers a surge in IFN-γ production [40] Additionally, engagement with TLR2, TLR3, TLR7 and TLR4 results in the release of marginal zone B-cells from the marginal zone [40]. TLR3 forms an endosomal homodimer and its natural ligand is double-stranded RNA [41]. TLR3 frequencies are low in naïve B-cells but can be found on plasma cells [42]. Additionally, the ligation of TLR3 on B-cells results in the production of IgA and IgG antibodies [42]. TLR4 forms homodimers, and its natural ligands are lipopolysaccharides [41]. TLR4 signaling is unique in that engagement of TLR4 can trigger the MyD88 pathway or the TRIF pathway, if the ligated TLR4 is internalized [43]. TLR4s are expressed on plasma cells and at low levels on naïve B-cells, although TLR4 can be upregulated if the BCR is stimulated [42]. TLR4 ligation on B-cells has also been shown to promote further development, activation and polarization of B-cells [42]. The ligation of TLR4 in combination with CD40/CD40L interaction results in the activation and proliferation of B-cells [42]. Additionally, a combined TLR9 and TLR4 ligation can induce class-switching of the B-cell to a IgG2b- or IgG3-producing B-cell, while a TLR4 ligation in combination with IL-4 will polarize the B-cell to produce IgG1 of IgE antibodies [42]. TLR5s natural ligand is flagellin and will form homodimers on the surface and studies have found TLR5 expression on plasma cells[42] [41]. TLR7 and TLR8 both form homodimers in the endosome and recognize single-stranded RNA [41]. Expression of TLR8 on germinal center, memory or naïve B-cells is limited [40].

However, TLR7 can be found on memory B-cell surfaces but not on naïve B-cells [40], [44]. Additionally, ligation of TLR7 on memory B-cells triggers proliferation [40]. Just like TLR4, TLR7 can be

upregulated when the B-cell is activated through its BCR and CD40 surface protein [42]. Furthermore, this interaction with T-cells is important for B-cell class-switching to IgG2a/c producing B-cells [42]. TLR9 forms homodimers in the endosome and its natural ligand is CpG DNA [41]. TLR9 ligation on naïve B-cells induces enhanced antigen presentation and also results in the upregulation of costimulatory proteins [42]. Ligation of TLR9 also induces class-switching to either IgG2a/c, IgG2b or IgG3 antibody-producing B-cells. TLR10 can form heterodimers with TLR2 and can recognize triacylated lipopeptides [45]. Studies have demonstrated that engagement of TLR10 results in the suppression of B-cell proliferation and cytokine production [45].

1.6 *Pseudomonas aeruginosa* and Pf bacteriophage

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found on the skin and typically does not result in infection in a host with an intact immune system. Although *P. aeruginosa* is ubiquitous, it can readily infect mucosal surfaces such as the eyes or lungs [46] [47]. Furthermore, *P. aeruginosa* has been implicated in hospital-acquired infections and can lead to chronic wound and lung infections in individuals with diabetes or cystic fibrosis, respectively [48]. The CDC antibiotic resistance report of 2019 found that there was a 29% reduction in multidrug-resistant cases of *P. aeruginosa* cases compared to their 2013 report [49]. Even though much progress has been made in decreasing the incidence of *P. aeruginosa* infections, cases rose by 32% in 2020 [50]. In 2020 there were approximately 28,000 cases of multi-drug resistant *P. aeruginosa* infections and of those cases 2,500 patients succumbed to the infection in the United States [50]. Additionally, *P. aeruginosa* is listed as a priority one pathogen by the World Health Organization [51].

One aspect of *P. aeruginosa* which contributes to its multidrug-resistant phenotype is its ability to form biofilms [48]. In the case of *P. aeruginosa*, biofilm production enhances protection against the host immune response and against penetration of antibiotics [48]. Biofilms are organized composites of various microbial components, such as individual bacteria, extracellular DNA, polysaccharides, and proteins [48]. Additionally, another element present in *P. aeruginosa* biofilms is Pf bacteriophages[52]. These

bacteriophages provide enhanced structural integrity to biofilms [52]. In addition, the negatively-charged Pf bacteriophage virions can bind positively-charged antibiotics [52]. Clinical studies have shown that *P. aeruginosa*-infected wounds with Pf bacteriophage present are typically chronic and are harder to treat than *P. aeruginosa* infected wounds without Pf bacteriophage present [53]. Furthermore, when mice were infected with *P. aeruginosa* with Pf bacteriophage present or *P. aeruginosa* without Pf bacteriophage present, dorsal wounds which were infected with the latter healed much quicker [53]. In addition to providing structural integrity to biofilms, the presence of Pf bacteriophage has the ability to skew the host immune response from an anti-bacterial response to an inappropriate anti-viral immune response [53].

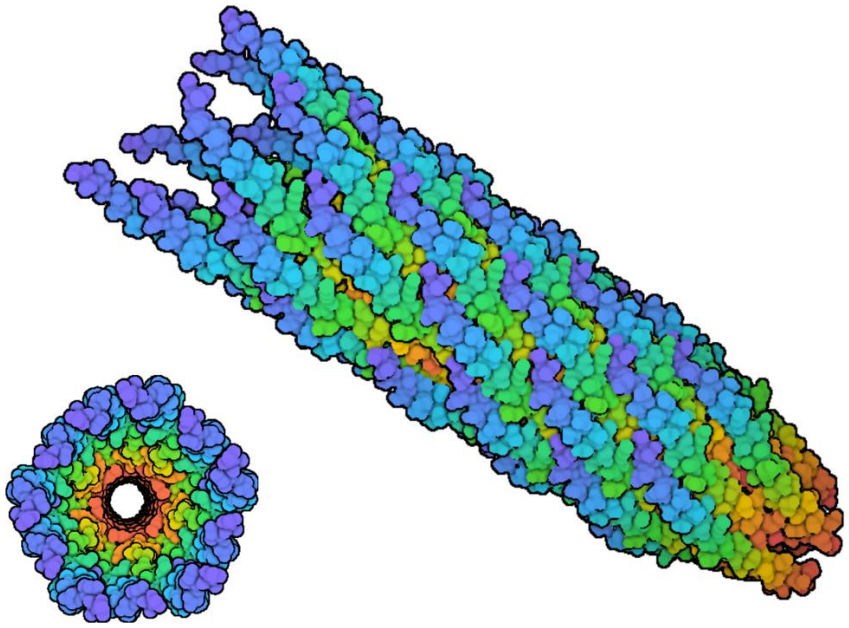

Figure 1-5 Crystal structure of Pf4 bacteriophage: Cross-sectional and cartoon diagram of Pf4 bacteriophage with surface Van Der Waals interactions. The diagram is color-coded to represent amino acid sequences. Negatively-charged amino acids are the primary amino acids exposed to the surface (PDB:6TUP) [54] .

Pf bacteriophages are temperate bacteriophages which belong to the inoviridae family [52]. Pf bacteriophage can exist as a prophage, meaning it is integrated into the *P. aeruginosa* chromosome [52]. As *P. aeruginosa* replicates, the Pf prophage will passively replicate [55]. If the bacterial host is in a stressful environment, such as a nutrient limited environment, this can cause the induction of Pf prophage to excise

from the bacterial chromosome [55]. This induction then leads to the transcription and assembly of single-stranded DNA (ssDNA) filamentous bacteriophages [55]. The fully formed Pf virions than can extrude out of the bacterial host typically leaving the host membrane intact and sparing the bacterial host from lysis [52]. Pf bacteriophage is coated primarily with a highly conserved major coat protein known as CoaB but also has a component of a minor coat protein known as CoaA [56]. The major coat protein arranges itself in a helical conformation and houses a ssDNA genome [52]. The diameter of Pf bacteriophage is approximately 6-7nm and the length can reach up to 2µm [52] (**Figure 1-5**). Because CoaB is highly conserved and because of Pf bacteriophage's direct contribution to enhanced *P. aeruginosa* virulence in the host, Sweere et al. developed a vaccine which included a consensus peptide derived from CoaB [53]. Initial Pf bacteriophage targeted vaccine strategies demonstrated that antibodies generated against the Pf bacteriophage enhanced clearance of *P. aeruginosa* from wound infections in mice [53].

1.7 Aims of dissertation

This dissertation aims to compare a novel TLR4 agonist with the commonly used adjuvant alum and characterize the humoral antibody response as well as adaptive cytokine response against Pf bacteriophage. This dissertation also seeks to broaden the application of the Pf bacteriophage vaccine by introducing intranasal administration of the vaccine and incorporate a distinct consensus peptide antigen from a genetically distinct clade of Pf bacteriophage. Finally, in this dissertation I explore and characterize how TLR4 agonist and TLR7 agonist influence the antigen-specific B-cell population of mice vaccinated with Pf targeted vaccine. The research presented herein explores how the incorporation of TLR4 agonists with a vaccine against Pf bacteriophage influences the immune response. The data presented here provide further evidence of how the route of administration can polarize an immune responses. Additionally, this research adds to the knowledge base on how TLR4 and TLR7 can impact antigen-specific B-cell populations.

Chapter 2: Adjuvanted vaccine creates functional antibodies against *Pseudomonas aeruginosa* Pf bacteriophage in C57 Bl/6 mice

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Abstract

Pseudomonas aeruginosa (*Pa*) is a World Health Organization priority 1 pathogen. *Pa* resulted in approximately 559,000 deaths globally in 2019, and has a multitude of host-immune evasion strategies which enhance its virulence. Most clinical isolates of *Pa* are infected by a phage called Pf that has the ability to misdirect the host-immune response and provide structural integrity to biofilms. Previous studies demonstrate that vaccination against the coat-protein (CoaB) of Pf4 virions (from *Pa* strain PaO1) can assist in the clearance of *Pa* from the dorsal wound model in mice. Here, a consensus peptide was derived from CoaB and conjugated to cross-reacting material-197 (CRM-197). This conjugate was adjuvanted with a novel synthetic Toll-like receptor (TLR) 4 agonist, INI-2002, and used to vaccinate mice. Mice vaccinated with CoaB-CRM conjugate and INI-2002 developed high anti-CoaB peptide-specific IgG antibody titers. Direct binding of the peptide-specific antibodies to whole-phage virus particles was demonstrated by ELISA. Furthermore, a functional assay demonstrated that antibodies generated from vaccinated mice disrupted the replicative cycle of Pf bacteriophage. The use of an adjuvanted phage vaccine targeting *Pa* is an innovative vaccine strategy with the potential to become a new tool targeting multi-drug resistant *Pa* infections in high-risk populations.

2.1 Introduction

Pseudomonas aeruginosa (*Pa*) is a Gram-negative bacterium that often infects diabetic ulcers, burn wounds, implanted medical devices, and the lungs of cystic fibrosis patients [57]. *Pa* is typically treated

with antibiotics, but emerging multi-drug resistant strains are problematic. Multi-drug resistant *Pa* was placed on the 2018 global priority 1 list by the World Health Organization [58], [59]. A surveillance study identified 12% of isolates tested in European intensive care units as multidrug-resistant and in 2019 a global multidrug-resistant surveillance study found that of 13 million bacterial infection related deaths approximately 4% were related to *Pa* infections [60], [61]

The search for an effective vaccine against *Pa* has been ongoing for roughly half a century. However, only three vaccine candidates reached phase III clinical trials, and each ultimately failed primary efficacy endpoints [62]. Most previous vaccine candidates have used *Pa* surface proteins or other bacterial expressed proteins as vaccine targets. Unfortunately, *Pa's* ability to rapidly adapt or modulate expression of target antigens has prolonged the arduous journey of finding an effective vaccine [62]. In addition to the ability to evade vaccine-mediated host defenses, *Pa* has an arsenal of strategies to evade natural host immunity potentially leading to serious and life-threatening chronic infection [63]. One such mechanism is *Pa's* ability to develop biofilms, which can lead to the reduction of surveillance by the adaptive immune system and antibiotic efficacy [64].

Most strains of *Pa* are infected by Pf integrated into the chromosome as a prophage [52]. *Pa* strain PAO1 is infected by the Pf4 prophage, which has been identified as a virulence factor and provides structural integrity to *Pa* biofilms [65]. Pf4 phage belongs to the Inoviradae family and is structurally a long, filamentous virion. The majority of the Pf4 virion is composed of repeated copies of the major coat protein CoaB packaging a circular single-stranded DNA genome [65]. Pf4 is lysogenic; it can passively replicate as a prophage without lysing its bacterial host. External signals (such as oxidative stress) received by the *Pa* host can result in excision of Pf4 prophage leading to the production of infectious virions [66]. Pf4 virions are internalized by immune cells via endocytosis where they activate Toll-like receptor (TLR) 3 signaling, leading to the production of type I interferons while also reducing TNF-α production [63]. This misdirected antiviral immune response to a bacterial pathogen reduces antibacterial immune responses, such as reduced phagocytic uptake by macrophages [63].

Because Pf4 virions are a key virulence factor of *Pa*, and Pf prophages are present in most *Pa* strains, Pf virions could be a viable vaccine target. CoaB is highly conserved, and previous studies identified a conserved portion of the coat protein that spans 699 isolates of *Pa* [63]. The N-terminus of this conserved portion is largely exposed to the environment and is predominantly comprised of negatively charged amino acids. Due to its highly conserved nature, a portion from this region was used to develop a 19 amino acid consensus peptide, thus creating a simple and novel vaccine antigen covering a majority of the *Pa* clinical isolates [63]. Previous murine vaccination studies demonstrated that a humoral immune response against the Pf4 phage results in protection against *Pa* and clearance of infection in a murine wound infection model [63].

Here, we optimized the peptide-carrier conjugate vaccine through the addition of synthetic adjuvants. An adjuvant is an additive in a vaccine that improves vaccine efficacy, resulting in longer lasting and more robust protection [67]. Some next generation adjuvants have the ability to target pattern recognition receptors (PRR) on immune cells. Adjuvants bound to PRRs can result in the production of specific cytokines and cell-surface proteins which can enhance adaptive immunity when co-administered with an antigen [67]. The lead adjuvant described herein, INI-2002, is a synthetic TLR4 agonist that enhances both cell-mediated and humoral immunity. Animals vaccinated with CoaB peptide conjugated to CRM and adjuvanted with INI-2002 had increased antigen-specific antibody production when compared to animals vaccinated with the CoaB conjugate adsorbed to Alhydrogel. Furthermore, the INI-2002 adjuvanted vaccine induced functional antibodies that bound directly to Pf4 phage and disrupted the phage replicative cycle. Creating a safe and effective vaccine that can mount a robust immune response against Pf4 phage is an important advancement towards the development and clinical testing of phage-based vaccines targeting multi-drug resistant *Pa* infections.

2.2 Materials and Methods

INI-2002 compound synthesis

INI-2002 was synthesized using a linear approach as follows.

A solution of 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (76.47 g, 0.23 mol) in methylene chloride (350 mL) and H_2O (350 mL) was treated with sodium bicarbonate (149.94 g, 1.79 mol) added in portions slowly. Benzyl chloroformate (79.17 g, 0.46 mol) was added in portions to control gas evolution and the reaction was stirred vigorously for 2.5 hours. The layers were separated, and the aqueous layer was extracted with methylene chloride (100 mL). The combined organic layers were washed with saturated aqueous sodium chloride, dried over anhydrous sodium sulfate, filtered and concentrated to approximately 100 mL. Methyl-*t*-butyl ether (200 mL) was added and the resulting mixture was stirred and cooled to 0 °C and the precipitate was collected by filtration, washed with cold methyl-*t*-butyl ether and dried in a vacuum oven to give 88.89 g (81 %) of 1,3,4,6-tetra-*O*-acetyl-2-(benzyloxycarbonylamino) 2-deoxy-β-D-glucopyranoside.

A solution of 1,3,4,6-tetra-*O*-acetyl-2-(benzyloxycarbonylamino) 2-deoxy-β-D-glucopyranoside (10 g, 20.8 mmol) and benzyl *N*-(2-hydroxyethyl)carbamate (4.48 g, 22.9 mmol) in anhydrous methylene chloride (80 mL), cooled to -15 °C, was treated dropwise with trimethylsilyl triflate (0.37 mL, 2.08 mmol). The reaction mixture was allowed to warm to room temperature over 5.5 hours. The reaction was quenched with saturated aqueous sodium bicarbonate (40 mL) and the layers were separated. The aqueous layer was extracted with methylene chloride (2x 20 mL) and the combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude product obtained was crystallized from methylene chloride/heptane to give 10.4 g (81 %) of 2-(benzyloxycarbonylamino)ethyl 3,4,6-tri-*O*-acetyl-2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside as a white solid.

A solution of 2-(benzyloxycarbonylamino)ethyl 3,4,6-tri-*O*-acetyl-2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (10 g, 16.3 mmol) in methanol (160 mL) was treated with ammonium hydroxide (20 equivalents) for 2 hours at room temperature. The reaction mixture was concentrated and dried under high vacuum overnight to give 8 g (100 %) of 2-(benzyloxycarbonylamino)ethyl 2-

benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside as a white solid, which was used without further purification.

A solution of 2-(benzyloxycarbonylamino)ethyl 2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (8 g, 16.3 mmol) in acetonitrile (180 mL) was treated with benzaldehyde dimethyl acetal (4.9 mL, 32.6 mmol) and camphorsulfonic acid (1.9 g, 8.2 mmol). The reaction was stirred for 3 hours, neutralized with saturated aqueous sodium bicarbonate, filtered, and concentrated *in vacuo*. The crude product was crystallized from ethyl acetate/heptane to give 7.1 g (75 %) of 2-(benzyloxycarbonylamino)ethyl 4,6-*O*-benzylidene-2-deoxy-2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside as a white solid.

A solution of 2-(benzyloxycarbonylamino)ethyl 4,6-*O*-benzylidene-2-deoxy-2-benzyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (1.5 g, 2.59 mmol) in anhydrous tetrahydrofuran (40 mL) was treated with triethylamine (0.54 mL, 3.89 mmol) and triphenylphosphine (1.09 g, 4.14 mmol). The reaction mixture was cooled to 0 °C and diisopropyl azodicarboxylate (0.82 mL, 4.14 mmol) was added. After 45 minutes at 0 °C, diphenylphosphoryl azide (0.89 mL, 4.14 mmol) was added. The reaction was allowed to gradually warm up to room temperature and stirring continued for 18 hours. The reaction mixture was concentrated *in vacuo* and the residue chromatographed on silica gel (gradient elution, 20→70 % ethyl acetate/heptane) affording 1.16 g (74 %) of 2-(benzyloxycarbonylamino)ethyl 3-azido-4,6-*O*-benzylidene-2-benzyloxycarbonylamino-2,3-dideoxy-β-D-allopyranoside as a white solid.

A solution of 2-(benzyloxycarbonylamino)ethyl 3-azido-4,6-*O*-benzylidene-2-benzyloxycarbonylamino-2,3-dideoxy-β-D-allopyranoside (2.95 g, 4.89 mmol) in anhydrous tetrahydrofuran (100 mL) was treated with a solution of 0.1 N sodium hydroxide (9.8 mL, 0.98 mmol) and a solution of 1.0 M of trimethylphosphine in tetrahydrofuran (7.8 mL, 7.82 mmol). The reaction stirred at room temperature for 18 hours. The reaction mixture was concentrated *in vacuo*. The residue was chromatographed on silica gel (gradient elution, 30→100 % ethyl acetate/heptane then 0→10%

methanol/chloroform) affording 2.37 g (84 %) of 2-(benzyloxycarbonylamino)ethyl 3-amino-4,6-*O*-benzylidene-2-benzyloxycarbonylamino-2,3-dideoxy-β-D-allopyranoside as a white solid.

A solution of 2-(benzyloxycarbonylamino)ethyl 3-amino-4,6-*O*-benzylidene-2-benzyloxycarbonylamino-2,3-dideoxy-β-D-allopyranoside (0.5 g, 0.87 mmol) in anhydrous methylene chloride (10 mL) was acylated with (*R*)-3-decanoyloxytetradecanoic acid (414 mg, 1.04 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (310 mg, 1.04 mmol) at room temperature for 2 hours. The reaction mixture was quenched with saturated aqueous sodium bicarbonate (5 mL) and the layers separated. The aqueous layer was extracted with chloroform (2 x 5 mL) and the combined organic layers were washed with water (5 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Chromatography on silica gel (gradient elution, 10→ 60% ethyl acetate/heptane) afforded 748 mg (90 %) of 2-(benzyloxycarbonylamino)ethyl 4,6-*O*-benzylidene-2-benzyloxycarbonylamino-3-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside as a colorless oil.

A solution of 2-(benzyloxycarbonylamino)ethyl 4,6-*O*-benzylidene-2-benzyloxycarbonylamino-3-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside (745 mg, 0.78 mmol) in anhydrous tetrahydrofuran (20 mL) was hydrogenated with 10 % palladium on carbon (220 mg) using a Parr hydrogenator at room temperature and 50 psig for 24 hours. The reaction mixture was filtered through Celite and the filtrate concentrated in vacuo. The resulting oil dissolved in methylene chloride (10 mL) was acylated with (*R*)-3-decanoyloxytetradecanoic acid (680 mg, 1.71 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (510 mg, 1.71 mmol) at room temperature for 2 hours. The reaction mixture was quenched with saturated aqueous sodium bicarbonate (10 mL) and the layers separated. The aqueous layer was extracted with methylene chloride (2 x 10 mL) and the combined organic layers washed with water (10 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Chromatography on silica gel (gradient elution, 20→80% ethyl acetate/heptane) afforded 732 mg (65 %) of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 4,6-*O*-benzylidene-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside as a glassy solid.

A solution of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 4,6-*O*-benzylidene-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside (400 mg, 0.282 mmol) in anhydrous methylene chloride (20 mL) cooled to 0 °C was treated with sodium cyanoborohydride (42mg, 0.655 mmol) followed by the addition of trifluoroacetic acid (0.06 mL, 0.786 mmol). The reaction mixture gradually warmed up to room temperature and continued to stir for 3 hours. The reaction was quenched with methanol (2 mL), concentrated in vacuo then reconstituted in methylene chloride and washed with a saturated solution of sodium bicarbonate. The layers separated and the aqueous layer was extracted with methylene chloride (2 x 10 mL) and the combined organic layers dried over anhydrous sodium sulfate and concentrated in vacuo. Chromatography on silica gel (gradient elution, 10→ 95% ethyl acetate/heptane) afforded 380 mg (93 %) of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 6-*O*-benzyl-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside as a colorless oil.

A solution of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 6-*O*-benzyl-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-β-D-allopyranoside (105 mg, 0.072 mmol) in anhydrous dimethylformamide (5 mL) was treated with sulfur trioxide triethylamine complex (78 mg, 0.43mmol). The reaction was heated to 50 °C for 5 h. An additional amount of sulfur trioxide triethylamine complex (100 mg, 0.55 mmol) was added and the reaction stirred at 50 °C for 18 h. The reaction mixture was concentrated *in vacuo*. Chromatography on C_{18} column (gradient elution, 5→ 20 % methylene chloride + 1 % triethylamine/methanol) afforded 90 mg (82 %) of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 6-*O*-benzyl-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-4-*O*-sulfoxy-β-D-allopyranoside triethylammonium salt as a white salt.

A solution of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 6-*O*-benzyl-2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-4-*O*-sulfoxy-β-D-allopyranoside triethylammonium salt (70 mg, 0.045 mmol) in a mixture of 2:1 anhydrous tetrahydrofuran: methanol (5 mL) was hydrogenated in the presence of 20 % palladium hydroxide on carbon (30 mg) and triethylamine (0.034 mL, 0.00024 mmol) using a Parr hydrogenator at room temperature and 50 psig pressure for 18 hours. The reaction mixture was

filtered through Celite and the filtrate was concentrated under vacuum. Chromatography on C_{18} silica column (gradient elution, 5→20 % methylene chloride + 1 % triethylamine/methanol), the purified material was dissolved in cold 2:1 chloroform-methanol (8mL) and washed with cold 0.1 N aqueous hydrochloride (1.6 mL). The lower organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was salted with (1 -2 equiv.) triethylamine to give 28 mg (43 %) of 2-[(*R*)-3-decanoyloxytetradecanoylamino]ethyl 2,3-di-[(*R*)-3-decanoyloxytetradecanoylamino]-2,3-dideoxy-4-*O*-sulfoxy-β-D-allopyranoside triethylammonium salt as a glassy solid: ^{1}H NMR ($CDCl_3/CD_3OD$): (ppm) 7.84 (t, *J* = 5.5 Hz, 1 H), 7.55 (d, *J* = 8.0 Hz, 1 H), 7.22 (d, *J* = 9.0 Hz, 1 H), 5.27 – 5.23 (m, 3 H), 4.65 (br s, 1 H), 4.59 – 4.55 (m, 2 H), 4.26 – 4.21 (m, 1 H), 4.19– 4.15 (m, 1 H), 3.85 – 3.79 (m, 2 H), 3.73 – 3.70 (m, 1 H), 3.51 – 3.43 (m, 2 H), 3.18 (q, *J* = 7.5 Hz, 7 H, CH_2 of triethylamine (~1.2 equiv.)), 2.62 – 2.19 (m, 12), 1.64 – 1.52 (m, 12 H), 1.37 – 1.26 (m, 100 H, including 10, CH_3 of triethylamine), 0.88 (t, *J* = 7.0 Hz, 18 H); HRMS (ESI-TOF) m/z: Calcd for $C_{80}H_{151}N_3O_{16}S$ $[M-H]^-$ 1441.0737, found 1441.0714.

CoaB conjugate to CRM

In order to conjugate CRM-197 (provided by Inimmune Corporation, Missoula MT) to CoaB (peptide sequence GVIDTSAVESAITDGQDM) (Anaspec), the linker N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS; Thermo Scientific) was dissolved in DMSO (Sigma) at a concentration of 25 mg/mL. To achieve 15-17 copies of GMBS to CRM-197, 85 equivalents of GMBS/DMSO solution to CRM-197 was incubated in a water bath for 30 minutes at 25°C. Remaining GMBS was removed by ultrafiltration using 30 kDa Amicon (Millipore) Ultra centrifugal filters and washed four times using 0.2 M sodium phosphate buffer (pH=7.2). MALDI TOF MS was used to determine the amount of GMBS added to CRM-197. The activated CRM-197 was added to 40 molar equivalents of peptide at a concentration of 1.5 mg/mL and incubated for two hours at 25°C. Remaining unattached peptide was removed by ultrafiltration using 30 kDa Amicon Ultra centrifugal filters and washed 4 times with 10mM phosphate Buffer. The peptide-CRM conjugate was sterile-filtered using a 0.22 µm PVDF syringe filter. MALDI TOF MS was used to determine peptide copy number.

Bacterial strains, plasmids, and growth conditions.

Strains, plasmids, and their sources are listed in **Table 1**. Unless indicated otherwise, bacteria were grown in lysogeny broth (LB) with gentamicin (10 ug/mL^{-1}) at 37°C with shaking. To induce expression of plasmid-encoded proteins, a final concentration of 0.1% arabinose was used.

Table 2-1 Bacterial strains, phage, and plasmids used in this study

Strain	Description	Source
P. aeruginosa		
PAO1	Wild-type	[68]
PAO1 ΔPf4*GmR*	Pf4 prophage deletion, replaced with GmR cassette. Att sites not retained.	[69]

P. aeruginosa and Pf4 Propagation

An isolated colony of *P. aeruginosa* strain PAO1 was selected from an LB agar plate and used to inoculate 500 mL of LB broth then incubated at 37°C/250rpm until cultures reached an optical density (OD_{600}) of 0.3. The cultures were then infected with Pf4 virions at a multiplicity of infection (MOI) of 10 virions :1 bacterium and incubated for 18 hours at 37°C. The culture was centrifuged at 16,000xg for 1 minute to remove cells and the supernatant was filtered through a sterile 0.22 µm syringe filter. Pf4 virion titer was determined by plaque forming units (PFU) using *P. aeruginosa* PAO1ΔPf4 as an indicator strain. Aliquots of 50 µL of the Pf4 lysate were stored at -20°C[70].

In vivo experiments

Animal studies were carried out in accordance with University of Montana's IACUC guidelines for the care and use of laboratory animals. For murine vaccine studies, groups of 6-10 female C57Bl/6 mice were vaccinated intramuscularly (right gastrocnemius) on days 0 and 14 with the indicated dose of INI-2002 (TLR4 adjuvant) with or without Alhydrogel (InvivoGen) and CoaB-CRM conjugate in a volume of 50 µL per injection. Compounds and antigen were diluted as needed in 2.5% glycine in 1X PBS. At 14 days following each vaccination, blood samples were collected via the submandibular vein (d14) or cardiac puncture (d28). For evaluation of cell-mediated immune response, spleens were harvested from a subset of mice on day 19 (5 days post booster vaccination). For some experiments, a third booster vaccination was administered on day 28, and blood was collected on day 42 for evaluation of humoral immunity.

Pf4 serum inhibition assay

P. aeruginosa ΔPf4GmR [70] was grown in LB at 37°C/250rpm to an OD_{600} 0.2. Pf4 virions were diluted in biological triplicate in 20 µL of sera from unvaccinated animals, sera from vaccinated animals, or 1X PBS to obtain a MOI of 0.01 and incubated at 37°C for 1 hour. Pf4 virions co-cultured with sera or 1X PBS were added to a *Pa* culture (*Pa* culture was grown to an OD_{600} of 0.3). Timepoints were collected directly prior to addition of Pf4 +/- sera and 5 minutes, 15 minutes, 45 minutes, 2 hours, 4 hours, and 16 hours after addition. At each time point, 250 µL of culture was removed and spun at 16,000xg for two minutes. Supernatant was transferred to fresh tube and frozen at -20°C. Supernatants were serially diluted 1:10 in 1X PBS and spotted in technical triplicate on lawns of ΔPf4GmR. Plaque-forming units (PFUs) were counted following 18 hours incubation at 37°C and analyzed using the unpaired Student's t-test (GraphPad Prism version 5.0 San Diego, CA). P values of <0.0001 were considered statistically significant.

ELISA for anti-CoaB peptide antibody quantification

Blood was collected from mice 14 days post-vaccination, and serum was isolated and diluted according to the expected antigen-specific antibody titers (between 1:20 and 1:500). Nunc MaxiSorp plates were coated

with 100 µL of CoaB peptide (GVIDTSAVESAITDGQDM) at 10 µg/mL overnight at room temperature. Plates were washed (1X PBS plus 0.05% Tween 20) and then blocked with 200 µl of SuperBlock (Scytek Laboratories) for 2 hours at 37°C. Next, serial diluted serum at a dilution factor of 1:3 was added and incubated at 37°C for 2 hours and then washed three times with 1X PBS plus 0.05% Tween-20 using a 30 seconds and aspiration. Following the washing step, plates were incubated with anti-mouse IgG, IgG1, IgG2b or IgG2c-HRP secondary antibody at a dilution of 1:1000 (Southern Biotech) for 1 hour at 37°C and then washed the same as above. Covered plates were then incubated with 100 µL of TMB substrate (BD) at room temperature for an hour followed by absorbance being read at 650nm using the Spectra Max 190 (Molecular Devices). Antibody titers were determined using XL fit software (IDBS) at an OD of 0.3. Negative values were assigned a point value of 0.5 in order to plot points and follow up with statistical analysis.

Phage ELISA

Blood was collected from mice 14 days post-vaccination, serum was isolated and diluted according to the expected antigen-specific antibody titers (between 1:20 and 1:500). Nunc MaxiSorp plates were coated overnight (20°C) with 100ul/well of whole phage virions collected from *P. aeruginosa* strain PAO1 at a concentration of 2 x 10^9 PFU/mL. Plates were then washed with KPL wash buffer (SeraCare) and blocked with 200 µL of blocking agent [1% BSA, 0.1% goat serum in 1X PBS] for one hour at 37°C. Plates were then incubated with serially-diluted serum at a dilution factor of 1:3 for 2 hours at 37°C. Plates were then washed (1X PBS plus 0.05% tween 20) and incubated with anti-mouse IgG, IgG1, or IgG2c-HRP secondary antibody at a dilution of 1:1000 (Southern Biotech) for 1 hour at 37°C. After another wash, 100 µL of TMB substrate (BD) was added, and plates were read at 650nm using the Spectra Max 190 (Molecular Devices). Antibody titers were determined by using XL fit software program at an OD of 0.3.

2.9 Cell-mediated immunity analysis -Spleens were harvested from five mice per group at five days post-booster vaccination (d19). Cells were processed by mechanical disruption of the spleens through a 100 µm filter (Foxx Life Sciences). Red blood cells were lysed by incubation with red blood cell lysis buffer (Sigma) for 5 minutes followed by washing in 1X PBS. Cells were plated in a sterile 96 well plate polystyrene flat bottom plate at $5x10^6$ cells/well in 200 µL complete Roswell Park Memorial Institute (RPMI) 1640 media (10% heat-inactivated fetal bovine serum, 0.1% 2-Mercaptoethanol, 1% Penicillin-streptomycin-L-glutamine).For secreted cytokines, splenocyte single cell suspensions were plated at $5x10^6$ in 96-well plates and incubated with 5 µg/mL of CRM or CoaB peptide for 72 hours at 37°C and 5% CO_2. Supernatants were harvested, and cytokine levels were measured by a MesoScale Discovery (MSD) U-PLEX Assay Platform (MesoScale Diagnostics) to detect mouse IFNγ, IL-17, TNFα, IL-2, IL-10 and IL-5 following manufacturer's instructions.

2.3 Results

2.3.1 Production and characterization of INI-2002-adjuvanted CoaB peptide-CRM conjugate vaccine.

For the antigen component of our vaccine, we identified a consensus peptide sequence derived from the coat protein (CoaB) of bacteriophages isolated from 669 *P. aeruginosa* strains [71] (**Figure 2-1A**). Cross-reacting material 197 (CRM), has been approved by the FDA as a carrier protein in several hapten-conjugate vaccines approved for use in humans[72], [73]. CRM is a non-toxic carrier protein derived from diphtheria toxin [74] and was selected to assist in amplifying the immunogenicity of the CoaB peptide by conjugation (**Figure 2-1B)**. The conjugate vaccine CoaB-CRM was prepared by covalently linking the 19 amino acid CoaB peptide to the carrier protein, CRM. This was accomplished using the heterobifunctional crosslinker GMBS (N-γ-maleimidobutyryl-oxysuccinimide ester). The production of the vaccine can be summarized in two major steps. Firstly, the activation of protein with GMBS, which renders it reactive toward the cysteine residue contained in the peptide sequence. Secondly, the conjugation of activated CRM protein with the peptide motif. The addition of GMBS to CRM results in a peak shift indicating activation of CRM. Activated CRM was determined through MALDI TOF MS (peak at approximately 60000 m/z),

minor peak at approximately 30000 m/z represents m/2 (double charged-product) (**Figure 2-1C**). The activated CRM was then incubated with CoaB peptide. The cysteine on the peptide should then form disulfide bonds with the linker. Incubation was then followed by ultrafiltration to remove unattached peptide. CoaB copy number (defined as the number of measured CoaB peptides per CRM molecule) was determined through MALDI TOF (**Figure 2-1D**). Throughout the following experiments, a copy number of approximately 10 was used.

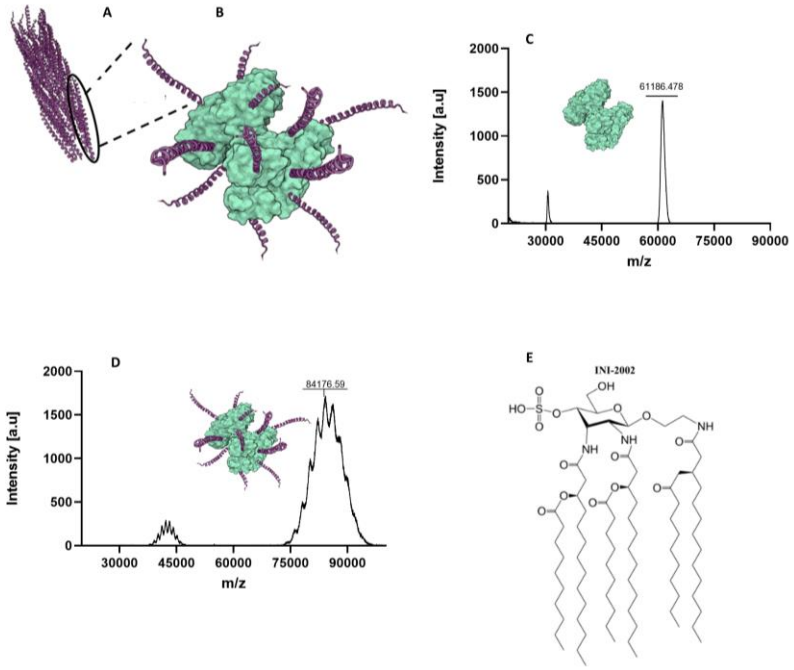


Figure 2-1. Components of Pf bacteriophage vaccine and characterization of CRM-CoaB conjugate. (a) Schematic of Pf bacteriophage crystal structure (PDB: 6TUP); (b) Schematic of CoaB peptide GVIDTSAVESAITDGQGDM conjugated to CRM-197 (PDB: 4AE0); (c) MALDI-TOF characterization of GMBS activated CRM-197; (d) MALDI-TOF characterization of CRM-CoaB conjugate; (e) Structure of TLR4 agonist, INI-2002.

To further enhance immunogenicity of the vaccine, we selected a synthetic TLR4 agonist as the adjuvant. Previous studies identified the importance of lipopolysaccharide (LPS) engagement of TLR4 in the clearance of *Pa* infections [75]. We hypothesized that the use of a TLR4 agonist would enhance an antibody response specific to CoaB. A component of LPS, lipid A, is the bioactive element and is the structural basis of our synthetic TLR4 agonist, INI-2002 (**Figure 2-1E**) [76].

3.2 INI-2002-adjuvanted CoaB-CRM vaccine enhanced CoaB-specific antibody titers

We first determined whether the addition of INI-2002 adjuvant increased anti-CoaB serum IgG antibody titers over that of CoaB-CRM alone or CoaB-CRM adjuvanted with Alhydrogel. We hypothesized that using two adjuvants together, INI-2002 and Alhydrogel, would further increase anti-CoaB antibody production. To test this hypothesis, C57BL/6 mice were vaccinated intramuscularly (IM) with 1 μg of CoaB-CRM alone, in combination with INI-2002, with Alhydrogel, or with both adjuvants on days 0 and 14. On day 28, sera were collected and CoaB peptide-specific IgG titers were measured by ELISA. Animals vaccinated with CoaB-CRM in combination with INI-2002 had significantly ($p<0.0001$) increased CoaB peptide-specific serum IgG titers when compared to all other groups (**Figure 2-2**). Surprisingly, IgG titers from animals vaccinated with CoaB-CRM in combination with both INI-2002 and Alhydrogel were not significantly higher than animals vaccinated with CoaB-CRM in combination with INI-2002 (**Figure 2-2**). We therefore selected INI-2002 as the lead adjuvant for further CoaB-CRM vaccination and characterization studies.

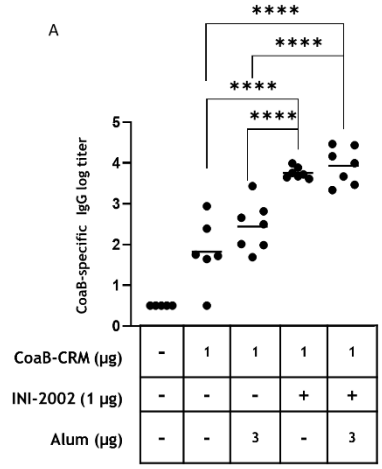


Figure 2-2. INI 2002-adjuvanted CoaB-CRM results in higher CoaB-specific titers compared to alum-adjuvanted CoaB-CRM. C57Bl/6 mice (10 or 15 per group) were injected once on day 0 and day 14 IM with 1 μg/mouse CRM-CoaB conjugate vaccine adjuvanted with 1 μg/mouse INI-2002 and/or with indicated dose of alum. Serum was collected on day 14 post-secondary vaccination. CoaB peptide specific IgG antibody titers were measured by ELISA. Symbols above individual points denote statistical

significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = p≤0.05, ** = p≤0.01, *** = p≤0.001, **** = p≤0.0001.

2.3.3 INI-2002 enhances humoral immunity to CoaB peptide in a dose-dependent fashion

Next, we optimized the dose of INI-2002 as an adjuvant for CoaB-CRM. We hypothesized that increasing the dose of INI-2002 would lead to an increase in anti-CoaB peptide antibody titers in a dose-dependent manner. Mice were vaccinated IM on days 0 and 14 with 1 µg CoaB-CRM and increasing doses of INI-2002 ranging from 0.0001 to 10 µg as noted in **Figure 2-3**. Serum was harvested on day 28 (14 days following the secondary vaccination) and anti-CoaB peptide IgG titers were determined by ELISA. Anti-CoaB IgG titers increased in a INI-2002 dose-dependent manner with titers plateauing at a dose of 0.1 µg INI-2002 (**Figure 2-3A**). Next, we evaluated both CoaB-specific IgG1 and IgG2c antibody titers as an indication of class switching and Th1/2 polarization of the T helper cell response [77] . Anti-CoaB IgG1 and IgG2c titers generally mirrored that of IgG with the peak titers generated with a 1 ug INI-2002 dose. Mice vaccinated with 1 ug of CoaB-CRM and 1 ug of INI-2002 had significantly (P=0.0006) higher IgG, IgG1, and IgG2 antibody titers when compared to naïve or CoaB-CRM + 0.0001 µg of adjuvant. (**Figure 2-3B and 2-3C**). The strong increases in both antigen-specific IgG1 and IgG2c suggest a mixed Th1/2 T helper response to the conjugate.

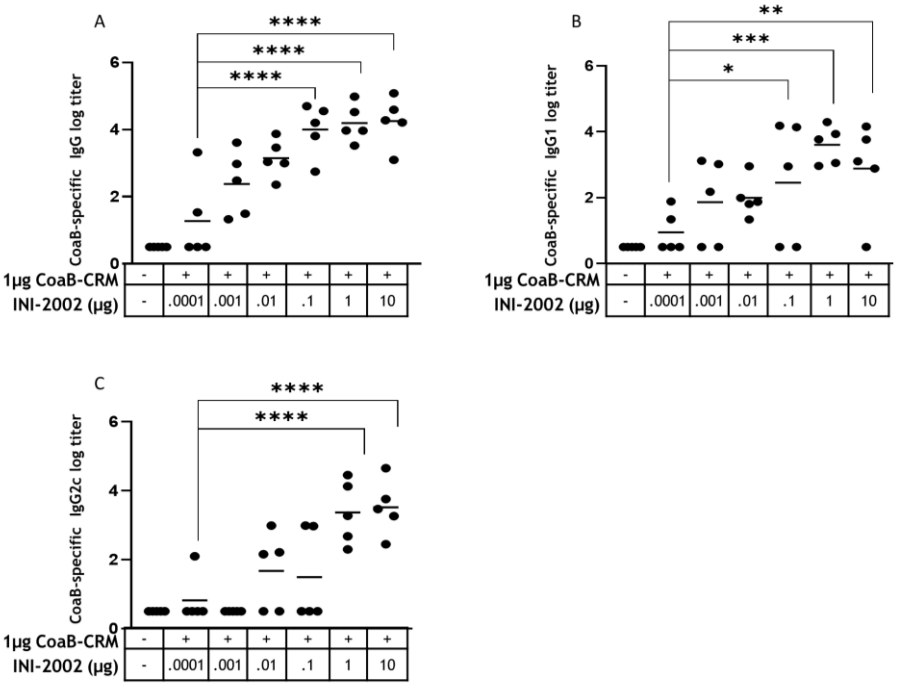


Figure 2-3. Anti-CoaB IgG, IgG1, and IgG2c antibody titers increase in a dose-dependent manner. C57 Bl/6 mice (10 or 15 per group) were injected once on day 0 and day 14 IM with 1 µg/mouse CRM-CoaB conjugate vaccine adjuvanted with INI2002 as indicated. Serum was collected on day 14 post-secondary vaccination. CoaB peptide specific IgG(a) IgG1(b), and IgG2c (c) antibody titers were measured by ELISA. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

2.3.4 INI-2002 adjuvant enhances humoral immunity across the antigen dose range and is antigen-dose sparing.

To determine if INI-2002 has an antigen-dose sparing effect and identify the optimal dose of CoaB-CRM, mice were vaccinated with 0.1 µg, 1.0 µg, or 10 µg of CoaB-CRM with or without 1 µg of INI-2002. Sera were collected at 14 days following the primary, secondary or tertiary vaccination to assess anti-CoaB antibody titers. Mice that received INI-2002 in combination with 0.1, 1 or 10 µg CoaB-CRM exhibited a statistically significant ($P<0.0001$) increase in anti-CoaB IgG antibody titers following a primary or secondary vaccination compared to dose-matched mice that received antigen alone (**Figure 2-4 A & B**). After three vaccinations, the addition of INI-2002 to CoaB-CRM resulted in a statistically significant boost when 0.1 or 1 µg CoaB-CRM was used when compared to unadjuvanted dose matched controls. However, the increase measured with 10 µg of CoaB-CRM did not reach significance (**Figure 2-4C**) when compared to the respective unadjuvanted group. The 0.1 µg dose of CoaB-CRM plus INI-2002 resulted in significantly ($p<0.0001$ post primary, $p<0.0001$ post-secondary) higher serum antibody titers compared to the 10 µg dose of CoaB-CRM (no adjuvant) post-primary and post-secondary but not post-tertiary. These data demonstrate at least a 100-fold antigen-dose sparing effect with the addition of INI-2002 adjuvant. Since 1 µg of CoaB-CRM in combination with 1 µg of INI-2002 resulted in significantly increased CoaB-specific antibody titers following a primary, secondary, or tertiary vaccination compared to antigen alone, this dose was selected as the lead vaccine formulation for further development.

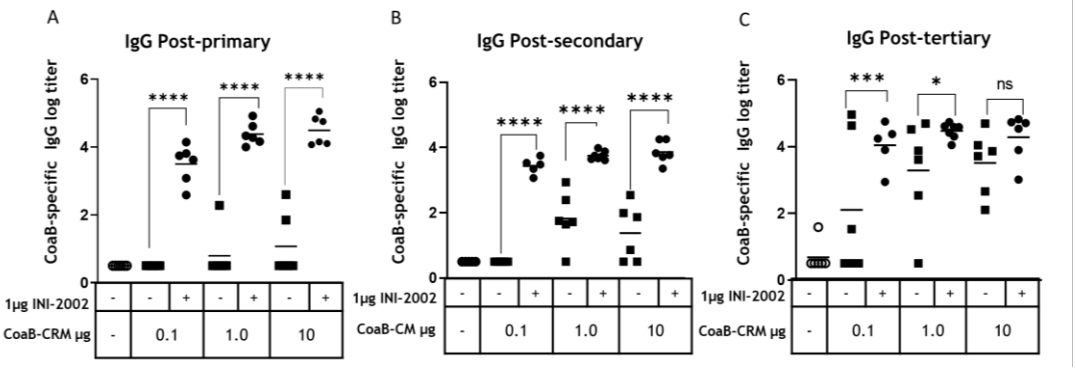


Figure2- 4. INI-2002 is antigen dose sparing and enhances CoaB specific antibodies across a wide range of CoaB-CRM doses. C57Bl/6 mice (10 or 15 per group) were vaccinated on day 0, day 14 and day 28 i.m. with CRM-CoaB conjugate as indicated with or without 1 µg INI-2002 . CoaB peptide specific IgG antibody titers were measured from serum collected on day 14 post-primary (a), day 14-secondary (b), and day 14 post-tertiary tertiary (c). Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

2.3.5 INI-2002 induces a balanced T-helper cell response with CRM

As noted above, the balanced anti-CoaB-specific IgG1 and IgG2c response suggested the lead vaccine combination induced a well-balanced T cell response to the associated conjugate. To explore the effects of INI-2002 on T cell help, the cell-mediated immune responses to the carrier protein (CRM) and peptide were assessed. Mice were vaccinated IM with 1 µg of CoaB-CRM and INI-2002 or CoaB-CRM alone on days 0 and 14. Spleens were harvested 5 days following the secondary vaccination, mechanically disaggregated, and re-stimulated with 5 µg/mL of CoaB-CRM or 5 ug/mL of the CoaB peptide for 72 hours. Culture supernatants were harvested and evaluated for secreted cytokines using a multiplex cytokine assay, MesoScale Discovery. Because the antigen is comprised of a well-established vaccine carrier protein (CRM197), we anticipated a robust cell-mediated immune response to the carrier and minimal-mediated responses to the peptide. Upon re-stimulation of splenocytes from vaccinated mice with the peptide alone, no increase in secreted cytokine was detected (**Supplemental Figure 1**). Splenocytes from animals vaccinated with 1 µg of CoaB-CRM plus 1 µg of INI-2002 and re-stimulated *ex vivo* with CoaB-CRM

exhibited an increased Th1 immune response, as demonstrated by increased IFNγ and TNFα cytokine production compared to their antigen alone and naïve counterpart (**Figure 2-5A,B**). Significantly increased IL-10 was also noted in antigen-plus-adjuvant-vaccinated animals, suggesting a balanced multi-functional T-cell response (**Figure 2-5C**)**.** These data, along with the IgG1 and IgG2c antibody titers (**Figure 2-3**), indicate that INI-2002 drives a balanced adaptive immune response when co-administered with CoaB-CRM.

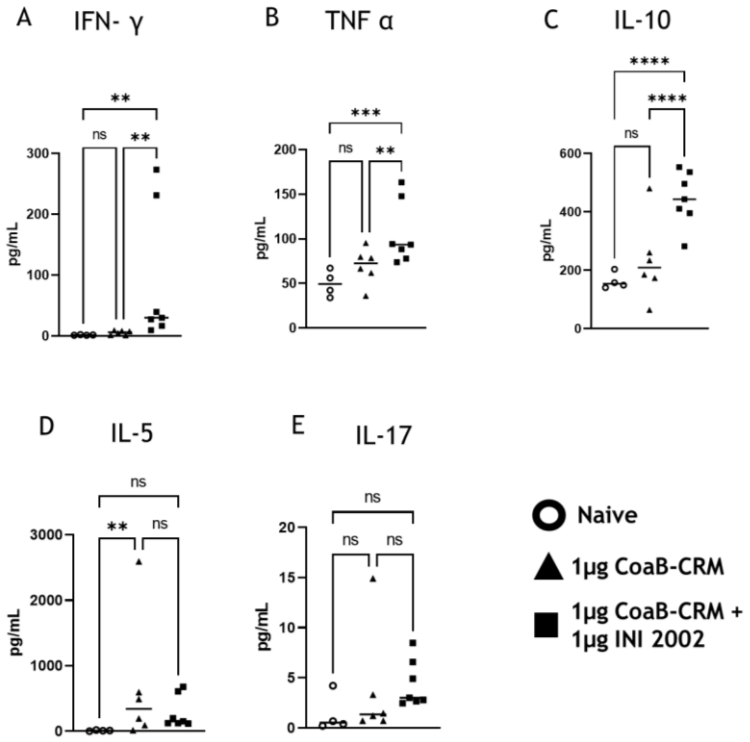


Figure 2-5. INI 2002 in combination with CoaB-CRM antigen promotes cell-mediated immunity to CoaB-CRM. At 5 days post-secondary vaccination mice were euthanized and spleens were harvested, disaggregated, and restimulated with 5 μg/mL of CoaB-CRM-197. The following cytokines: IFNγ (a), TNFα (b), IL-10 (c), IL-5 (d). IL-17 (e) were measured by MSD cytokine array. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = p≤0.05, ** = p≤0.01, *** = p≤0.001, **** = p≤0.0001.

2.3.6 Antibodies generated from CoaB-CRM-vaccinated animals recognize native Pf4 virions

The above data demonstrate that serum antibodies generated by vaccination of mice with CoaB-CRM or CoaB-CRM and INI-2002 bind to CoaB peptide. Next, we evaluated if the anti-CoaB peptide

antibodies were capable of binding to whole Pf4 virions. *P. aeruginosa* strain PAO1 was used to generate and purify Pf4 virions as described in the Materials and Methods. Pf4 virions were coated on ELISA plates and Pf4-specific IgG, IgG1 and IgG2c titers were measured by ELISA. Serum from mice vaccinated with the lead vaccine candidate had significantly higher Pf4-specific IgG ($p<0.0001$) (**Figure 2-6A**), IgG1 ($p<0.0005$) (**Figure 2-6B**) and IgG2c ($p<0.036$) (**Figure 2-6C**) antibody titers compared to mice vaccinated with CoaB-CRM alone or naïve mice. These data demonstrate that antibodies generated from CoaB peptide-vaccinated animals recognized and bound the Pf4 virions. Interestingly, while CoaB-CRM (no adjuvant) induced CoaB peptide-specific antibodies (see **Figures 2-2 and 2-4**), these antibodies did not measurably bind Pf4 virions suggesting that INI-2002 adjuvant altered both the titer and specificity of the polyclonal antibody response.

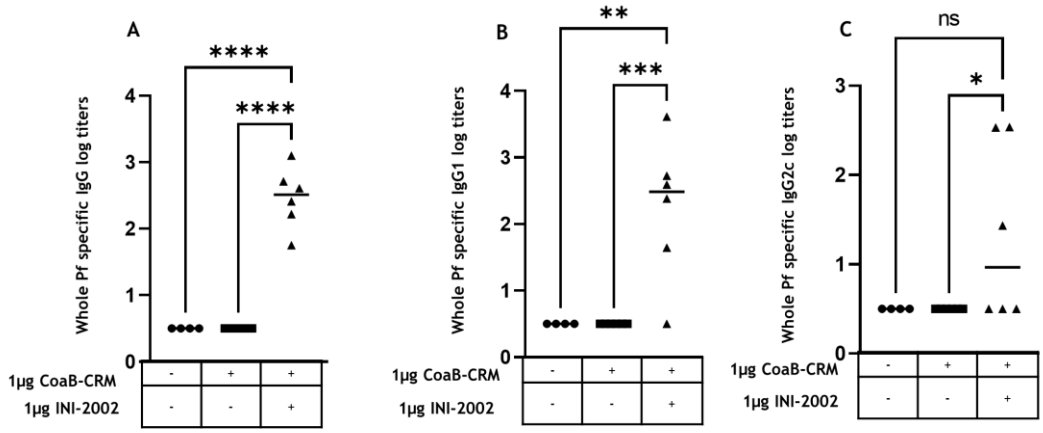


Figure 2- 6. Vaccination with CoaB-CRM + INI-2002 produces whole phage-specific antibodies Mice were vaccinated, IM, with 1 µg CoaB-CRM and 1 µg INI-2002 or 1 µg of CoaB-CRM alone on days 0 and 14. Mice were bled on day 28 and serum antibody titers against whole Pf virions were measured by ELISA. Whole phage specific A) IgG, B) IgG1, and C) IgG2c antibody titers were measured. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p\leq0.05$, ** = $p\leq0.01$, *** = $p\leq0.001$, **** = $p\leq0.0001$.

2.3.7 Anti-Pf4 antibodies disrupt the Pf4 replication cycle

Sweere et al. previously demonstrated that CoaB peptide-specific antibodies can protect mice from *P. aeruginosa* challenge [63]. To further these important findings, we sought to explore possible

mechanisms for this protection. We tested whether the antibodies present in serum from CoaB-CRM + INI-2002-vaccinated mice could disrupt the Pf4 replicative cycle. Sera from vaccinated mice, naive mouse serum, or 1X PBS were co-cultured with Pf4 virions for 1 hour. These cultures were then added to a culture of ΔPf4GmR (strain lacking the Pf4 prophage) at an OD_{600} of 0.3 to achieve an MOI of 1 virion per 1 x 10^4 bacterial cells and incubated for 18 hours. Supernatant was collected and spotted on lawns of PAO1 ΔPf4. The successful replication of Pf4 bacteriophages can be enumerated by the formation of plaques on PAO1 ΔPf4 lawns. Plaques were counted and plaque-forming units per mL (PFU/mL) were calculated. Cultures incubated with serum from animals vaccinated with INI-2002-adjuvated CoaB-CRM showed a significant ($p<0.001$) reduction in PFU/μL compared to cultures incubated with naive mouse serum (**Figure 2-7**). These data indicate that antibodies generated in mice immunized with CoaB-CRM + INI-2002 produced functional antibodies against Pf4, preventing antibody-bound virions from infecting *Pa* and disrupting the Pf4 phage replicative cycle.

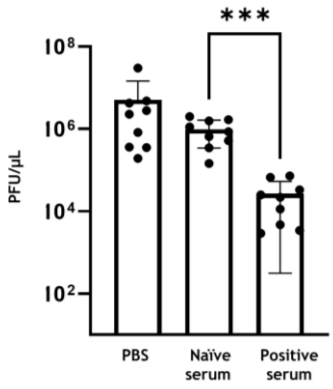


Figure 2-7. CoaB-specific antibodies disrupt Pf replication cycle. Pf bacteriophage was incubated with either PBS, sera from naïve animals, or sera from vaccinated animals (CoaB-CRM + INI-2002) and then spotted on a lawn of ΔPf:Pao1 for determination of PFUs. Each data point represents an average (PFU/mL) from technical triplicates and all data points collectively represent three separate experimental repeats. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p\leq0.05$, ** = $p\leq0.01$, *** = $p\leq0.001$, **** = $p\leq0.0001$

2.3.8 Discussion

The search for a vaccine against *Pa* infections has been ongoing for over 50 years with only three vaccine candidates reaching phase III clinical trials. One such vaccine candidate was Aerugen®, a vaccine comprised of Toxin A from *Pa* conjugated to *O*-polysaccharides. Intranasal administration of Aerugen® showed protection in murine burn wound and lung infection models, but unfortunately in clinical trials there was no significant difference between individuals treated with Aerugen® and those receiving the placebo [78], [79]. One other vaccine which showed promise was a bivalent vaccine derived from *Pa* flagellum. Unfortunately, this vaccine showed only 34% protection in acute infections and 55% protection in chronic infections during a phase III clinical trial [79]. *Pa* proteins offer an attractive vaccine target, but ultimately the challenge of these potential targets lies in *Pa*'s ability to reduce expression of certain proteins or mutations that arise in genes encoding *Pa* proteins to avoid detection by the adaptive immune system [62]. In addition, *Pa* evades the host immune system through the use of biofilms to sequester *Pa* from immune surveillance. As an alternative vaccine strategy we employed a CoaB peptide derived from a consensus sequence of the coat protein from Pf4 phage. This phage infects about 60% of *Pa* strains, enhances *Pa* virulence, contributes to biofilm formation, and increases *Pa* chronicity [65]. The development of an adjuvanted vaccine enhancing the humoral immunity to Pf4 phage could provide an alternative vaccine approach against *Pa* infections. The addition of a novel synthetic TLR4 agonist adjuvant, INI-2002, results in: a balanced immune response, significantly higher humoral immunity, and disruption of the Pf4 replication cycle [24], [25].

Another major finding reported here is that incorporating INI-2002 adjuvant allows for antigen-dose sparing without a reduction in antibody titer. With the incorporation of INI-2002, seroconversion is achieved after a primary injection at all antigen concentrations tested; whereas, in the CoaB-CRM antigen alone, seroconversion was only achieved after multiple vaccinations with 10-100 times higher antigen doses. Reducing antigen dose per vaccine means increased quantity of available vaccine during high demand. Additionally, reducing antigen concentration has been associated with increased affinity of antibodies and less likelihood of T-cell exhaustion post-vaccination compared to higher doses of antigen

[80], [81]. Dose-sparing vaccine strategies, such as the incorporation of INI-2002, can enhance protection while also limiting costs and improving vaccine efficacy. The efficient binding of antibodies from the CoaB-CRM plus INI-2002 group to whole Pf4 virions, and the lack of measurable binding from CoaB-CRM control serum, suggest an increased affinity and/or breadth of polyclonal responses with the addition of INI-2002 adjuvant.

Here, we showed that INI-2002-adjuvanted CoaB-CRM vaccination resulted in a balanced CoaB-specific adaptive immune response. Our collective data showed that when mice were vaccinated with 1μg of CoaB-CRM and 1μg of INI-2002, there was a balanced IgG1/IgG2c antibody production specific for the CoaB peptide and native phage. Additionally, vaccinated animals had a significant increase in TNF-α production (a pro-inflammatory cytokine), which has been implicated in enhanced clearance of *P a* in the lung [82]. Furthermore, IL-10 (an anti-inflammatory cytokine), which was also significantly increased in vaccinated animals, has been implicated in moderating inflammation and reducing tissue damage in *Pa* lung infections [83]. This balanced immune response produced by the vaccine may lead to reduction of tissue damage while simultaneously enhancing an appropriate inflammatory response.

The use of a peptide-based vaccine is not an entirely novel concept for *Pa*. Previous pre-clinical studies have used peptide-based vaccines derived from *Pa* proteins such as elastase and flagellar proteins to generate high antibody titers [84], [85]. Where our vaccine differs from previous studies is the targeting of a Pf4 bacteriophage, a highly conserved virulence factor for *Pa*. Sweere et al., previously demonstrated that a humoral immune response against the Pf4 phage results in protection against *Pa* and clearance of infection in a murine wound infection model [63]. A monoclonal antibody targeting the CoaB demonstrated increased protection in direct comparison to the CoaB-KLH + alum-adjuvanted vaccine indicating that an optimized vaccine could have additional benefits. Optimizing the CoaB-CRM conjugate and including a synthetic TLR4-based adjuvant, INI-2002, increased humoral immunity, promoted IgG2c antibody responses, and significantly increased binding of antibodies to Pf4 phage. The antibodies that bound Pf4 phage also had the functional capability to disrupt the replicative cycle of Pf4. We hypothesize that Pf4

phage-specific antibodies bind to the highly-conserved and exposed negatively-charged surface domain of Pf4 and reduce the efficiency in which Pf4 can enter and infect a *Pa* host. *In vivo*, this could result in reduced biofilm integrity, enhanced immune recognition and clearance of *Pa* harboring Pf4, and give the host a chance to clear the infection before a biofilm can be established.

Although animals dosed with our lead vaccine produced high phage-specific antibody titers with the ability to disrupt Pf4 phage replicative cycle, our study has some limitations; one such limitation is the use of a murine models. Although mice can generate valuable data regarding how the vaccine can influence the immune system, the ability to translate the data to the human immune response is limited. Additionally, CoaB epitope sequence homology data across a broad range of *Pa* clinical isolates suggests that targeting 2-4 clades of Pf may be necessary to improve the breadth of immunity in future studies[86].

For future work we wish to further characterize the immune-stimulatory properties of our peptide-based vaccine, and we will determine if the CoaB-CRM antigen in combination with INI-2002 will confer protection in a *Pa* challenge in both small and large animal models. As previous studies have shown, antibodies generated against the Pf4 phage are important in clearance of *Pa* from the host [63]. The antibody-mediated clearance can be attributed to multiple effector functions of antibodies, which can be addressed in future studies. Specifically, identifying which antibody subtypes have the ability to generate the best protection against *Pa* infection could guide efforts to further optimize a vaccine for enhanced breadth and durability through expansion of protective antibody subtypes.

As previous efforts to develop an effective vaccine against *Pa* have failed, novel approaches are required that look beyond traditional approaches to antigen targets. Our data demonstrate that CoaB-CRM in combination with INI-2002 generates high levels of phage-specific functional antibodies which have the potential to generate protection against a *Pa* challenge. Previous studies have suggested that engaging TLR4 enhances *Pa* bacterial clearance [75]. The use of INI-2002 may have additional benefits beyond increased antigen-specific antibody titers in a *Pa* infection setting. Preliminary data suggest that Pf4-specific antibodies can accelerate and promote clearance of *Pa*-infected wounds [63], thus enhancing Pf4-specific

antibodies through the use of INI-2002 as an adjuvant will likely increase cure rates or may prevent *Pa* colonization and biofilm formation. Thus, the creation of a vaccine which incorporates for the first time, a phage-based antigen in combination with a novel synthetic TLR4 agonist opens the door to vaccine approaches against multi-drug resistant *Pa* infections.

Chapter 3: Development of mucosal and multivalent vaccine formulations

3.1 Introduction

Hospital-associated cases of *Pseudomonas aeruginosa* infections rose by 32% from 2019 to 2020 [50]. Multi-drug resistant *P. aeruginosa* infections can occur at mucosal sites such as the eyes, sinus, and lung [52], [46]. Individuals with cystic fibrosis (CF) face challenges with lung colonization of antibiotic-resistant strains of bacteria such as *P. aeruginosa*, which has been found to colonize up to 60% of adults with CF[52]. Furthermore, *P. aeruginosa* infections account for up to 20% of ventilator-associated pneumonia [87]. *P. aeruginosa* infections are also the most common cause of ocular bacterial keratitis from the use of contact lenses [88].

Because *P. aeruginosa* infections occur frequently at mucosal sites, vaccines that enhance mucosal immunity against *P. aeruginosa* infections are needed [89]. The mucosal immune system serves to protect vulnerable tissues such as the lung and gut and is comprised of epithelial cells, immune cells and proteins such as secreted IgA antibodies [90]. IgA is the primary antibody found in the mucosa such as the gut and primarily functions to neutralize the pathogens ability to attach to epithelial cells [91]. Intranasal administration of vaccines against various *P. aeruginosa* proteins can enhance production of IgA antibodies and, promote clearance of *P. aeruginosa* lung infections in mice [89], [92]. Furthermore, the intranasal administration of TLR4 adjuvants has been shown to promote not only IgA production but also IL-17A cytokine production which resulted in the clearance of *P. aeruginosa* in a lung challenge mouse model [89]. It is for these reasons we have elected to design an intranasal vaccine using our CoaB-CRM antigen in combination with a TLR4 agonist. In chapter 3, we have identified an optimal intranasal dose of CoaB-

CRM antigen followed by optimal intranasal dose discovery of our TLR4 adjuvant INI-2004. INI-2004 was selected because of its ability to mount a mucosal response and its potency (data not yet published). The selected doses of INI-2004 and CoaB-CRM increases mucosal IgA and sera IgG antibody levels and induced a balanced cytokine response.

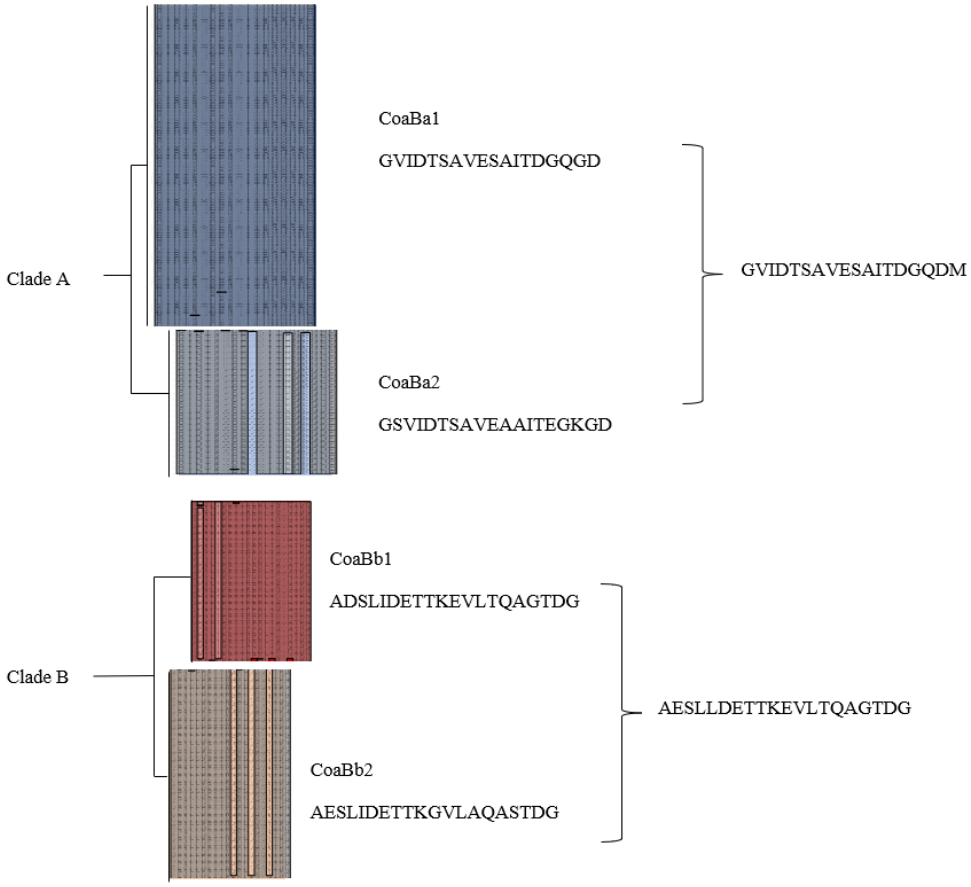


Figure 3.1 Major Coat protein peptide can be divided into two clades: Schematic of a phylogenetic tree depicting major clades and minor clades of CoaBa and CoaBb. Consensus sequence of CoaBa1 minor clade is GVIDTSAVESAITDGQGD, consensus sequence of CoaBa2 minor clade is GSVIDTSAVEAAITEGKGD. Consensus sequence of CoaBb1 minor clade is ADSLIDETTKEVLTQAGTDG and consensus sequence of CoaBb2 minor clade is AESLIDETTKGVLAQASTDG. Consensus sequence of major clade CoaBa is GVIDTSAVESAITDGQDM and consensus sequence of major clade CoaBb is AESLLDETTKEVLTQAGTDG.

Humanity witnessed first-hand the importance of vaccines inducing cross-protective immunity against variants of SARS CoV-2; as such, it is important to consider the diversity of Pf bacteriophage in

designing a broadly protective vaccine [93]. Clades are genetic groupings of organisms (such as virus) which can be divided based on protein sequence homology [94]. These sequence differences can visually be demonstrated using a phylogenetic tree [94]. In the case of Pf bacteriophage, the acidic epitope of CoaB can be grouped into two major clades, Clade A and Clade B (Figure 3.1).

To this point we have established a robust immune response against a CoaBa- derived peptide (data found in previous chapter), but because Pf bacteriophage CoaB acidic epitope can be divided into two genetically distinct clades, we wanted to incorporate a Pf peptide conjugate from Clade B. Creating heterogeneous conjugates to broaden the immune response in other disease systems have been used before[95], [96]. With this backdrop we decided to create a multivalent vaccine strategy wherein both CoaBa and CoaBb were conjugated to the same CRM-197 protein and compare it to an admixed (CoaBa and CoaBb conjugated separately to CRM-197) vaccine approach. At the conclusion of these studies, we identified that antibodies specific for one clade do not cross-react with peptide from the other clade. Furthermore, mice vaccinated with peptide-conjugates from both clades in combination with INI-2002 generated robust antibody titers specific for peptides from both clades. The collective data featured in this chapter provide evidence that further modifications of Pf bacteriophage-targeted vaccine enhances mucosal immunity as well as broaden immunity against diverse Pf CoaB epitopes.

3.2 Materials and Methods

INI-2002 compound synthesis

Previously described in material and methods section from Chapter 2.

INI-2004 compound synthesis

Previously described [97]

CoaBa conjugate to CRM

Previously described in materials and methods from Chapter 2

CoaBb conjugate to CRM

In order to conjugate CRM-197 (provided by Inimmune Corporation, Missoula MT) to CoaB (peptide sequence AESLLDETTKEVLTQAGTDG) (Anaspec), the linker N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS; Thermo Scientific) was dissolved in DMSO (Sigma) at a concentration of 25 mg/mL. To achieve 15-17 copies of GMBS to CRM-197, 85 equivalents of GMBS/DMSO solution to CRM-197 was incubated in a water bath for 30 minutes at 25°C. Remaining GMBS was removed by ultrafiltration using 30 kDa Amicon (Millipore) Ultra centrifugal filters and washed four times using 0.2 M Sodium phosphate buffer (pH=7.2). MALDI TOF MS was used to determine amount of GMBS added to CRM-197. The activated CRM-197 was added to 40 molar equivalents of peptide at a concentration of 1.5 mg/mL and incubated for two hours at 25°C. Remaining unattached peptide was removed by ultrafiltration using 30 kDa Amicon Ultra centrifugal filters and washed 4 times with 10mM Phosphate Buffer. The peptide-CRM conjugate was sterile filtered using a 0.22 μm PVDF syringe filter. MALDI TOF MS was used to determine peptide copy number.

Multivalent CoaBb and CoaBa conjugate to CRM

Methods were previously described in Chapter 2 methods section but with the following modification: For the preparation of multivalent peptide-CRM conjugate, an equimolar solution of peptides was prepared first in 0.2M NaPB pH 7.2, before activated CRM was added to the mixed peptide solution. The relative excess of peptide was held constant at 40 molar equivalents.

Admixed CoaBb and CoaBb conjugate to CRM

Methods were previously described in Chapter 2 methods section but with the following modifications: Admixed preparations were made by adding monovalent conjugates at a 1:1 ratio.

P. aeruginosa and Pf4 Propagation

Previously described in materials and methods from Chapter 2.

C57Bl/6 treatment In vivo experiments

Animal studies were carried out in accordance with University of Montana's IACUC guidelines for the care and use of laboratory animals. For murine vaccine studies, groups of 6-10 female C57Bl/6 mice were vaccinated intramuscularly (right gastrocnemius) on days 0 and 14 with the indicated dose of INI-2002 (TLR4 adjuvant) and CoaB-CRM conjugate in a volume of 50 µL per injection. Compounds and antigen were diluted as needed in 2.5% glycine in 1X PBS. At 14 days following each vaccination, blood samples were collected via submandibular vein (d14) or cardiac puncture (d28). In the case of intranasal administration, mice were given 80-100mg/kg of Ketamine and 80-100mg/kg of Xylazine via intraperitoneal administration followed by 10µL per nare of antigen +/- INI-2004 for a total of 20µL per mouse. For evaluation of the cell-mediated immune response, spleens were harvested from a subset of mice on day 19 (5 days post booster vaccination). For some experiments, a third booster vaccination was administered on day 28, and blood was collected on day 42 for evaluation of humoral immunity.

ELISA for anti-CoaB peptide antibody quantification

Previously described in materials and methods section from Chapter 2

Phage ELISA

Previously described in materials and methods section from Chapter 2

Cell-mediated immunity analysis

Previously described in material and methods section from Chapter 2

3.3 Results

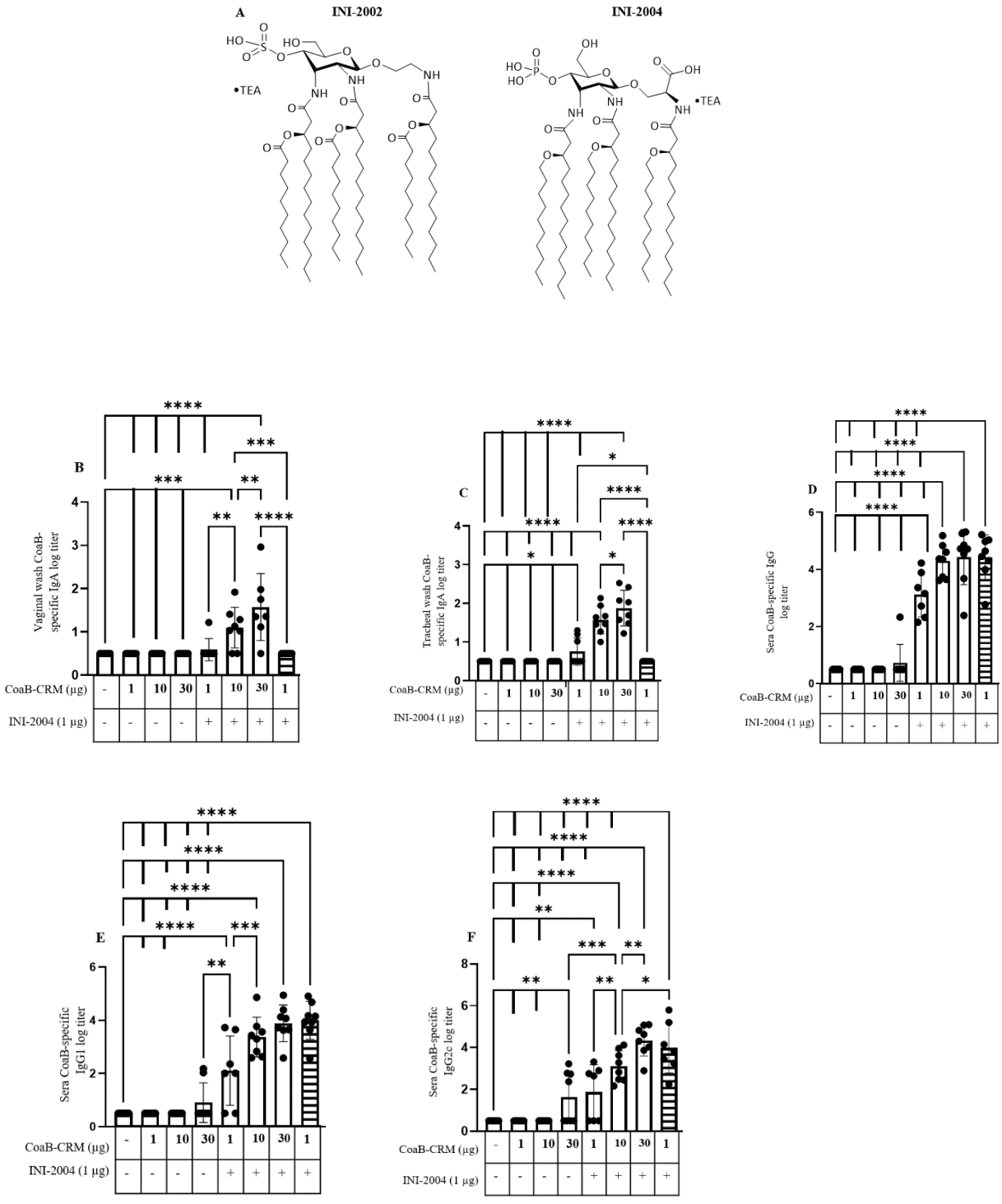


Figure 3-2. Intranasal administration of the TLR4 agonist INI-2004 enhances CoaB-specific IgA production in mucosa and CoaB-specific IgG in sera. C57 Bl/6 mice (8 per group) were administered vaccine on days 0 and 14 intranasally or intramuscularly (indicated by the horizontal strips) with CRM-CoaB conjugate vaccine adjuvanted with 1 µg/mouse INI-2004 as indicated. Serum

and washes were collected on day 14 post-secondary vaccination. (a) structures of INI-2002 and INI-2004, CoaB peptide specific (b) IgA from vaginal washes, (c) IgA from tracheal washes, (d) IgG from sera, (e) IgG1 from sera, and (f) IgG2c from sera antibody titers were measured by ELISA. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

3.3.1 Intranasal administration of the TLR4 agonist INI-2004 enhances CoaB-specific IgA production in mucosa and CoaB-specific IgG in sera.

Because *P. aeruginosa* has the ability to infect various mucosal sites, we wanted to further characterize how the Pf-targeted vaccine affected mucosal antibody responses via intranasal route of administration [52], [87], [88]. To this end we selected INI-2004, a similar TLR4 agonist to INI-2002 (**Figure 3-2A**). We selected INI-2004 because other lipid A mimetics with a similar structure as INI-2004, when administered intranasally, have induced pro-inflammatory cytokine production in the lung and induced production of mucosal-associated IgA antibodies [98]. Additionally, INI-2004 when compared to INI-2002 is more potent and is currently in Phase I clinical trials for IN delivery for seasonal allergies (data not shown). Mice were vaccinated with various doses of CoaBa-CRM and 1 µg of INI-2004 strictly intranasally or intramuscularly (indicated by the horizontal stripped bar); 14 days post-secondary vaginal washes and tracheal washes were collected as well as sera. Mice administered intranasally with both INI-2004 and 10µg, 30µg of CoaB-CRM had statistically higher CoaB-specific IgA antibodies from both the tracheal washes and vaginal washes than groups without adjuvant as well as the group receiving 1µg of INI-2004 and 1µg of CoaB-CRM via intramuscular administration (**Figure 3-2B,C**). Additionally, mice receiving 10µg, 30µg of CoaB-CRM in combination with INI-2004 intranasally as well as mice receiving 1µg of CoaB-CRM in combination with INI-2004 intramuscularly had robust anti-CoaB IgG titers which were significantly higher when compared to the unadjuvanted groups (**Figure 3-2D**). Mice which received 1µg, 10µg, 30µg of CoaB-CRM intranasally and mice receiving 1µg of CoaB-CRM intramuscularly in combination with INI-2004 had statistically higher CoaB-specific IgG1 and IgG2c production than mice which received antigen alone. The data also suggest that within these same adjuvanted groups the production of IgG1 and IgG2c is balanced

(**Figure 3-2E,F**). These data demonstrate that higher doses of CoaB-CRM administered with INI-2004 intranasally not only result in murine production of CoaB-specific IgA statistically 10µg dose (P=0.0004), 30µg dose (P<0.0001) higher than mice receiving 1µg of CoaB-CRM in combination with INI-2004 intramuscularly but also produce CoaB-specific IgG just as well as with an intramuscular administration.

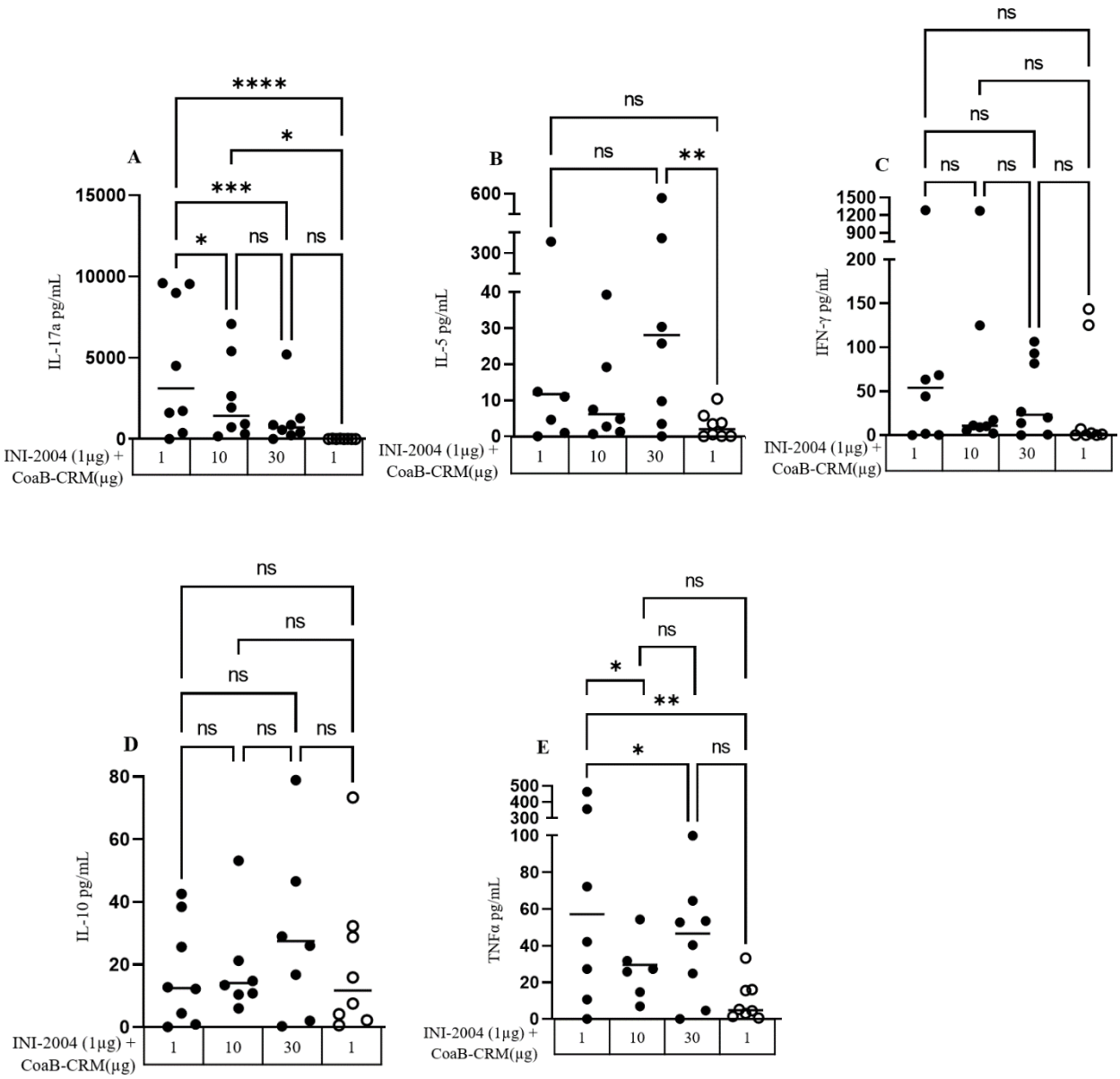


Figure 3-3. Intranasal administration using a potent TLR4 agonist with lowest dose of CoaB-CRM enhances production of pro-inflammatory cytokines while higher dose of CoaB-CRM enhances anti-inflammatory cytokine IL-5. At 14-day post-secondary vaccination mice were euthanized. Cervical lymph nodes from intranasally administered mice and inguinal and popliteal lymph nodes were harvested from mice vaccinated intramuscularly (open circles), disaggregated, and restimulated with 5 µg/mL

of CoaB-CRM-197. The following cytokines: IL-17a (a), IL-5 (b), IFNγ (c), IL-10 (d), TNFα (e) were measured by MSD cytokine array. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3.3.2 Intranasal administration of INI-2004 and 1µg CoaB-CRM enhances production of Th1 and Th17 associated cytokines while 30µg of CoaB-CRM enhances Th2 associated cytokine IL-5.

In addition to identifying effects of intranasal administration of INI-2004 on the humoral immune response, we also sought to understand how intranasal administration of INI-2004 influenced the cell-mediated response. We hypothesized that the antigen would enhance production of pro-inflammatory cytokines in a dose-dependent manner. To this end, single-cell suspensions re-stimulated with CoaB-CRM, were analyzed for presence of pro-inflammatory and anti-inflammatory cytokines. Generally, mice vaccinated with 1µg of INI-2004 and 1µg CoaB-CRM intramuscularly trended lower in measured cytokine responses. Surprisingly, mice administered 1µg of CoaB-CRM and 1µg of INI-2004 intranasally had statistically higher TNFα and IL-17 production compared to the other groups (**Figure 3-3A,E**). Moreover, although not statistically significant; the production of IFNγ trended higher in 1µg of CoaB-CRM and 1µg of INI-4001 administered intranasally (**Figure 3-3C**). We were also surprised to find that IL-5 production was highest from the group which received 30µg of CoaB-CRM and 1µg of INI-2004 intranasally (**Figure 3-3B**). This is an interesting observation, since at the 30µg dose of CoaB-CRM resulted in a balanced CoaB-specific IgG1 and IgG2c response. These data collectively suggest that the 1µg dose of both INI-2004 and CoaB-CRM administered intranasally will enhance production of pro-inflammatory cytokines.

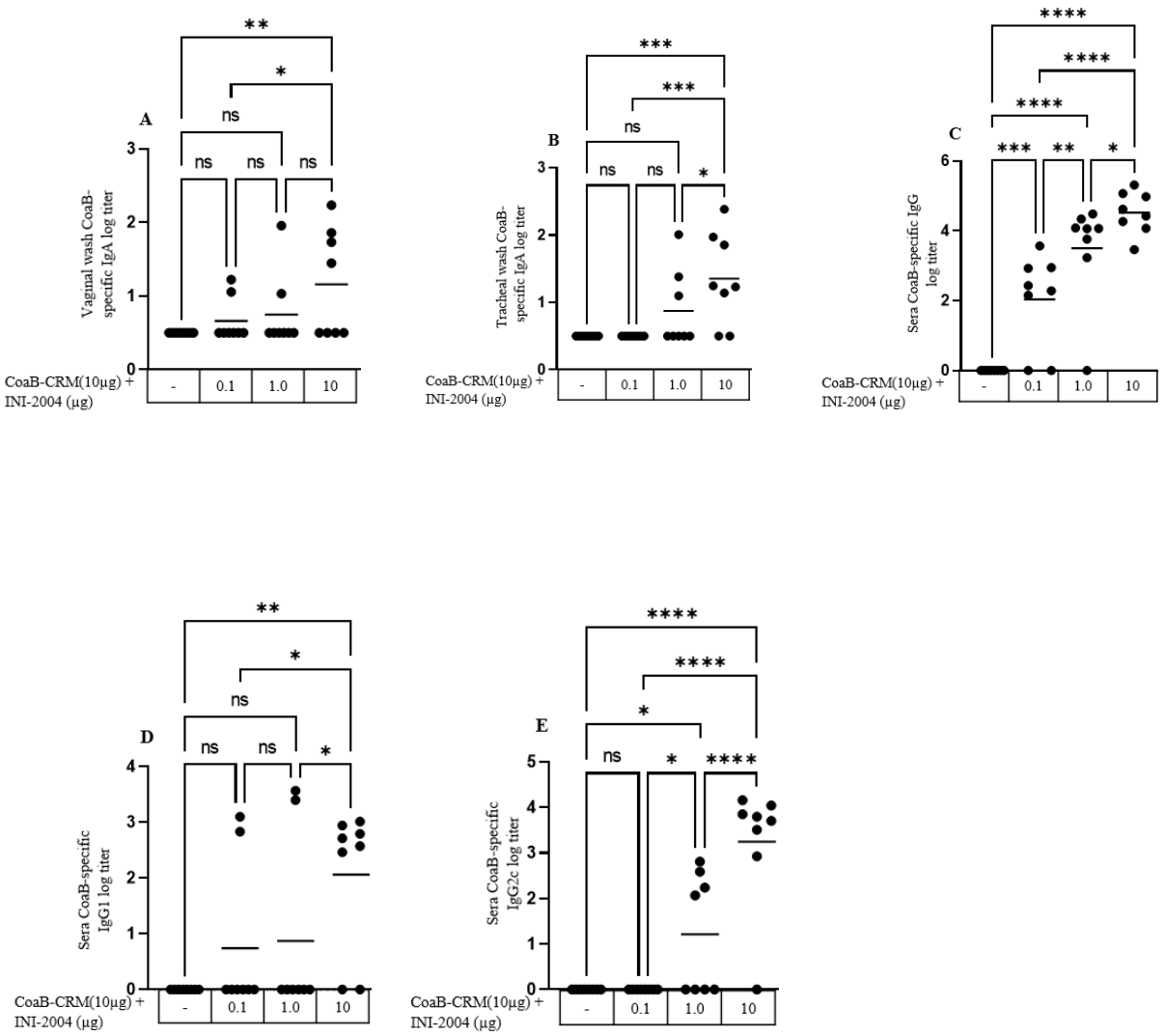


Figure 3-4. 10µg dose of INI-2004 administered intranasally enhances CoaB-specific IgG in sera as well as mucosa CoaB-specific IgA. C57 Bl/6 mice (8 per group) were administered once on day 0 and day 14 intranasally with 10µg of CRM-CoaB conjugate vaccine adjuvanted with INI-2004 as indicated. Serum and washes were collected on day 14 post-secondary vaccination. (a) IgA from vaginal washes (b) IgA from tracheal washes (c), and IgG from sera (d) IgG1 from sera and (e) IgG2c from sera antibody titers were measured by ELISA. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

3.3.3 Intranasal administration of INI-2004 enhances CoaB-peptide specific serum IgG and mucosal IgA titers.

The observation that both 10µg and 30µg of CoaB-CRM administered intranasally resulted in high CoaB-specific mucosal IgA and sera IgG suggests that the higher doses of antigen can achieve higher titers of CoaB-specific antibodies. To further guide on which dose of CoaB-CRM would be selected for sequential INI-2004 dose studies we looked to the cell-mediated cytokine response. As previously shown, higher doses of CoaB-CRM resulted in reduced pro-inflammatory cytokine production. Because the 10 µg dose resulted in higher magnitude of CoaB-specific antibodies and the cell-mediated response was more balanced at this dose, we selected 10µg of CoaB-CRM as our antigen dose for the INI-2004 dose-response studies. We hypothesized antibody responses would be dose-dependent and would increase with increased INI-2004 dose. Mice were either vaccinated with 0.1,1, or 10µg of INI-2004 in combination with CoaB-CRM and harvested at14 days post-secondary treatment. Mice treated with 10µg of INI-2004 in combination with CoaB-CRM had significantly higher CoaB-specific IgA antibody responses compared to other groups from both vaginal and tracheal washes (**Figure 3-4A,B**). Additionally, mice vaccinated with the highest dose in this study had statistically higher sera-derived CoaB-specific IgG, IgG1 and IgG2c compared to the other groups of treated mice (**Figure 3-4C,D,E**). These data collectively suggest that mice administered intranasally with 10µg of CoaB-CRM and 10µg of INI-4001 will have a robust production of not only mucosal CoaB-specific IgA but also sera-derived CoaB-specific IgG, IgG1 and IgG2c.

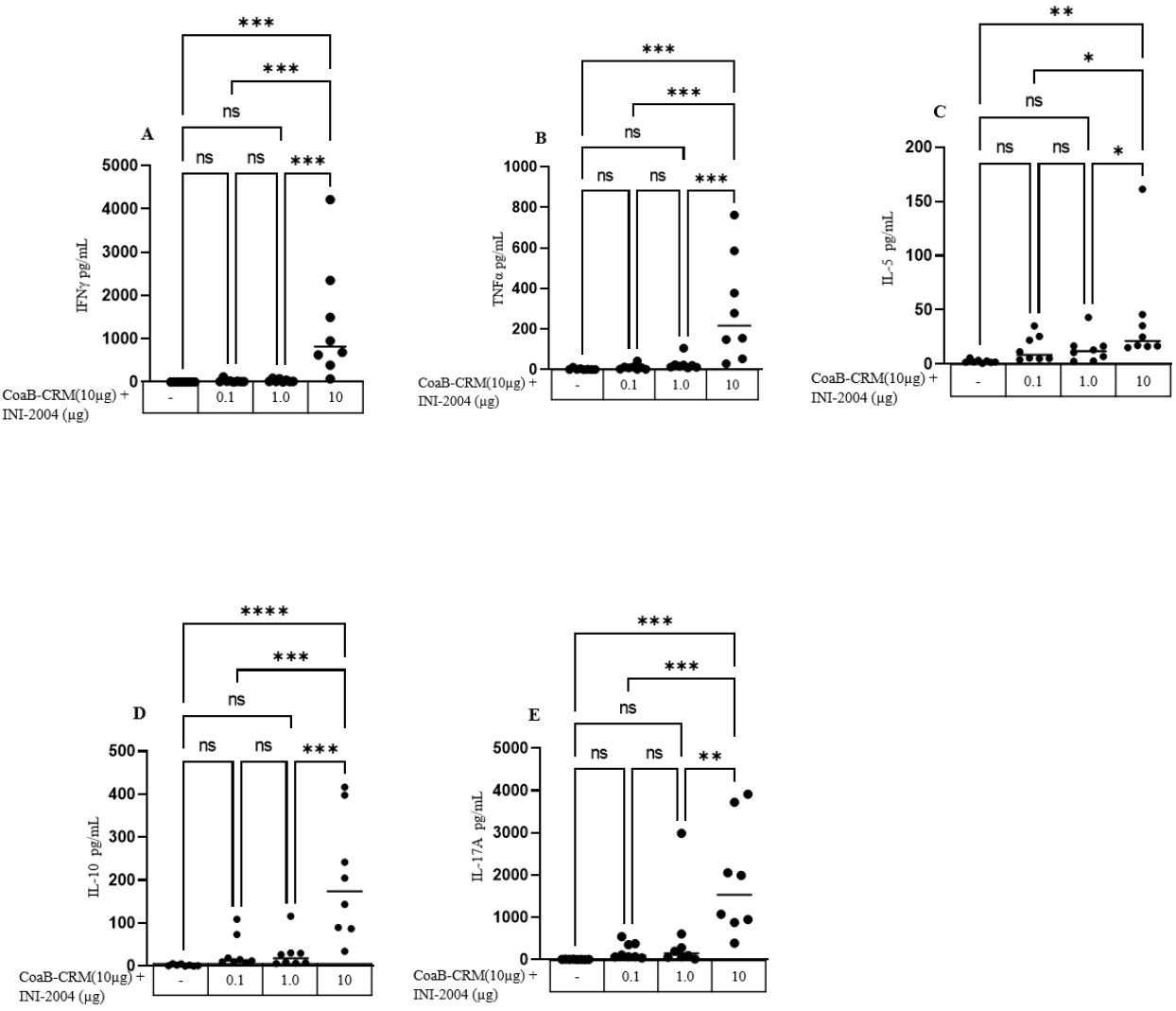


Figure 3-5. Intranasal administration using potent TLR4 agonist with highest dose of CoaB-CRM enhances production of balanced T-helper response. At 14-day post-secondary vaccination mice were euthanized and cervical lymph nodes were harvested from mice vaccinated intranasally, disaggregated, and restimulated with 5 µg/mL of CoaB-CRM-197. The following cytokines: IFNγ (a), TNFα (b), IL-5 (c),IL-10 (d), IL-17a (e) were measured by MSD cytokine array. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3.3.4 Intranasal administration using highest dose of potent TLR4 agonist enhances production of balanced T-helper cell response

We then asked how does INI-2004 dose influence the cell-mediated response? Cervical lymph-nodes were disassociated and single cell suspensions were re-stimulated with CoaB-CRM. Finally, we used a five-panel Mesoscale discovery cytokine kit to identify differences in cytokine production from treated groups. Mice treated with a 10μg dose of INI-2004, in combination with 10μg CoaB-CRM, had statistically significant increases in TNFα, IFN-γ, as well as IL-17a production compared to all of the other treated groups (**Figure 3-5A,B,E**). Surprisingly, mice treated with CoaB-CRM in combination with 10μg of INI-2004 not only had high Th1 and Th17 immune cytokine response but also had statistically significant increase ($P<0.05$) in IL-10 and IL-5 cytokine production compared to the other groups (**Figure 3-5C,D**). These data recapitulate the balanced humoral response seen in Figure 3-4D,E and demonstrate that mice treated intranasally with 10μg of CoaB-CRM in combination with 10μg of INI-2004 produce a balanced T-helper cell-mediated response and balanced humoral response.

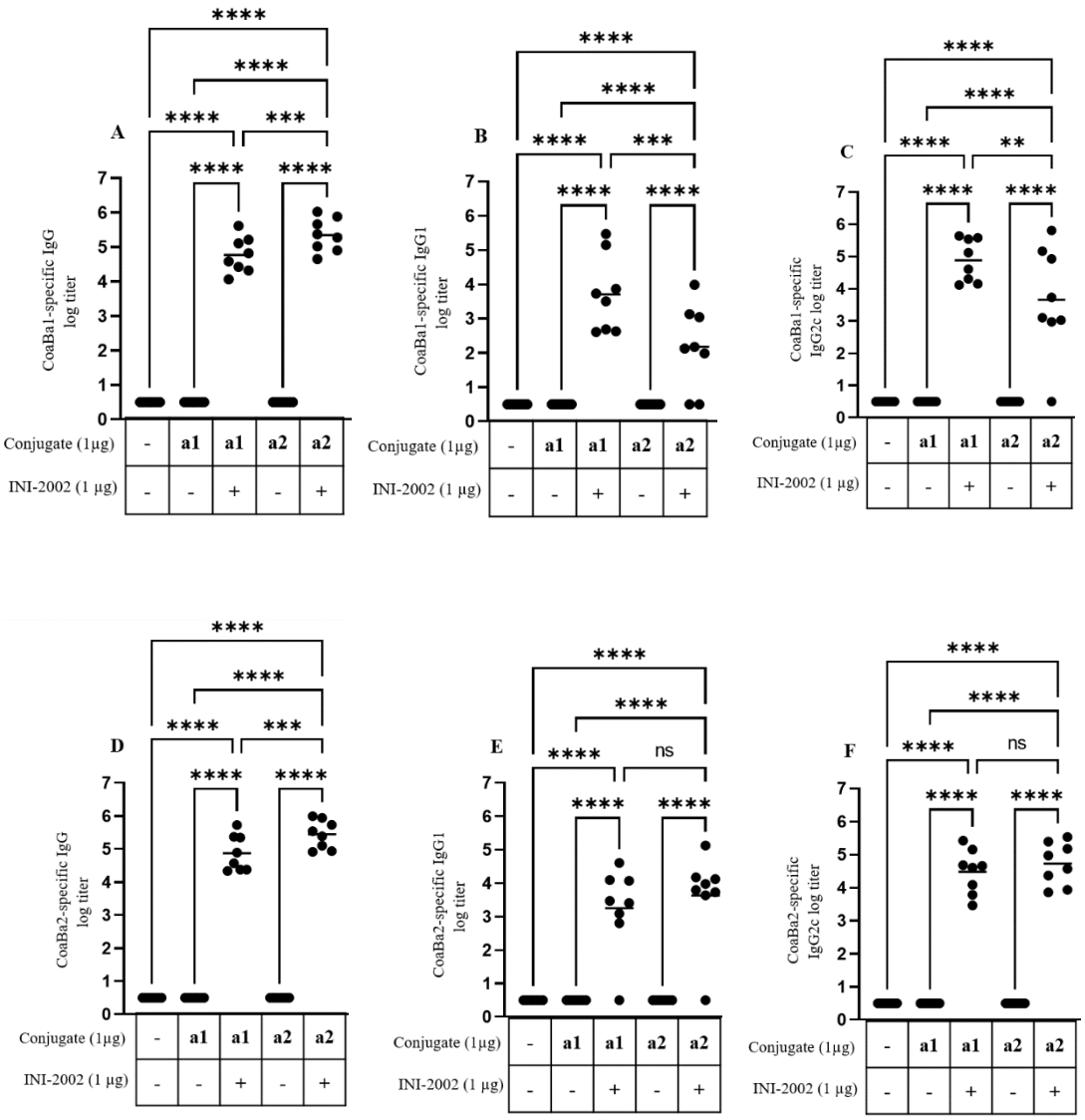


Figure 3-6, Mice vaccinated with Pf peptide from clade A generate antibodies reactive to other peptides within the same clade. Mice were vaccinated twice, IM, with 14 days between vaccinations. After the second vaccination, CoaBa1 IgG (A), IgG1 (B) and IgG2b (C) were measured as well as CoaBa2 IgG (D), IgG1 (E) IgG2c (F). Asterisks denote statistical significance; color of asterisks correspond to comparison group. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3.3.5 Mice vaccinated with Pf peptide from clade A generate antibodies reactive to other peptides within the same clade.

After establishing dose responses of CoaB-CRM and both TLR4 agonists for multiple routes of vaccine administration, we next sought to identify how genetic variance of Pf bacteriophage could influence vaccine efficacy. To this end, we found that Pf bacteriophage CoaB-peptide can genetically be categorized into two major clades with each clade containing two minor clades. For these next studies, we elected to vaccinate via intramuscular administration to establish proof-of-concept before introducing other vaccination routes. We first asked the question if antibodies generated could cross-react with CoaB from within the same clade. We hypothesized that mice vaccinated with INI-2002 and CoaB-CRM would generate antibodies that would cross-react with CoaB-peptide from the same major clade. Results showed that mice receiving 1µg INI-2002 in combination with CoaBa1 or CoaBa2 had significantly higher CoaBa1-specific IgG antibodies than groups that received antigen alone. CoaBa1-specific IgG antibodies from mice vaccinated with peptide CoaBa2 cross-reacted with peptide CoaBa1 and exhibited high titers against CoaBa1 (**Figure 3-6A,D**). All groups receiving 1µg INI-2002 in combination with antigen had significantly higher CoaBa1-specific IgG1 and IgG2c antibodies than groups that received antigen alone (**Figure 3-6B,C**). After establishing that CoaBa2-specific antibodies could cross-react with CoaBa1 we examined whether the reverse was true. Here, mice vaccinated with 1µg INI-2002 in combination with CoaBa2-CRM demonstrated significantly higher CoaBa2-specific IgG antibodies than groups which received antigen alone **(Figure 3-6D**). CoaBa2-specific serum IgG antibodies from mice vaccinated with CoaBa2 with INI-2002 cross-reacted with peptide CoaBa1 and exhibited high titers (**Figure 3-6D**). Mice vaccinated with INI-2002 in combination with conjugate CoaBa1-CRM or CoaBa2-CRM had high CoaBa2-specific IgG1 and IgG2c antibodies (**Figure 3-6E,F**). These data collectively provide evidence that mice vaccinated with peptides from clade A generate antibodies that can react to different peptides from within the same clade.

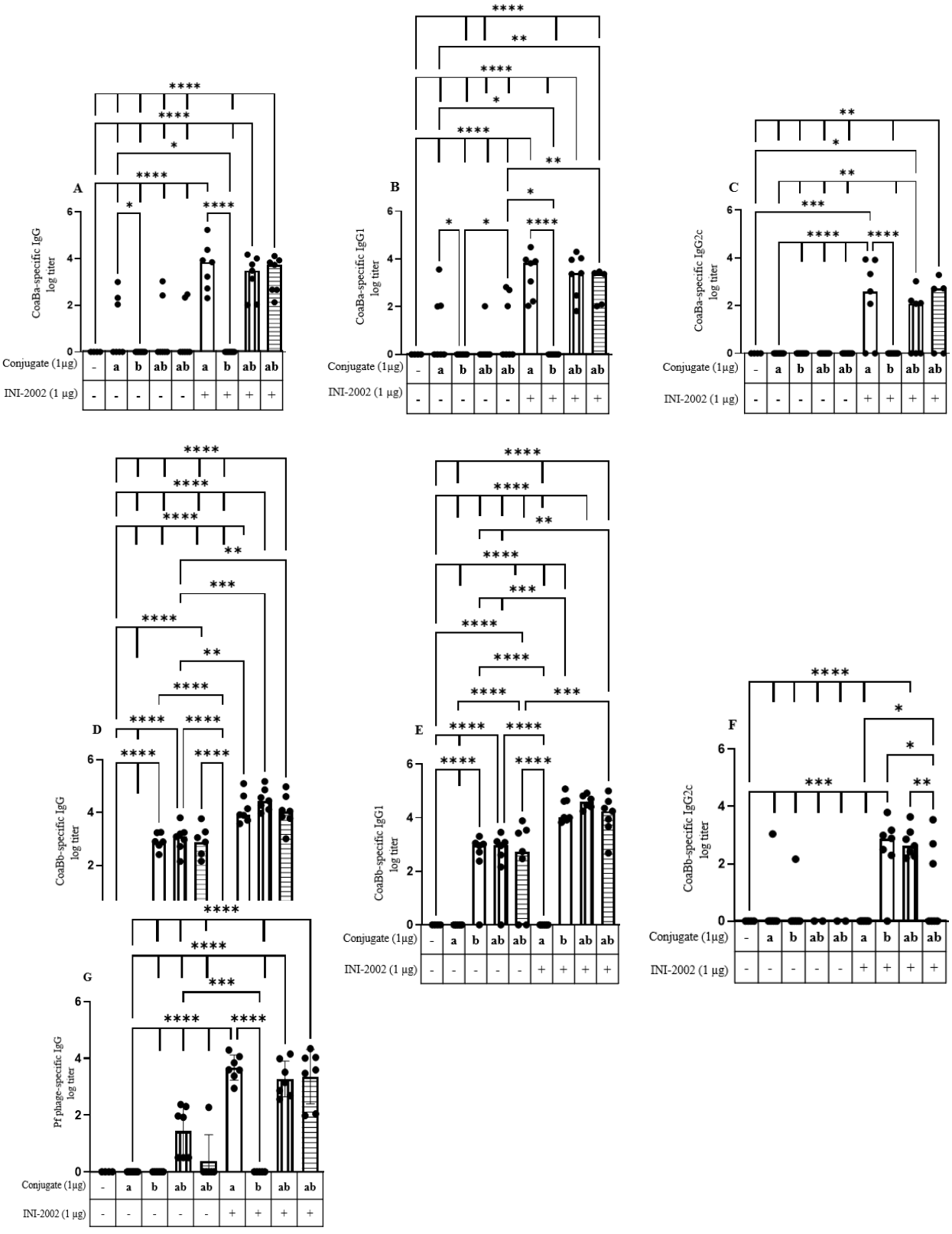

A
CoaBa-specific IgG log titer
B
CoaBa-specific IgG1 log titer
C
CoaBa-specific IgG2c log titer
D
CoaBb-specific IgG log titer
E
CoaBb-specific IgG1 log titer
F
CoaBb-specific IgG2c log titer
G
Pf phage-specific IgG log titer
Conjugate (1µg) - a b ab ab a b ab ab
INI-2002 (1 µg) - - - - - + + + +

Figure 3-7 Mice vaccinated with multivalent or admixed conjugate generate antibodies specific for both clades. Mice were vaccinated twice, IM, with 14 days between vaccinations. Multivalent antigen-treated mice are indicated with vertical striped bars and mice vaccinated with an admixed conjugate are indicated by horizontal striped bars. After the second vaccination, CoaBa IgG (A), IgG1 (B) and IgG2b (C) were measured as well as CoaBb IgG (D), IgG1 (E) IgG2c (F), and Pf4-specific IgG (G) antibodies. Asterisks denote statistical significance; color of asterisks correspond to comparison group. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3.3.6 Mice vaccinated with multivalent or admixed conjugate generate antibodies specific for both CoaBa and CoaBb epitopes. After establishing that antibodies from mice vaccinated with INI-2002 in combination with CoaB recognize antigen from within the same clade we hypothesized that CoaBa specific antibodies would not cross-react with CoaBb peptide and vice versa. To test this hypothesis, we created two distinct antigen (multivalent and admixed) conjugates to determine if either conjugation strategies would generate both CoaBa- and CoaBb-specific antibodies. The multivalent conjugate is produced by conjugating both CoaBa and CoaBb peptide to the same CRM protein. On the other hand, the admixed conjugate has CoaBa peptides conjugated to separate CRM proteins and mixing equal amount CoaBb-CRM peptide for each antigen. Mice vaccinated with INI-2002 in combination with CoaBa alone, admixed or multivalent all produced statistically higher CoaBa-specific IgG than the antigen-alone counter-parts. Furthermore, mice vaccinated with CoaBb in combination with INI-2002 did not produce antibodies which could cross-react with CoaBa peptide. When we compared adjuvanted admixed and adjuvanted multivalent vaccinated mice, both groups did not differ in CoaBa-specific IgG response (**Figure 3-7A**). When we measured CoaBa-specific IgG1 and IgG2c, groups that received adjuvant also had statistically higher antibody response than their respective antigen alone groups. Furthermore, there was no cross-reactivity between CoaBb-specific IgG1 or IgG2c antibodies with CoaBa peptide and no difference in IgG1 or IgG2c antibody response between admixed treated or multivalent treated mice (**Figure 3-7B,C**). After measuring CoaBa-specific antibodies from the various treated groups, our next step was to measure CoaBb-specific antibodies. Once again, mice treated with INI-2002 had much higher CoaBb-specific IgG, IgG1 and IgG2c than unadjuvanted groups (**Figure 3-7D,E,F**). Mice that were vaccinated with CoaBa, independently of

adjuvant, did not produce antibodies which could cross-react with CoaBb peptide. Mice vaccinated with either admixed or multivalent in combination with INI-2002 also had similar CoaB-specific IgG, IgG1 and IgG2c antibody responses. Interestingly, CoaBb-specific antibody titers from CoaBb , admixed , and multivalent (without adjuvant) were higher than CoaBa-specific antibodies for the antigen (no adjuvant) treated groups, suggesting that peptide CoaBb is more immunogenic than peptide CoaBa (**Figure 3-7A,B,D,E**). Finally, when we tested IgG responses against whole phage virions from clade-A, mice that received CoaBa, admixed conjugate, or multivalent conjugate in combination with INI-2002, had a significant boost in IgG phage-specific antibody response compared to antigen alone. Mice vaccinated with CoaBb conjugate independent of adjuvant-status did not produce antibodies which could cross-react with Clade-A phage (**Figure 3-7G**). These data suggest that antibodies generated against CoaBa will not cross-react with CoaBb-peptide and vice versa, regardless of incorporation of adjuvant. These data also suggest the use of either multivalent or admixed preparations in combination with INI-2002 will produce similar antibody responses.

3.3.7 Discussion

In the previous chapter we demonstrate that vaccinating mice using INI-2002 in combination with CoaB-CRM resulted in high CoaB-specific antibody titers as well as a balanced T-helper cell response. Additionally, antibodies generated from vaccinated mice were able to recognize Pf4 virions and disrupt the Pf4 replication cycle. Generating sera CoaB-specific IgG antibodies has been associated with improved clearance of PaO1 dorsal burn-wounds [53]. This work has the potential to broaden the application of this vaccine to include vaccine-strategies which can lead to enhanced mucosal immunity. Enhancement of Pf-specific mucosal antibodies could have the potential to clear *P. aeruginosa* infections in patients with cystic fibrosis. Furthermore, the alteration of the original conjugate to a heterogeneous conjugate could lead to a broader immune response against Pf bacteriophage.

Intranasal vaccines have been extensively used in pre-clinical settings with some reaching FDA approval [99],[100]. Intranasal vaccines have the ability to enhance mucosal immunity as well as systemic

immunity against pathogens[100]. An intranasal Pf-targeted vaccine for cystic fibrosis patients could provide a boost in mucosal immunity in the lung and enhance clearance of *P. aeruginosa* infections. As previously mentioned, Pf bacteriophage promotes chronicity of *P. aeruginosa* infections and is associated with reduced lung function in cystic fibrosis patients [101]. It is for these reasons we explored intranasal administration of our TLR4-adjuvanted CoaB-CRM conjugate vaccine, and looked for hallmarks of enhanced mucosal and systemic immunity.

In chapter 3, we found that vaccinating mice with 10µg or 30µg doses of CoaB-CRM conjugate in combination with INI-2004 intranasally could generate high CoaB-specific IgA in the mucosa and high CoaB-specific IgG in the sera. However, the administration of the adjuvanted-conjugate intramuscularly was unable to mount a similar mucosal response. In order to provide further clarification as to which CoaB-CRM dose to proceed with, we considered the cell-mediated cytokine profiles of the treated groups. We noted that at the 10µg dose of antigen IL-17 production was high; a cytokine that has been associated with improved clearance of *P. aeruginosa* in the lung [102]. Using the 10µg of CoaB-CRM we identified the appropriate dose of INI-2004. At the 10µg dose of INI-2004, mice had the highest production of CoaB-specific mucosal IgA and CoaB-specific serum IgG. Furthermore, these mice had high production of antigen-specific Th1 and Th17 cytokines, including IFNγ, TNFα as well as IL-17. Both IFNγ and TNFα production have been associated with improved phagocytic uptake and killing of *P. aeruginosa* [103]. Additionally, mice receiving 10µg of INI-2004 had high production of IL-10 and IL-5. IL-10 production during *P. aeruginosa* infection has been associated with less lung damage and control of infection, while IL-5 is associated with the maturation of IgA secreting B-cells [104],[105]. This balanced T-helper response could have the potential to induce clearance of *P. aeruginosa* while also reducing lung damage in an active *P. aeruginosa* lung infection.

In chapter 2 we found that CoaB-specific IgG antibodies produced as a result of intramuscular injection have the ability to disrupt the Pf bacteriophage replication cycle. In these studies, we are not aware of the functional aspect of antibodies produced after intranasal administration. Furthermore, at this point

we are unable to verify if intranasal administration of 10μg of INI-2004 with 10μg of CoaB-CRM will prove to be protective against *P. aeruginosa* infections. We hypothesize that our lead intranasal vaccine will enhance protection against *P. aeruginosa* and reduce lung damage; as part of our future directions, we will test this hypothesis by challenging intranasally-vaccinated mice with *P. aeruginosa.* Additionally, we will test the functional aspects of antibodies produced using phage replicative disruption assays as well as phagocytosis assays.

The biomedical community recently witnessed the importance of cross-protective and broadly neutralizing antibodies in the form of SARS-CoV-2 Omicron variants' immune evasion in individuals immunized or infected with SARS-CoV-2 ancestral strains[93]. In other disease systems such as *Streptococcus pneumoniae* bacterial infections, creating heterogeneous conjugate vaccines which incorporate polysaccharide from various serotypes have been shown to induce cross-protection [96]. In this same vein, the acidic CoaB epitope Pf bacteriophage can be largely be divided into two main clades, Clade-A and Clade-B. In chapter 2 we have demonstrated that incorporation of INI-2002 in combination with CoaBa-CRM, can produce CoaBa-specific antibodies in vaccinated mice. However, an unknown was whether CoaBa antibodies could cross-react with antigen from Clade-B. For this reason, we synthesized conjugates which incorporated consensus peptides from Clade-A and Clade-B with the purpose of broadening the immune response.

We first asked if antibodies generated against antigen from Clade-A could cross react with a different antigen within Clade-A. We hypothesized that antibodies could cross-react with peptide from within the same clade and the data presented provided evidence to support our hypothesis. We next asked if antibodies generated against a specific clade could cross-react with the opposite clade. We hypothesized that antibodies generated against one clade could not cross-react with antigen from the other, and the evidence provided in this chapter supported our hypothesis. With the understanding that antibody cross-reaction is not attainable with the current vaccine strategy, we modified our antigen to broaden the antibody response. We synthesized a CoaB-CRM conjugate to include a consensus peptide from Clade-A and Clade-

B, and this multivalent conjugate was then tested against an admixed conjugate. We hypothesized the multivalent conjugate in combination with INI-2002 would enhance production of CoaB-specific antibodies relative to the admixed counterpart. The data suggest that both conjugates gave a similar CoaB-specific IgG response. The admixed formulation is more precise in the ratio between peptides from both clades compared to a multivalent conjugate where it's unknown if equal loading of both peptides is achieved. With this understanding, the admixed conjugate would be selected for future studies.

A surprising aspect is when we compared CoaBa-specific IgG antibody response with CoaBb-specific IgG antibody response from admixed or multivalent antigen alone groups. Here, CoaBb antibody responses were typically higher than CoaBa antibody responses. Furthermore, mice vaccinated with CoaBb alone had statistically higher CoaBb-specific antibody response than naïve control groups. These data suggest peptide-CoaBb is inherently more immunogenic than peptide-CoaBa. Interestingly, when we compare peptide-immunogenicity CoaBb is 30% hydrophobic while CoaBa is 38.89% hydrophobic and both peptides have an overall charge of -4. Additionally, using a MHC class I immunogenicity predictive tool; CoaBa had a immunogenicity score of 0.13112 and CoaBb had an immunogenicity score of 0.07666, suggesting that CoaBa is more immunogenic than CoaBb [106].

Although evidence provided in this chapter suggests antibodies generated from a heterogenous peptide conjugate creates antibodies reactive to both Pf clades there are limitations to the studies presented. INI-2002 does enhance CoaBa and CoaBb antibody titers, but at this point the functionality and neutralizing capabilities of these antibodies are unknown. In future studies we will test functionality of these antibodies using the previously described Pf bacteriophage replicative disruption assay. Validation of generated CoaBb-specific antibodies binding to Clade-B phage is yet to be determined. Propagating Clade-B Pf bacteriophage and determining antibody-mediated Pf bacteriophage recognition would also be a future study. In addition to these *in vitro* assays, another future study would be to determine if antibodies generated from mice vaccinated with admixed CoaBab-CRM in combination with INI-2002 provides protection against *P. aeruginosa* infections. Collectively, data in this chapter provide evidence that further

modifications to a Pf bacteriophage-targeted vaccine can create a broader antibody response, which has broader implications as a potential therapy against *P. aeruginosa* infections.

Chapter 4: Characterization of adjuvant influence on CoaB-specific B-cells

4.1 Introduction

The adaptive immune response is comprised of the cell-mediated and the humoral response. A major component of the humoral response is the B-cell, which has the ability to generate antigen-specific, pathogen-neutralizing antibodies[107]. B-cells typically generate in the bone-marrow where they exit with mature B-cell receptors or BCRs [107]. BCRs on individual B-cells are antigen-specific and develop via a well characterized immunological process known as V (variable) D (diversity) J (joining) recombination. VDJ recombination allows for the extreme diversity of antigen-specific B-cell populations, which is an intrinsic factor in the adaptive-humoral response. This process is described in Chapter 1 in greater detail [108].

As previously described in Chapter 1, once a B-cell first encounters antigen, the B-cell migrates to the spleen or lymph node. Once in the secondary lymphoid organ, the B-cell can migrate to a germinal center where it can go through an additional immunological process known as somatic hypermutation[108]. During the process of somatic hypermutation, mutations are accumulated on the BCR. This results in either gained affinity to the antigen or loss of affinity to an antigen [108]. If it is the latter, the B-cell undergoes apoptosis. If it is the former, the B-cell can go through the process of somatic hypermutation once again or exit the germinal center as a memory or plasma cell [108].

Toll-like receptors or TLRs belong to a broader family of receptors known as pattern recognition receptors or PRRs [109]. These receptors recognize conserved microbial patterns known as pathogen-associated molecular patterns or PAMPS [109]. Recent studies have revealed the importance of TLR

engagement in determining antibody response and specificity to antigens [109]. The majority of TLRs are expressed on the surface of B-cells, such as TLR4 and TLR7 [109]. TLR4 and TLR7 natural ligands include lipopolysaccharides (LPS) and single-stranded ribonucleic acid, respectively [110].

The incorporation of adjuvants in vaccines has historically been used since the 1930's with the incorporation of alum salts to enhance immune responses[7]. The development of cell-receptor targeting-adjuvants has become the next-generation of adjuvants, with some reaching FDA approval such as Cervarix which includes the incorporation of a TLR4-targeting adjuvant [111]. Although there is extensive evidence of safety and efficacy of TLR adjuvants in vaccines, the exact mechanisms of how these adjuvants influence the humoral response is still largely unknown. In this chapter we explore two TLR adjuvants, INI-2002 (TLR4 agonist) and INI-4001 (TLR7/8 agonist) and find that both these adjuvants, in large part, affect the humoral immune response differently than mice which are vaccinated with antigen alone. The aim of this chapter is to elucidate the difference between two TLR adjuvants with the objective that further understanding of these adjuvants can lead to inform future vaccine design.

4.2 Materials and Methods

INI-2002 compound synthesis

Previously described in material and methods section from Chapter 2.

INI-4001 compound synthesis

Previously described in materials and methods [112]

CoaBa conjugate to CRM

Previously described in materials and methods from Chapter 2

In vivo experiments

Previously described in materials and methods from Chapter 2.

ELISA for anti-CoaB peptide antibody quantification

Previously described in materials and methods section from Chapter 2

Tetramer and Decoy production

7.4μM of SA-PE (Prozyme PJRS25) was incubated with 100μM of biotinylated peptide (Anaspec) for 30 minutes at room temperature. To purify tetramer, PBS was used to wash the fraction twice using 100 kDa Amicon Ultra spin filters (Millipore, UFC510024) spun at 14,000g for 10 min. To remove the tetramer fraction, the filter tube was flipped over and centrifuged at 1,000g for 2 minutes. Tetramers were stored at 1 μM in 1x DPBS at 4°C or 1x DPBS containing 50% glycerol at −20°C prior to use. Control PE650 and tetramers were created by mixing a biotinylated control antigen with streptavidin-PE preconjugated with DyLight 650 (PE650) following the manufacturer's instructions for DyLight conjugation (Thermo Fisher Scientific, 62266). The concentration of each tetramer was calculated by measuring the absorbance of PE at 565 nm combined with an extinction coefficient of 1.96 $\mu M^{-1}cm^{-1}$.

Enrichment

CoaB peptide-specific B-cells were enriched using previously-described protocols[113].

FACS

For surface staining, single-cell suspensions were prepared from inguinal and popliteal lymph nodes from the side of injection at 14 days post-secondary vaccination followed by an enrichment protocol as previously described [113]. Bound and flow through single cell suspensions were stained using 50μL of master mix with the following antibodies: BV421-CD138 (clone 281-2, Biolegend 142507), BV510-Ly6G (clone RB6-8C5, Biolegend 108437), BV510-F4/80 (clone BM8, Biolegend, 123135), BV510-CD3e (clone 145-2c11 Biolegend, 100353), Ghost violet510-live/dead stain (Tonbo, 13-0870-T100) , BV650-IgM (clone IL/41 BD 743326), BV786-CD38 (clone 90/CD38 BD 740887) , FITC-GL7 (clone GL7 Biolegend144604) , PE-Cy7-B220, AF700-IgD (clone 11-26c.2a, Biolegend 405730). Following surface staining, samples were centrifuged for five minutes at 300xg and supernatants discarded. Prior to single-cell sorting, 20,000 Fluorescent AccuCheck counting beads (ThermoFisher) were added to

calculate cell numbers downstream. Fluorescence-activated cell sorting was performed on a BD FACSAria 4-laser (637nm, 488nm, 405nm, 561nm) and data was analyzed using FlowJo software (BD Biosciences). CoaB peptide-specific cells were gated on SSC-A/SSC-W- duplet discrimination, followed by B220+,Ly6G-, F4/80-, CD3e-,FVD- B-cells that bound CoaB-PE tetramers but not PE650 tetramers. Single cells were sorted into individual wells of 96-well PCR plates (Eppendorf) containing 10 µL/well of ice-cold lysis buffer containing 0.25 µL 12.5 U RNase out (Thermo Fisher Scientific), 2.5 µL 5x SuperScript IV First Strand Buffer (Thermo Fisher Scientific), 0.625 µL 0.1M DTT (Thermo Fisher Scientific), 0.3125 µL 10% Igepal detergent (Millipore Sigma), and 6.625 µL DEPC treated water. Plates were sealed with adhesive PCR plate seals (Thermo Fisher Scientific), centrifuged briefly and immediately frozen on dry ice before storage at −80°C.

PCR of B-cell receptor heavy chain and analysis

Three µL of reverse transcription reaction mix consisting of 1.5 µL of 50 µM random hexamers (Thermo Fisher Scientific), 0.4 µL 25 mM dNTPs (Thermo Fisher Scientific), 0.5 µL 10 U SuperScript IV (Invitrogen) RT was added to each well containing a single sorted B cell in 10 µL lysis buffer and incubated at 42°C for 10 minutes, 25° for 10 minutes 50°C for 1 hour followed by 94°C for 5 minutes. A total of 13µL of DEPC-treated water was added to cDNA. After cDNA synthesis, 2 µL of cDNA was added to 19 µL of PCR reaction mix containing 0.21 µL 0.5 U HotStarTaq Polymerase (Qiagen), 0.075 µL 50 µM 3' reverse primers, 0.115 µL 50 µM 5' forward primers, 0.24 µL 25 mM dNTPs, 1.9 µL 10x Buffer (Qiagen), and 16.5 µL water. The PCR program for heavy chain was 50 cycles of 94°C for 5 minutes, 94°C for 30 seconds, and 50°C for 30 seconds, followed by 72°C for 55 minutes with a final extension time of 10 minutes at 72°C. After completion of the first round of PCR, 2 µL of the first round PCR product was added to 19 µL of the second round PCR reaction so that the final reaction contained 0.21 µL 0.5 U HotStarTaq Polymerase, 0.075 µL 50 µM 3' reverse primers, 0.075 µL 50 µM 5' forward primers, 0.24 µL 25 mM dNTPs, 1.9 µL 10x Buffer, and 16.5 µL water. PCR programs were the same as the first round of PCR. Four µL of second round PCR product was run on a 2% agarose gel (Thermofisher) to

confirm presence of heavy chain band. Five µL from PCR reactions showing the presence of heavy or light chain amplicons was mixed with 0.025 µL of Exonuclease I (New England Biolabs) and 0.250 µL of shrimp alkaline phosphatase (Sigma-Aldrich) plus 9.725 µL of DEPC-treated water and incubated at 37°C 30 minutes, followed by 95°C for 5 min to hydrolyze excess primers and nucleotides. Hydrolyzed second round PCR products were sequenced by Genewiz with the respective reverse primer and sequences were analyzed using IMGT/V-Quest to identify *V*, *D* and *J* gene segments. Unproductive sequences and sequences with less than 85% V gene similarity were excluded.

4.3 Results

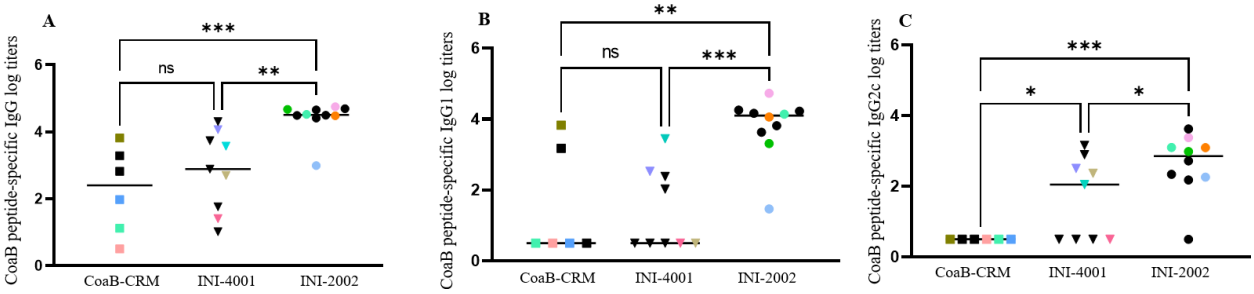


Figure 4-1. INI-2002 adjuvanted CoaB-CRM results in higher CoaB peptide-specific titers compared to INI-4001 adjuvanted CoaB-CRM and CoaB-CRM alone. C57 Black/6 mice (4 or 5 per group) were injected once on day 0 and day 14 i.m. with 1 µg/mouse CRM-CoaB conjugate vaccine adjuvanted with 1 µg/mouse INI-2002 or 10µg/mouse INI-4001. Mice were bled and serum was collected on day 14 post-secondary vaccination. CoaB peptide specific IgG (a) IgG1 (b), and IgG2c (c) antibody titers were measured. Symbols above individual points denote statistical significance. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = p≤0.05, ** = p≤0.01, *** = p≤0.001, **** = p≤0.0001

4.3.1 INI-2002 adjuvanted CoaB-CRM results in higher CoaB peptide-specific titers compared to INI-4001 adjuvanted CoaB-CRM and CoaB-CRM alone.

We first determined the antibody response between groups vaccinated intramuscularly with either the TLR4 or TLR7/8 agonist. We hypothesized that both adjuvanted groups would boost CoaB peptide-specific IgG antibodies. To test this hypothesis, we vaccinated mice with 1 µg CoaB-CRM alone or in

combination with either 1 µg of INI-2002 or 10 µg of INI-4001. Surprisingly, mice vaccinated with INI-4001 did not produce CoaB peptide-specific antibody IgG titers statistically significantly higher than mice vaccinated with CoaB-CRM alone. Mice vaccinated with INI-2002 were able to produce statistically higher IgG antibody titers versus both CoaB alone (p=0.0062) and INI-4001 (p=0.0094) adjuvanted groups (**Figure 4-1A**). We then wanted to further identify differences in CoaB peptide-specific IgG1 and IgG2c between vaccinated groups. Once again, mice vaccinated with INI-4001 did not have statistically higher CoaB peptide-specific IgG1 antibody titers between CoaB treated mice and INI-4001 treated mice (**Figure 4-1B**). However, there was a statistically significant (p=0.02) difference between these two groups in titers of CoaB peptide-specific IgG2c antibody titers (**Figure 4-1C**). Mice vaccinated with INI-2002 had not only statistical difference between CoaB treated mice but also INI-4001 treated mice in both CoaB peptide-specific IgG1 and IgG2c antibody titers (**Figure 4-1B,C**). These data suggest that vaccinating with INI-4001 gives a varied CoaB peptide-specific IgG antibody titers and most of the vaccinated mice produced higher levels of CoaB peptide-specific IgG2c antibodies suggesting a Th1 polarizing response. On the other hand, mice vaccinated with INI-2002 had high CoaB peptide-specific IgG antibody titers and there was a balanced production of both CoaB peptide-specific IgG1 and CoaB peptide-specific IgG2c titers suggesting a balanced Th1 and Th2 response. These results were consistent with those reported in Chapter 2 and 3 of this dissertation.

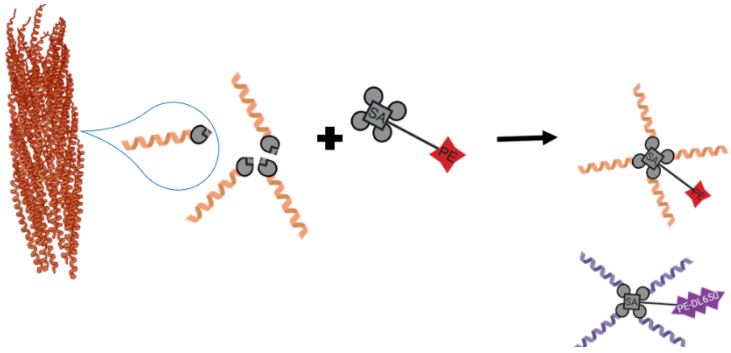
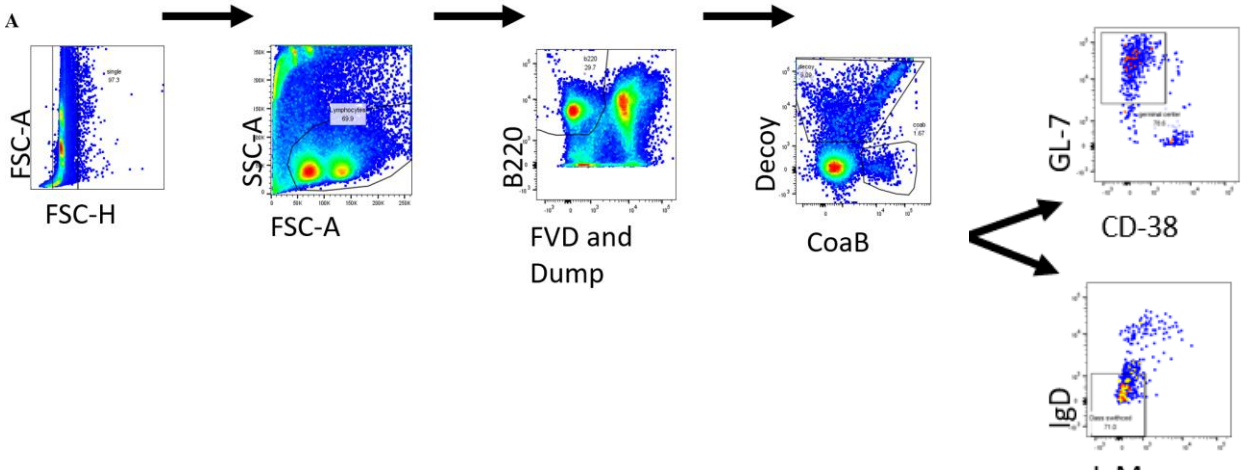
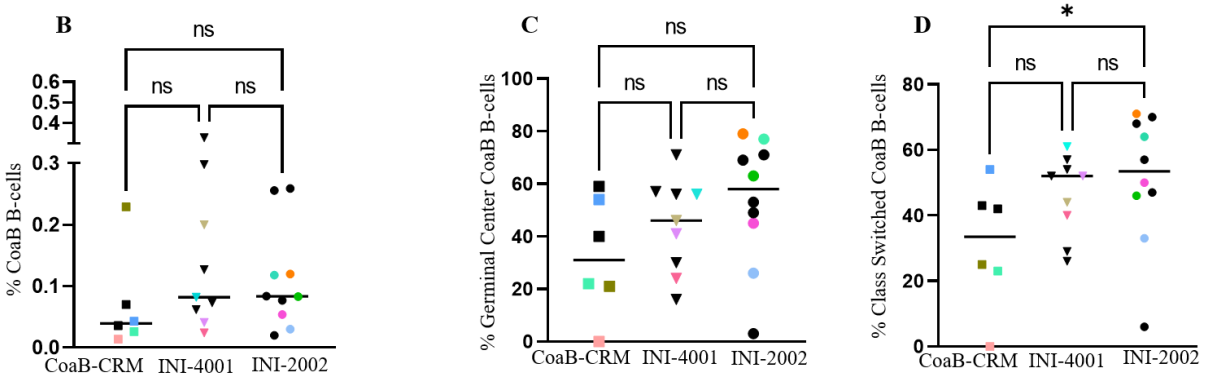
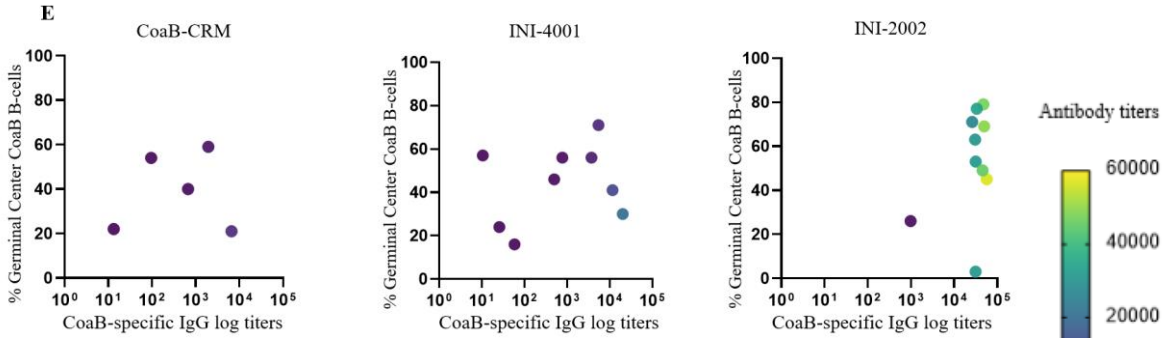


Figure 4-2. Creation of CoaB specific tetramer and decoy. Tetramers were synthesized using consensus peptide derived from *Pseudomonas aeruginosa* Pf bacteriophage (GVIDTSAVESAITDGQDM). Peptides were conjugated to biotin and biotinylated

peptides were then incubated with streptavidin conjugated to phycoerythrin. Decoy was synthesized using an irrelevant biotinylated peptide (DSLIDETTKEVLTQAGTDGC) incubated with streptavidin conjugated phycoerythrin-Dylight 650. Each tetramer us assumed to be armed with approximately four biotinylated peptides per streptavidin.







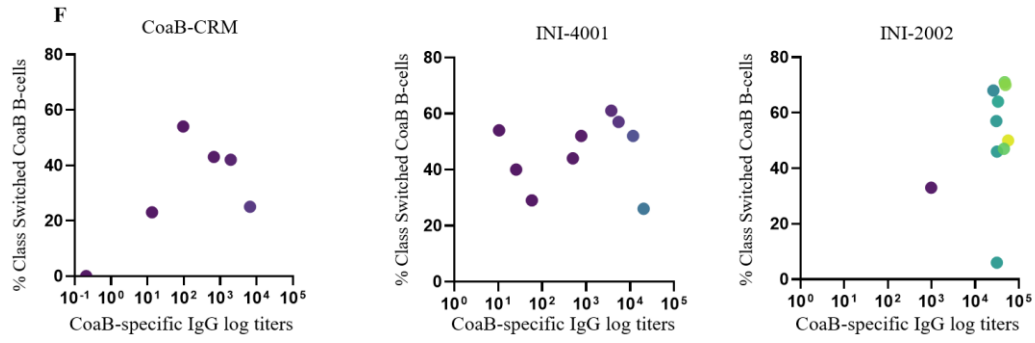


Figure 4-3. INI-2002 adjuvanted CoaB-CRM results in increased class-switching of CoaB specific B-cells. C57 Black/6 mice (4 or 5 per group) were injected once on day 0 and day 14 i.m. with 1 µg/mouse CRM-CoaB conjugate vaccine adjuvanted with 1 µg/mouse INI-2002 or 10µg/mouse INI-4001. Inguinal and popliteal lymph nodes from the site of injection were collected on day 14 post-secondary vaccination followed by single cell suspension and stain. A) Gating strategy to identify CoaB specific B-cells, CoaB specific B-cells found in the germinal center and class switched CoaB specific B-cells. B) Percent of CoaB peptide-specific B-cells between CoaB-CRM alone, CoaB-CRM in combination with INI-4001 or in combination with INI-2002. C) Percent of CoaB peptide-specific B-cells found in the germinal center. D) Percent of class-switched CoaB specific B-cells. E) Bubble plot demonstrating relationship between frequency of CoaB peptide-specific B-cell found in the germinal center and antibody titers of individual mice. F) Bubble plot demonstrating relationship between frequency of class-switched CoaB peptide-specific B-cell found and antibody titers of individual mice. Ordinary one-way ANOVA followed by Fisher's LSD were used to determine statistical analysis where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

4.3.2 INI-2002 adjuvanted CoaB-CRM results in increased class-switching of CoaB specific B-cells.

Next we identified frequencies of CoaB- specific B-cells found in both the inguinal and popliteal lymph nodes from vaccinated treated animals (**Figure 4-3A**). We hypothesized that since CoaB IgG antibody titers were highest in adjuvanted groups especially with the addition of INI-2002 (**Figure 4-1**), that there would be an increased in frequencies of CoaB-specific B-cells in adjuvant-treated groups. Surprisingly, there was not a difference in CoaB specific B-cell frequencies between CoaB-CRM alone and adjuvanted groups (**Figure 4-3B**). We then wanted to identify if adjuvants would influence trafficking of CoaB-specific B-cells to the germinal center in the lymph node. We found that within the CoaB alone group there was variability in CoaB-specific B-cells found in a germinal center. Although not statistically significant, when CoaB-CRM was combined with INI-2002 the majority of CoaB peptide-specific B-cells were found in the germinal center. There was a slight boost in CoaB-CRM specific B-cells found in the germinal center from mice treated with INI-4001. This boost was not statistically significant compared to the CoaB-alone group (**Figure 4-3C**). We then asked if the use of adjuvants could influence frequency of class-switched CoaB-specific B-cells in the lymph nodes. Mice treated with CoaB-CRM alone had a mean of about 20% class switched CoaB-specific B-cells. Mice vaccinated with INI-4001 had an increase in frequency of class-switched CoaB-specific B-cells, although this was not statistically higher than the CoaB-

CRM alone treated group. Mice treated with INI-2002 had an average frequency of approximately 60% of class-switched CoaB specific B-cells and was statistically higher than CoaB-CRM alone treated group (p=0.02) (**Figure 4-3D**). We then wondered if there could be an association between CoaB peptide-specific IgG titers of individual mice with the frequencies of CoaB peptide-specific B-cells found in the germinal center or between frequencies of class-switched CoaB peptide-specific B-cells. When we plotted individual mice, we found mice vaccinated with INI-2002 with high titers of CoaB peptide-specific IgG also had high frequencies of class-switched CoaB peptide-specific B-cells and CoaB specific B-cells in the germinal center (**Figure 4-3E,F**).

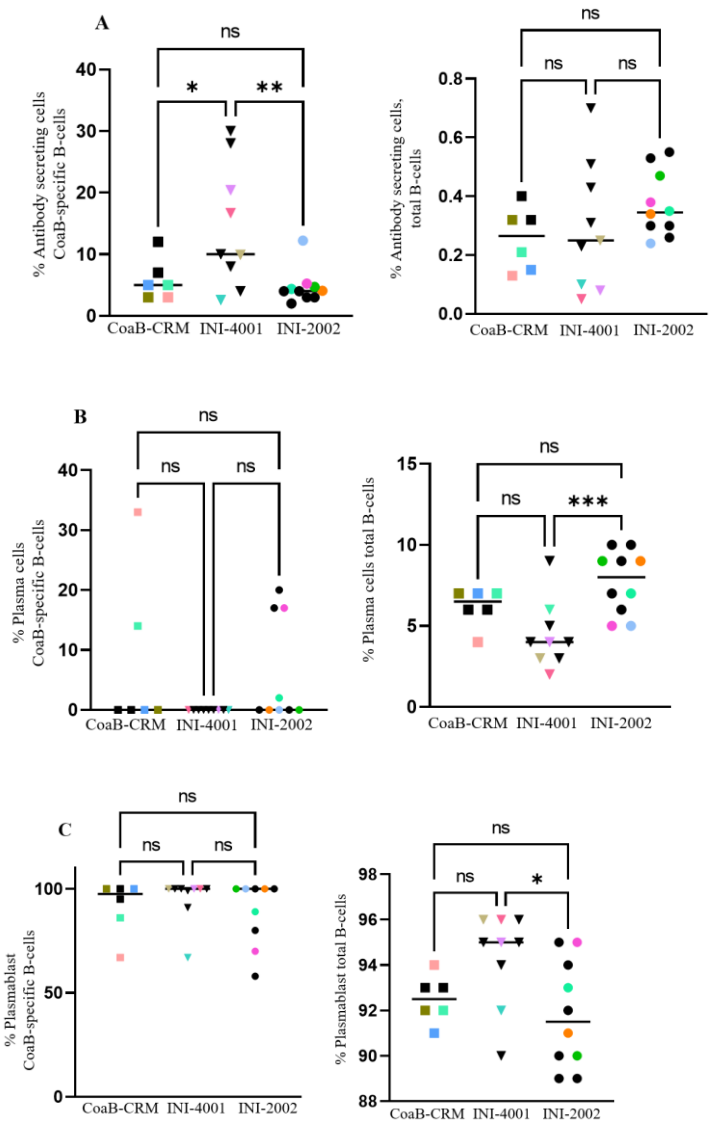


Figure 4-4. **Mice vaccinated with INI-4001 have an increased frequency of CoaB peptide-specific antibody secreting cells , while INI-2002 vaccinated mice have increased frequency of total plasma cells** C57 Black/6 mice (4 or 5 per group) were injected once on day 0 and day 14 i.m. with 1 µg/mouse CRM-CoaB conjugate vaccine adjuvanted with 1 µg/mouse INI-2002 or 10µg/mouse INI-4001. Inguinal and popliteal lymph nodes from the site of injection were collected on day 14 post-secondary vaccination followed by single cell suspension and stain. **.** A) Frequencies of CoaB specific antibody secreting cells and total antibody secreting cells from individual vaccinated mice. Antibody secreting cells were gated on IgD-, CD38+ cell population. B) Frequencies of CoaB specific plasmacells and total plasmacells cells from individual vaccinated mice. Plasma cells were gated on IgD- CD38+ CD138+ cell population C) Frequencies of CoaB specific plasmablasts and total plasmablasts from individual vaccinated mice. Plasmablasts were gated on IgD- CD38+ CD138- cell population.

4.3.3 Mice vaccinated with INI-4001 have an increased frequency of CoaB peptide-specific antibody secreting cells, while INI-2002 vaccinated mice have increased frequency of total plasma cells

We then wanted to further identify how these adjuvants influenced frequencies of antibody secreting cells, specifically the short lived plasmablasts and the longer-lived plasma cells. We hypothesized that since mice vaccinated with INI-2002 had the highest anti-CoaB IgG titers (**Figure 4-4A**) that these mice would also have higher frequencies of antibody secreting cells in the lymph node. We were surprised to find that mice vaccinated with INI-2002 had frequencies of CoaB peptide-specific antibody secreting cell similar to mice vaccinated with CoaB-CRM alone and had lower frequencies than mice vaccinated with INI-4001. Mice vaccinated with INI-4001, on the other hand, had statistically higher CoaB peptide-specific antibody secreting cells compared to mice vaccinated with CoaB-CRM alone. Although there were statistical differences of CoaB peptide-specific antibody secreting cell frequencies between the treated groups, when we looked at all antibody secreting cells from the total population of B-cells this difference was lost (**Figure 4-4A**). We then wanted to further identify if the antibody-secreting cells produced were plasmacells or plasmablasts, to this end we used cell surface marker CD138 to further categorize antibody secreting cells. Expectedly, there were lower frequencies overall of plasma cells and much higher frequencies of plasmablasts with all treated groups having similar frequencies (**Figure 4-4B,C**). But when we look at total frequencies of plasma cells from the antibody- secreting cells mice vaccinated with INI-2002 had statistically higher frequency of plasma cells than mice vaccinated with INI-4001. Based on these

data we can conclude the use of adjuvant INI-4001 increases frequencies of CoaB peptide-specific antibody-secreting cells compared to INI-2002 and un-adjuvanted group, while INI-2002 increases frequencies of overall plasma cells in the draining lymph nodes.

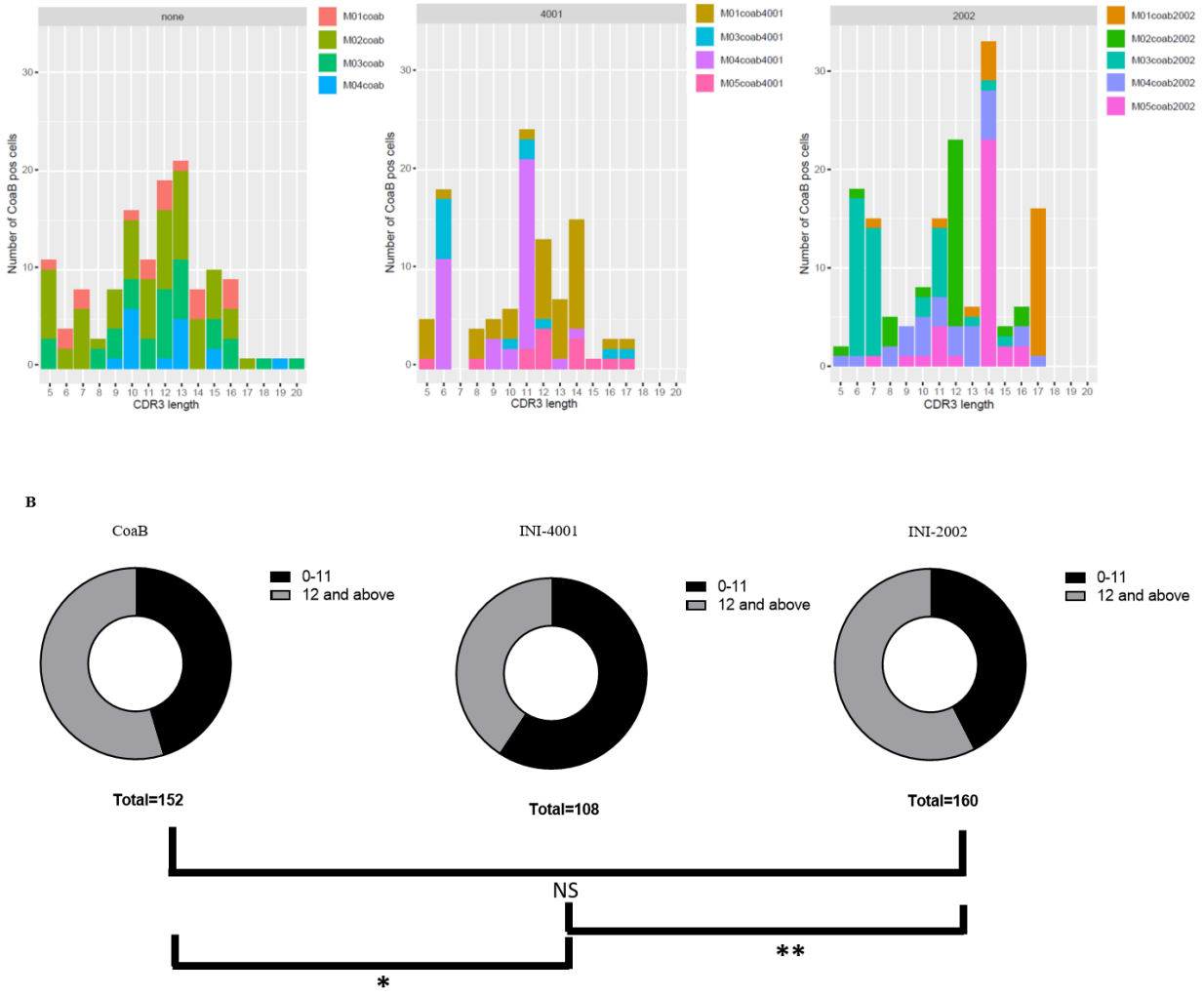
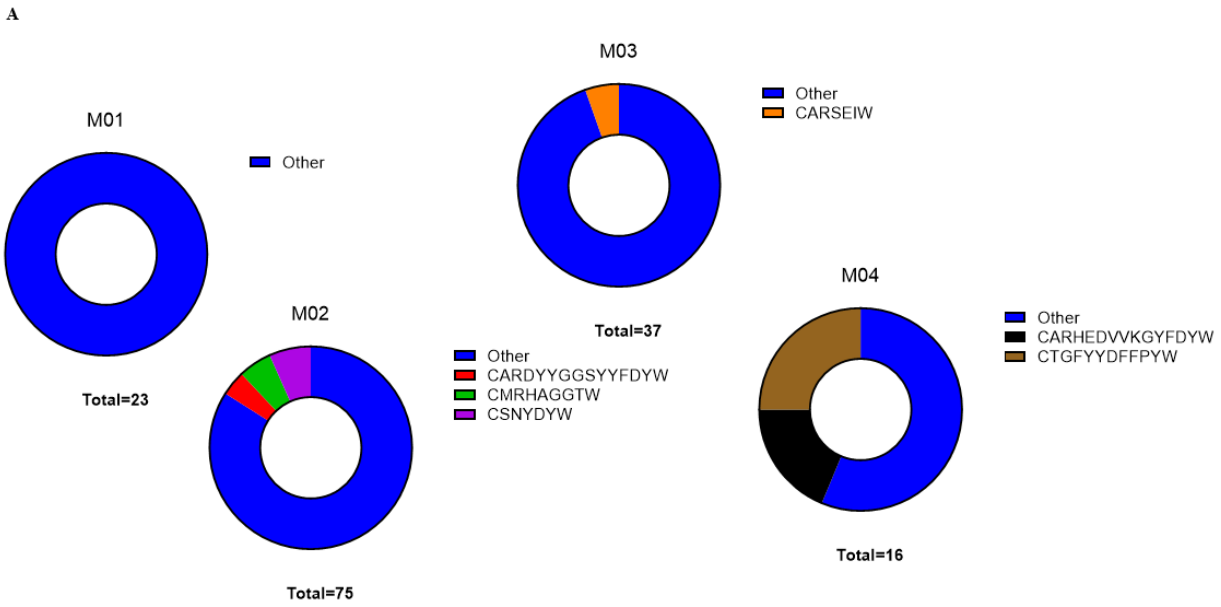


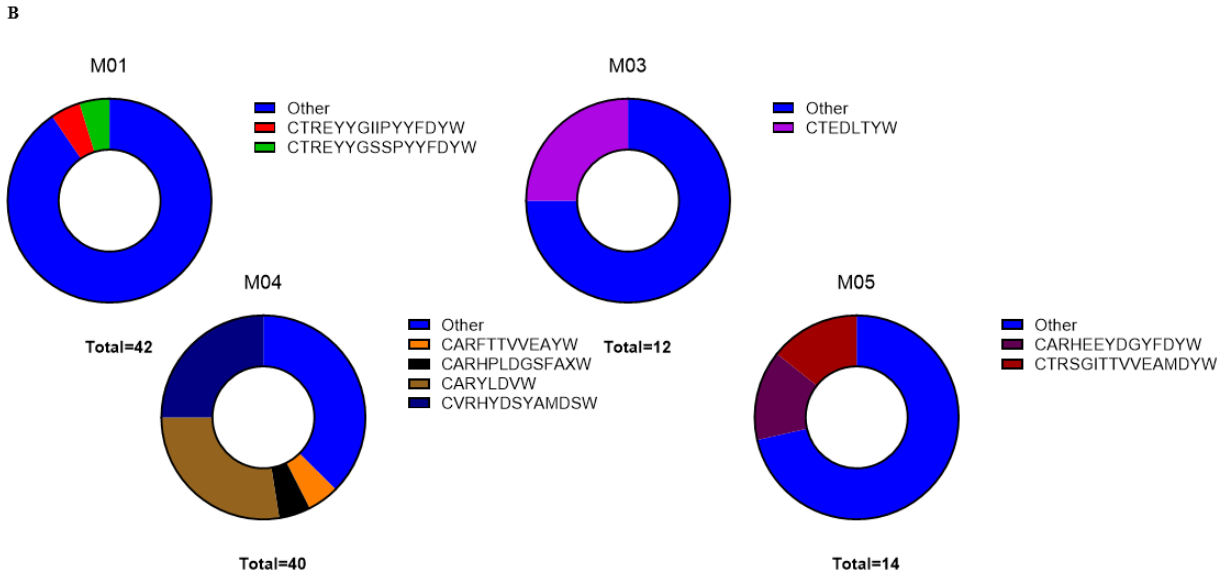
Figure 4-5 Vaccination with INI-4001 results in shorter heavy chain CDR3 length in CoaB peptide-specific B-cell receptors

(A) Distribution of heavy chain CDR3 lengths in individual mice from the treated groups. (B) Frequencies of sequences containing short (0-11) and long (12<) heavy chain CDR3 length. Total amount of sequences from CoaB specific B-cells derived from treated mice are listed below the pie chart. Distributions were compared using Chi-square test where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

4.3.4 Vaccination with INI-4001 results in shorter heavy chain CDR3 lengths in CoaB peptide-specific B-cell receptors

After characterizing CoaB peptide-specific cells by cell-surface marker expression, we wanted to further dissect the B-cell response to adjuvants. To do this, we sorted individual CoaB peptide-specific B-cells into 96-well plates and performed single-cell sequencing of B-cell receptor (BCR) heavy chains. Our first question was, how might adjuvants influence the third complementarity-determining region (CDR3) length of the BCR? We focused on the CDR3 region specifically, because the CDR3 region of the BCR has the most antigenic contact of the three CDRs'[114]. Additionally, we focused on length of the CDR3 region because increased CDR3 length has been associated with increased antibody specificity in other disease systems [115]. When we first looked at CDR3 length in sequences of individual mice treated with CoaB-CRM alone, there was an even distribution in lengths across all CoaB peptide-specific cells. There was a similar response in mice treated with INI-4001 except for mouse 4 which predominately expressed CoaB peptide-specific B-cells with CDR3 lengths of 6 and 11. With the exception of mouse 4, mice treated with INI-2002 expressed CoaB peptide-specific B-cells with preferential CDR3 lengths, i.e., mice 1,2,3,5 expressed lengths 17,12,6,14 respectively (**Figure 4-5A**). We then hypothesized that the incorporation of adjuvant would result in overall CDR3 lengths higher than 11 amino acids long. The length of 11 was selected because mice predominately produce BCRs with a CDR3 length of 11 [116]. When we grouped sequences based on treatment and CDR3 lengths below and above 11, we saw most sequences from mice treated with INI-4001 produced shorter CDR3 regions than groups treated with CoaB-CRM alone ($p=0.0275$) or INI-2002 ($p=0.0071$) (**Figure 4-5B**). These data suggest mice treated with either INI-4001 or INI-2002 do not produce CoaB peptide-specific B-cells with greater CDR3 lengths more so than mice vaccinated with antigen alone, but instead mice treated with INI-4001 produces shorter CDR3 lengths. Additionally, some individual mice treated with INI-2002 or INI-4001 had preferential CDR3 lengths in CoaB peptide-specific B-cells; this could be a result of clonal expansion occurring within the CoaB peptide-specific B-cell population.


A
M01
Other
Total=23
M02
Other
CARDYYGGSYYFDYW
CMRHAGGTW
CSNYDYW
Total=75
M03
Other
CARSEIW
Total=37
M04
Other
CARHEDVVKGYFDYW
CTGFYYDFFPYW
Total=16


B
M01
Other
CTREYYGIIPYYFDYW
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M03
Other
CTEDLTYW
Total=12
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CARHPLDGSFAXW
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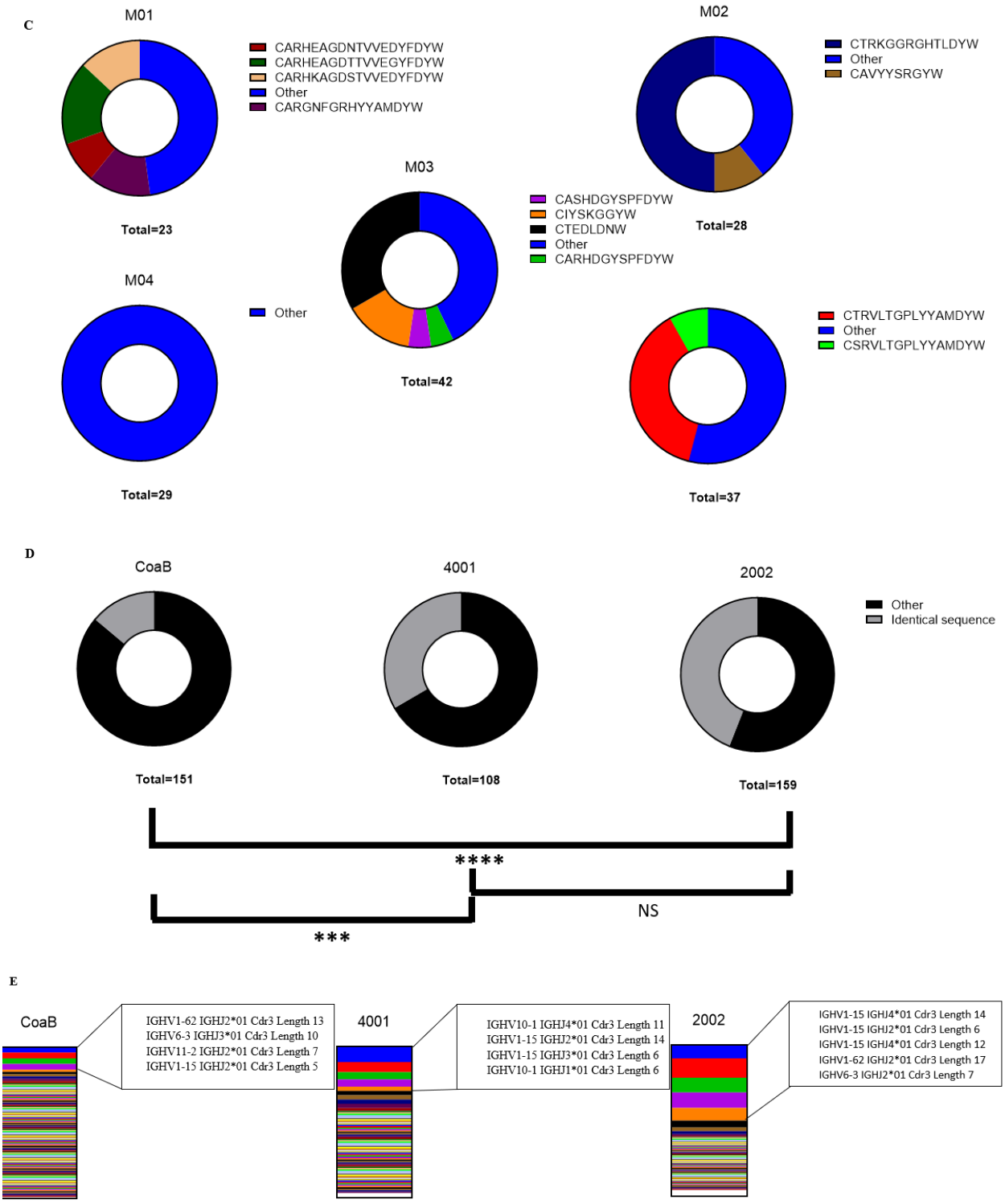


Figure 4-6 Mice vaccinated with adjuvant produce a higher frequency of clonal CoaB peptide-specific B-cells with similar CDR3 regions than mice vaccinated with antigen alone. Pie charts of CDR3 amino acid sequences grouped by repeated sequences vs. unrelated sequences in individual mice treated with: (A) CoaB-CRM alone, (B) INI-4001, (C) INI-2002. (D) Pie charts of proportion of unrelated CDR3 sequences to proportion of sequences with identical sequences between treated groups.

Distributions were compared using Chi-square test where * = p≤0.05, ** = p≤0.01, *** = p≤0.001, **** = p≤0.0001 (E) Proportion bars indicating proportions of B-cells belonging to the same clone. Each color represents frequency of a specific clone and a clone was defined as identical V and J gene as well as CDR3 length of the heavy chain.

4.3.5 Mice vaccinated with adjuvant produce a higher frequency of clonal CoaB peptide-specific B-cells with identical CDR3 regions than mice vaccinated with antigen alone.

After evaluating the adjuvants' influence on CDR3 length we next wanted to determine if adjuvants influenced clonality of CoaB peptide-specific B-cells. When we looked at the CDR3 amino acid sequences of CoaB-CRM treated alone group we found all but mouse 4's CDR3 sequences to be highly varied in CoaB peptide-specific B-cell populations from individual mice (**Figure 4-6A**). We then next looked at CDR3 sequences from mice treated with INI-4001 and found most mice had CoaB peptide-specific B-cells which had a higher proportion of sequences with identical CDR3 amino acid sequences compared to sequences with unrelated CDR3 amino acid sequence (**Figure 4-6B**). This phenomenon was also mostly observed again with mice treated with INI-2002 (**Figure 4-6C**). The differences between mice treated with adjuvant versus those treated with CoaB-CRM alone was even more pronounced when we grouped the sequences by treatment condition. Mice which received INI-4001 or INI-2002 had a statistically higher proportion of CoaB peptide-specific B-cells with identical CDR3 sequences than unadjuvanted (**Figure 4-6D**). We then asked if adjuvant could influence the expansion of clonal CoaB peptide-specific B-cells. Clones were defined as sequences which had identical heavy chain V and J gene segments combined with same length of CDR3 region. We found that about a third of total sequences from CoaB peptide-specific B-cells from mice vaccinate with INI-4001 were from a CoaB peptide-specific B-cell clone compare to mice treated with CoaB-CRM alone. Additionally, 44% of total sequences from CoaB peptide-specific B-cells from mice vaccinated with INI-2002 were from a CoaB peptide-specific B-cell clone compared to mice treated with CoaB-CRM alone which had 14% of total sequences (**Figure 4-6E**). These data collectively suggest that vaccination using adjuvants INI-4001 or INI-2002 boosts CoaB peptide-specific B-cell clonal expansion with INI-2002 having the greatest proportion of clonally expanded CoaB peptide-specific B-cells.

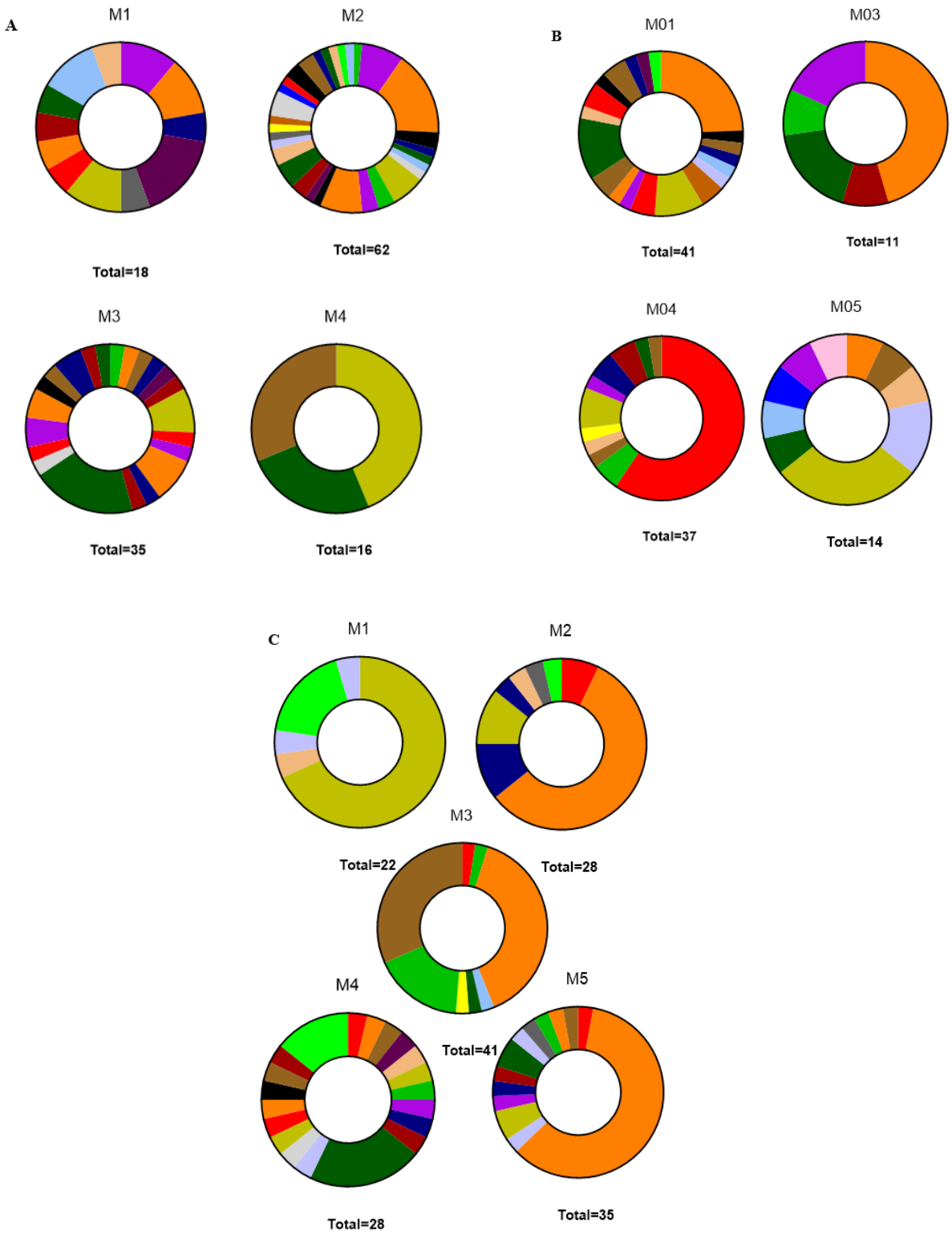
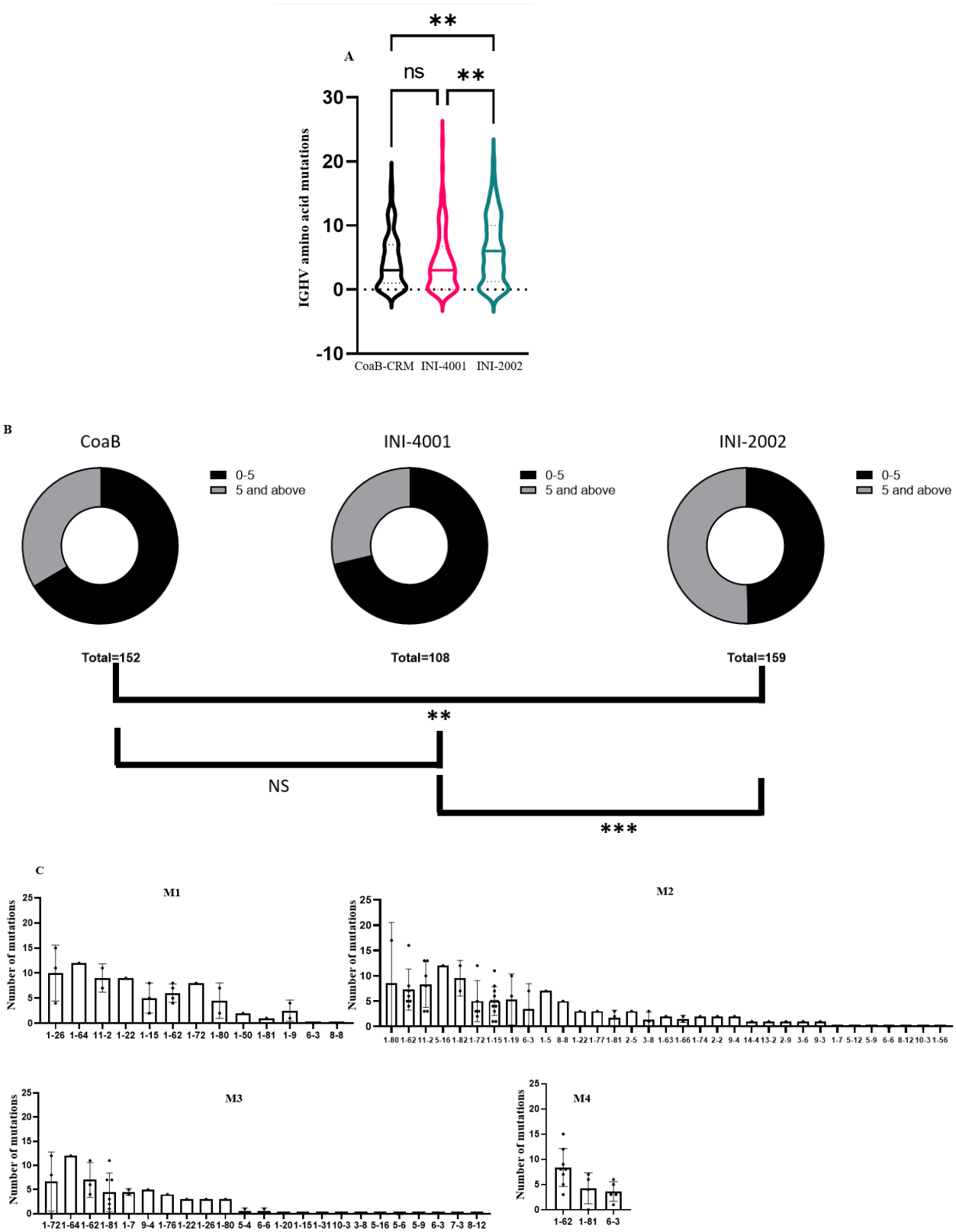


Figure 4-7. IgH family and allele usage is more selective and less broad with the use of adjuvants Single CoaB specific B-cell from inguinal and popliteal lymph nodes from individual mice were sorted in 96-well plates. mRNA from single cells were

converted to cDNA followed by a two-step nested amplification of IgH. A) IgH family and allele usage from four individual mice vaccinated with CoaB-CRM alone. B) IgH family and allele usage from four individual mice vaccinated with CoaB-CRM and INI-4001. C) IgH family and allele usage from four individual mice vaccinated with CoaB-CRM and INI-2002.

4.3.6 IGVH family and allele usage is more selective and less broad with the use of adjuvants

We then examined how individual CoaB-specific B-cells differed between the treated groups and how adjuvants may influence immunoglobulin heavy variable heavy chain (IGVH) family and allele usage. We hypothesized that mice treated with adjuvants would result in selection of IGVH allele usage in sequencing results. To test our hypothesis, we sorted individual CoaB peptide-specific B-cells from popliteal and inguinal lymph nodes from individually treated mice. We then converted mRNA from individually sorted cells to cDNA and followed cDNA synthesis with a nested PCR to amplify the heavy chain. Samples were then sent for Illumina sequencing followed by sequence identification using IgBLAST. IGVH gene expression from mice treated with CoaB alone were highly varied for mice 1,2,3, however, mouse 4 had three distinct IGVH alleles overexpressed, including 1-62, 1-81, 6-3 (**Figure 4-7A**). On the other hand, the majority of mice treated with CoaB-CRM in combination with either adjuvant had overexpression of specific IGVH alleles in individual mice. For example, the four mice vaccinated with INI-4001 each had one IGVH that was overexpressed. M1 and M3 from the INI-4001 treated group overexpressed 1-15 while M4 and M5 overexpressed VH 10-1 and 1-62, respectively (**Figure 4-7B**). The majority of mice treated with INI-2002 overwhelmingly had an overexpression of IGVH 1-15, except for M1 which had an overexpression of 1-62, and M4 which responded more similarly to mice treated with CoaB alone (**Figure 4-7C**). These data suggest that adjuvants bias towards selection of certain VH alleles when compared to the antigen alone group.


A
**
ns
**
IGHV amino acid mutations
30
20
10
0
-10
CoaB-CRM
INI-4001
INI-2002
B
CoaB
INI-4001
INI-2002
0-5
5 and above
Total=152
Total=108
Total=159
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NS
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C
M1
M2
M3
M4
Number of mutations

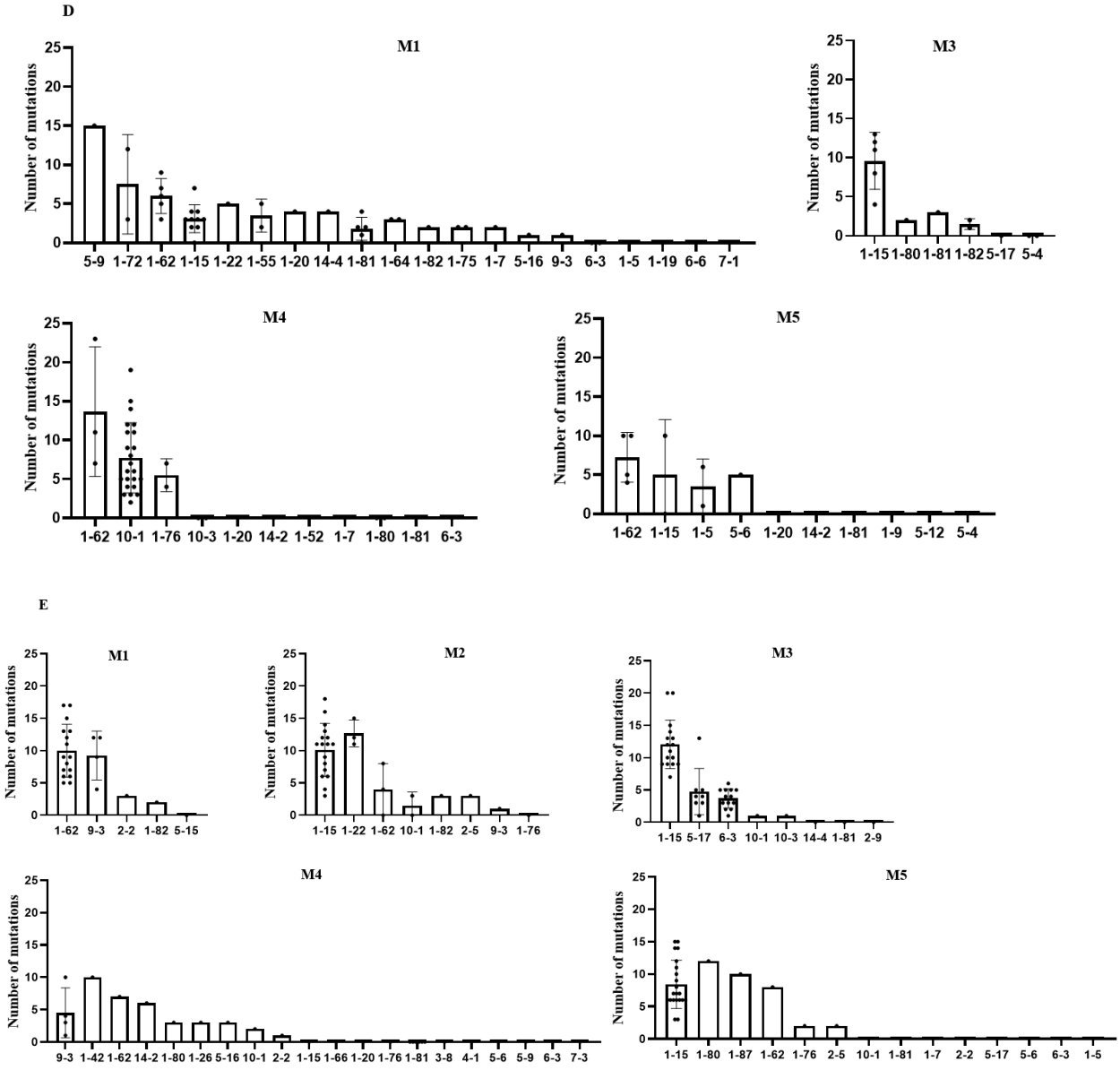

Figure 4-8. INI-2002 adjuvant results in more somatic hypermutation in CoaB-specific B-cells than INI-4001 or CoaB alone. (A)Violin plots depicting number of IGHV amino acid mutations accumulated in CoaB peptide-specific B-cells. Statistical significance was calculated using Kruskall-Wallis with Dunn's multiple corrections tests where ** = p≤0.01. (B) Frequencies of sequences containing low mutations (0-5) and high mutations (5<). Total amount of sequences from CoaB specific B-cells derived from treated mice are listed below the pie chart. Distributions were compared using Chi-square test **p=0.028, ***p=0.0004. Frequencies of alleles with accumulated mutations from individual mice treated with (C) CoaB-alone, (D) INI-4001, (E) INI-2002.

4.3.7 INI-2002 adjuvant results in more somatic hypermutation in CoaB-specific B-cells than INI-4001 or CoaB alone. Following determination of VH allele usage in CoaB peptide-specific B-cells we then

wanted to identify differences of accumulated mutations between the treated groups. We hypothesized that groups which received adjuvant would accumulate more mutations than the mice treated with CoaB-CRM alone. The evidence suggests that vaccinating mice with INI-2002 results in more CoaB peptide-specific B-cell amino acid IGHV mutations than mice treated with INI-4001 or CoaB-alone (**Figure 4-8A**). Furthermore, mice vaccinated with INI-4001 did not show difference CoaB peptide-specific B-cell amino acid IGHV mutations than mice treated with CoaB-CRM alone. The majority of CoaB-specific B-cell sequences in CoaB alone and INI-4001 treated groups had low mutation counts per sequence (0-5), with no statistical difference between CoaB alone and INI-4001 treated groups. IN-2002, on the other hand, had more sequences with higher mutations (5<) than CoaB alone ($p=0.028$) and INI-4001 treated mice ($p=0.0004$) (**Figure 4-8B**). We then asked if overexpressed alleles accumulated more mutations. CoaB peptide-specific B-cells from mice vaccinated with INI-2002 which had undergone VH allelic selection tended to accumulate more mutations compared to mice vaccinated with INI-4001 or CoaB-CRM alone (**Figure 4-8C,D,E**). These data suggest that vaccinating with INI-2002 results in highly mutated IGHV sequences from CoaB peptide-specific B-cells.

4.3.8 Discussion

Adjuvant effects on antigen-specific B-cells is a largely unexplored area, especially when examining the comparative effects of TLR agonists. In this body of work we sought to further understand how different TLR agonist may influence antigen-specific B-cells, in the hopes that the better understanding of adjuvant effects can lead to improved adjuvant selection for future therapies. There are a multitude of examples in the literature of the use of synthetic TLR4 and TLR7/8 agonists boosting immunity against pathogens such as Influenza virus, SARS-CoV-2 and even metastatic cancer [117]–[119]. What largely remains unanswered is how the engagement of TLR ligands affects the humoral response and specifically the antigen-specific B-cell population. Chapters 2 and 3 established INI-2002's ability to enhance a balanced immune response in the form of multi-functional T-cells as well as high CoaB peptide-specific IgG1 and IgG2c antibodies, but antibody titers are just one component of the humoral response. In this

chapter we have leveraged single-cell sequencing techniques and analysis to further broaden our understanding of adjuvant effect on the humoral response.

INI-2002's ability to create a balanced production of CoaB peptide-specific IgG1 and IgG2c antibody titers from vaccinated mice was recapitulated in this study (**Figure 4-1A,B,C**). We also expected to see a skewed IgG2c production in [120]. What was unexpected was the similar CoaB peptide-specific B-cell frequencies between the treated groups (**Figure 4-3B**). Because CoaB peptide-specific antibody titers were high in mice treated with INI-2002, we expected there were more CoaB peptide-specific B-cells. Although mice treated with INI-2002 and INI-4001 trended toward having higher frequencies of CoaB peptide-specific B-cells, ultimately there was no statistically significant difference between INI-2002 or INI-4001 and unadjuvanted groups. TLR engagement of draining lymph node dendritic cells has been associated with an increased magnitude of antigen-specific IgG; engagement of INI-2002 with TLR4 on dendritic cells could explain the increase of CoaB peptide-specific IgG independent of CoaB specific B-cell frequency [121].

Interestingly, mice vaccinated with INI-4001 had a higher frequency of CoaB peptide-specific antibody-secreting B-cells than mice vaccinated with INI-4001 or CoaB-CRM alone (**Figure 4-4A**). Engagement of the TLR7 receptors on B-cells has been associated with downstream proliferation effects [44]. It is interesting that mice vaccinated with INI-4001 did not show a significant difference in frequency of CoaB peptide-specific B-cells, but of those B-cells a larger proportion were IgD-, CD38+ antibody-secreting cells; it could be that INI-4001 stimulates antibody-secreting cells. Another interesting finding was although mice vaccinated with INI-2002 had similar frequencies of total antibody-secreting cells as mice vaccinated with INI-4001 or CoaB-CRM alone; the proportion of total antibody secreting cells were mostly plasma cells (**Figure 4-4B**). This is significant because engagement of TLR4 on plasma cells has been associated with augmentation of antibody secretion and proliferation [109], [122]. TLR4 receptor ligation by INI-2002 on plasma cells could also contribute to the overall magnitude of IgG response seen in mice vaccinated with INI-2002.

Previous studies have indicated an increased length of CDR3 to be important in increased specificity of the B-cell receptor to antigen [123], [124]. Length of the CDR3 region can change due to increase in somatic hypermutation, the accumulation of inserted amino acids during VDJ recombination with selection for D2 and D3 gene families, and finally VH replacement [124]. It is for these reasons we wanted to determine if INI-4001 or INI-2002 vaccination resulted in the increased length of the CDR3 in the BCR of CoaB peptide-specific B-cells, and if so, which of the three mechanisms listed could be the major contributor. We hypothesized that mice vaccinated with adjuvant would have longer CDR3 BCR lengths, and it would be because of accumulation of amino acid mutations. On average, mice produce CDR3 lengths of 11 amino acids long; this is why we selected 12 and above to be considered a long CDR3 [116]. To our surprise, mice vaccinated with INI-4001 had shorter CDR3 regions than mice treated with INI-2002 and CoaB-CRM alone (**Figure 4-5A**). We then wondered if at the individual level mice who had longer CDR3 regions also had the most mutations accumulated. However, this was not the case; mouse M01 from the INI-2002-treated group had the longest predominant CDR3 length (17 amino acids) and had the 4th lowest accumulation of mutations (202); while M03 had the shortest predominant CDR3 length (6-7 amino acids) and the highest accumulation of mutations (281). It could be that shorter CDR3 results in improved antigen contact with CDR1,2, and 3, leading to selection and expansion of these B-cells.

CDR3 is a highly variable segment of the BCR, thus similarities of CDR3 amino acid sequence between antigen specific B-cells can give an indication of clonal expansion [125], [126]. When we analyzed sequences from the individual mice within each treated group we noticed differences in shared CDR3 sequences between mice vaccinated with adjuvant and mice treated with CoaB-CRM alone. Adjuvanted mice had more shared CDR3 amino acid sequences than unadjuvanted, and mice receiving INI-2002 had more occurrences of shared CDR3 amino acid sequence than mice treated with INI-4001. The increased magnitude of shared CDR3 amino acid sequence in TLR stimulated mice, compared to unstimulated mice, observed in our study aligns with what has been previously reported in the literature [126]. These shared CDR3 amino acid sequences give an indication of clonal expansion of antigen-specific B-cells [127]. The

higher frequency of shared CDR3 amino acid sequences in mice vaccinated with INI-2002 suggests that INI-2002 enhances clonal expansion of antigen-specific B-cells (**Figure 4-6C**). Kotagiri et al., and Nouri et al., established the definition of clonal, antigen-specific B-cells as common IGHV genes, IGHJ genes, and the same length of CDR3 [127], [128]. The higher proportion of clonal antigen-specific B-cells from total sequences generated from all mice treated with INI-2002 provides further evidence that vaccination with INI-2002 increases the magnitude of clonal expansion compared to INI-4001 and antigen alone. In our data set we also see a relationship between mice which had a high frequency of antigen specific B-cells found in the germinal center and shared CDR3 amino acid sequences.

In the INI-2002 treated group, mice 1,2,3, and 5 had frequencies of ~50-80% of antigen-specific B-cells in the germinal center (**Figure 4-3B**) and had about half of sequences contain shared CDR3 regions (**Figure 4-6C**) as compared to mouse 4 which had ~25% antigen specific B-cells in the germinal center, no shared CDR3 amino acid sequences and the lowest titers of antigen-specific antibodies. These data suggest that if there is a successful response to INI-2002 there will be a likely clonal expansion of antigen specific B-cells in the germinal center. The correlation of INI-4001 adjuvant influence on shared CDR3 and frequency of antigen-specific B-cells is less clear. Mouse 4 had more than half of sequences contain shared CDR3 amino acid sequences, while mouse 1 had the least amount of shared CDR3 amino acid sequences out of all mice treated with INI-4001 (**Figure 4-5B**). However, both mouse 1 and mouse 4 had approximately 40% of antigen-specific B-cells in the germinal center (**Figure 4-3B**). Furthermore, mouse 5 had about a quarter of sequences containing shared CDR3 amino acid sequences while antigen-specific B-cell frequency in germinal center was about 25%. These data suggest that use of INI-4001 agonist gives a more varied humoral response as compared to the INI-2002 agonist. The data are significant because in previous chapters we observed INI-2002's influence on mounting an increased magnitude of functional CoaB peptide-specific IgG; that combined with chapter 4 data suggest that INI-2002 vaccination results in clonally-expanded functional antigen-specific B-cells.

We hypothesized that mice vaccinated with antigen alone would have a similar VH allele profile as naïve mice, but when we compared VH allele usage from total sequences of treated groups to naïve populations, we found sequences from CoaB-alone treated mice shared 1-26, and 1-80 allele usage in the top ten most used alleles [116]. Sequences from INI-4001-treated mice shared 1-80, 1-82, 6-3 and 1-55 with naïve populations in the top ten most-used alleles and sequences from INI-2002 treated mice shared 1-80 and 1-26 with naïve populations in the top ten most-used alleles[116]. Interestingly, IGVH1-15 (not predominately used in naïve mice) is used to some degree in 61% of all mice from the various treated groups; with half of mice treated with INI-4001 selecting for IGVH1-15, and most of mice treated with INI-2002 selecting for IGHV1-15. These data suggest that vaccination with INI-2002 results in a more consistent expression of CoaB peptide-specific B-cells with IGVH1-15 allelic expression as compared to mice vaccinated with INI-4001, which results in more varied allelic selection, and mice vaccinated with CoaB-CRM alone, which largely do not undergo allelic selection.

In addition to CoaB peptide-specific B-cell consistent allelic selection found in INI-2002 vaccinated mice compared to the other treated groups, there were also marked differences in the accumulation of amino acid mutations in the IGHV region. We see that overall, mice vaccinated with INI-2002 accumulated more amino acid mutations in IGHV regions of CoaB peptide-specific B-cells. We also see that of the sequences which had mutations, the majority had greater than 5 mutations accumulated per sequence as compared to the other treated groups. Furthermore, mice 1,2,3,5, which had undergone allelic selection, also had highly mutated IGHV regions (**Figure 4-8A,B,C,D,E**) . These data, in combination with frequencies of CoaB peptide-specific B-cells found in the germinal center, suggest that mice which had higher frequencies of CoaB peptide-specific B-cells found in the germinal center are undergoing somatic hypermutation more frequently than CoaB peptide-specific B-cells from mice treated with INI-4001 or CoaB-CRM alone. A higher level of somatic hypermutation has been implicated in improved disease resolution in disease systems such as SARS-CoV-2 infections [129]. It could be possible that vaccination

with INI-2002 results in the production of not only a higher magnitude of IgG antibodies but also quality antibodies with higher affinity to antigen.

Although we have provided extensive evidence on how adjuvant with INI-4001 and INI-2002 enhance the humoral response; this body of work does contain limitations which should be addressed in future studies. We are confident in the rigor of techniques used to isolate CoaB peptide-specific B-cells, nonetheless, future studies should include cloning and production of monoclonal antibodies from select sequences to confirm the identity and specificity of sorted B-cells. Additionally, even though accumulation of mutations is generally indicative of higher quality antibodies, antibody affinity should be validated using Bio-Layer Interferometry assays [130]. Other future directions include head-to-head comparison of INI-4001 and INI-2002 adjuvanted CoaB-CRM in a *P. aeruginosa* challenge study.

Extensive characterization of TLR adjuvant effects on antigen B-cell receptors is limited. Herein, we have isolated CoaB peptide-specific B-cells from mice vaccinated with CoaB-CRM alone, INI-4001, or INI-2002 and found differences in sequences from these cells as well as B-cell population differences between the treated groups. Generally, mice adjuvanted with INI-2002 had a higher magnitude of CoaB peptide-specific IgG. This higher magnitude of CoaB peptide-specific IgG could possibly be due to increased frequency of plasma cells, which are known to secrete more antibodies upon TLR4 stimulation [109]. Furthermore, these CoaB peptide-specific B-cells have undergone extensive clonal expansion and are highly mutated, indicating these populations have undergone somatic hypermutation. Mice vaccinated with INI-4001, on the other hand, had a lower frequency of plasma cells and fewer IGHV mutations, but still had IGHV allelic selection in individual mice. Less mutations in IGHV regions are a characteristic of memory B-cells; it is possible INI-4001 may influence differentiation of antigen-specific B-cells into memory cells soon after secondary vaccinations [129]. Together, these data provide further description of how INI-4001 and INI-2002 influence the humoral response. The value of the study is that further characterization of adjuvant effects could lead to more accurate adjuvant selection for various disease systems.

Chapter 5: Concluding remarks and future directions

The Gram-negative bacterium, *P. aeruginosa* has been listed as a priority 1 pathogen by the WHO [131]. Infections caused by multidrug-resistant *P. aeruginosa* can lead to serious health complications and death [52]. *P. aeruginosa* can establish infections in mucosal tissues such as the eyes, lungs and urinary tract, and is a common culprit of nosocomial infections in healthcare facilities [132]. *P. aeruginosa* adaptability and host-immune evasion capabilities have created challenges in developing effective vaccines and therapeutics against infections [133]. Virulence factors such as the presence of temperate Pf bacteriophage can further exacerbate disease by sequestering positively-charged antibiotics and enhancing integrity of biofilms. Previous work demonstrated that a Pf-targeted vaccine improves clearance of *P. aeruginosa* infections [53].

Sweere et al. demonstrated that Pf-specific monoclonal antibodies applied topically to mouse dorsal wounds infected with *P. aeruginosa* resulted in improved clearance compared to wounds that received a polyclonal sera control [53]. Although the monoclonal antibodies were shown to be effective in enhancing clearance of *P. aeruginosa*, this therapeutic tool is limited. There is overwhelming evidence of adjuvants' abilities to enhance effective and long-lasting immunity against pathogens [7]. Next-generation adjuvants such as INI-2002 engage the TLR4 and can boost immune responses against specific pathogens. Furthermore, the engagement of TLR4 in a *P. aeruginosa* infection result in improved clearance of the infection; it is for these reasons we elected INI-2002 to compare against alum in our initial studies [75]. When comparing magnitudes of CoaB-specific antibody titers, there was a statistically higher magnitude in mice vaccinated with INI-2002 than with alum. We selected 1µg of INI-2002 because of the consistently high magnitude of IgG, IgG1 and IgG2c CoaB-specific antibody titers compared to other doses. We also identified that both the 1µg and 10µg dose increased CoaB-specific antibodies in mice equally but to be antigen-dose sparing we selected 1µg to be our lead dose of CoaB-CRM. In addition to optimizing dose based on magnitude of IgG antibody response, we also identified the lead dose of INI-2002 and CoaB-

CRM lead to a balanced T-helper response based on lymphocyte cytokine expression. Notably, mice vaccinated with our lead vaccine produced statistically higher IFN-γ and had statistically higher IgG2c CoaB-specific antibody responses; both of which are Th1-associated immune responses. Additionally, mice vaccinated with INI-2002 had an increase in class-switched CoaB-specific B-cells. These data suggest that INI-2002 promotes the induction of a Th1 response which recapitulates what has been previously demonstrated with other TLR4 agonists [134]. Inducing a Th1 response has been associated with improved lung function in *P. aeruginosa*-infected lungs of cystic fibrosis patients [135]. The use of a Th1-inducing TLR4 agonist, such as INI-2002, has the potential to improve prognosis in *P. aeruginosa* infected lungs. We also tested the functionality of antibodies produced and identified CoaB-specific antibodies from mice vaccinated with lead CoaB-CRM vaccine. These were able to bind to whole Pf virions and disrupt the replication cycle. This is significant because disruption of the replication cycle gives insight into the functionality of generated antibodies. During a *P. aeruginosa* infection, antibody against Pf bacteriophage can potentially bind Pf bacteriophage extruding from a *P. aeruginosa* bacterium. This could, in turn, prevent Pf bacteriophages from infecting neighboring *P. aeruginosa* which may lack the Pf prophage and ultimately lead to reduced biofilm formation. As previously mentioned, Pf bacteriophages' negatively charged coat protein results in the formation of liquid crystal structures which strengthen the integrity of biofilms [52]. It is possible CoaB-specific antibodies generated after vaccination with INI-2002 and CoaB-CRM could bind Pf bacteriophage and disrupt the organization of liquid crystalline structure in the biofilm, thus reducing the integrity of the biofilm.

Because *P. aeruginosa* chronic infections occur frequently in mucosal sites, we optimized a Pf-targeted vaccine for intranasal administration. In our initial study, we identified intramuscular administration of INI-2004 boosted sera IgG but not CoaB-specific IgA at mucosal sites. However, intranasal administration of INI-2004 not only boosted mucosal CoaB-specific IgA but also sera CoaB-specific IgG. This observation is consistent with other intranasal vaccine studies in which intranasal administration of vaccine enhances not only site-specific and antigen-specific IgA but also systemic

antigen-specific IgA and IgG [136]. This is important in the context of creating a vaccine against *P. aeruginosa*, where various mucosal sites in the body such as the urogenital tract and eyes can be entry points for *P. aeruginosa* [136]. Additionally, we also identified 10µg of CoaB-CRM and 10µg of INI-2004 resulted in a high magnitude of CoaB-specific IgA IgG, IgG1 and IgG2c antibody production while also creating a balanced T-helper cell response. High IgA production post intranasal administration is of significance since it has been previously demonstrated that administration of vaccines at mucosal sites can lead enhanced opsonization and killing of bacteria at peak IgA production [137]. CoaB-specific IgA could have the functional capabilities of increased opsonization and killing of *P. aeruginosa* bacteria in a lung challenge setting.

Recent analysis of Pf bacteriophage structural protein's DNA sequences has revealed Pf bacteriophage can be divided into two distinct groups [138]. With this in mind, to broaden therapeutic applications of the Pf bacteriophage targeted vaccine, we also incorporated an additional divergent acidic CoaB-epitope (CoaBb) to the previously tested CoaBa epitope. Initially, we tested and found that antibodies generated against CoaB-peptide from within the same clade are cross-reactive. However, antibodies cannot cross-react between the two clades. We also found that mice vaccinated with peptide-conjugate from both CoaB-epitopic clades, generated antibodies against both of these clades. This is significant because in a recent study *P. aeruginosa* strain LES, which contains a prophage from Clade B, was found in 50% of clinics in the UK; and strain PA14, which contains a prophage from Clade A, represented 5.5% of *P. aeruginosa* infections in the UK [139]. Our vaccine has the capability of generating a humoral response against both clades encountered. Additionally, when testing both formulation strategies we found that both admixed and multivalent conjugates had no difference in magnitude of the CoaBa/b-specific IgG, IgG1 and IgG2c response. Because synthesis of the admixed formulation is facile processes compared to the multivalent formulation synthesis, the admixed formulation would be the lead formulation for future studies.

After optimizing and broadening the application of our Pf-phage targeted vaccine, we further characterized how adjuvants influence antigen-specific B-cell populations. In this study we found that adjuvant effects did not result in an overall increase in CoaB-specific B-cells frequencies. However, INI-2002 effects drove class-switching of CoaB-specific B-cells and also increased total plasma cell. Furthermore, mice vaccinated with INI-2002 produced more clonal CoaB-specific B-cell populations. These clonal CoaB-specific B-cell populations have undergone somatic hypermutation as evidenced by the highly mutated IGHV regions. Extrafollicular responses generated by activated B-cells canonically precede the formation of germinal center reactions, and germinal center formation is essential for creating highly mutated V-region B-cells and affinity maturation [24]. Previous studies have discovered that TLR4 engagement of immature B-cells can lead to maturation of these B-cells [140]. Additionally, studies have indicated TLR4 engagement induces activation and proliferation of mature B-cells [140]. It possible that INI-2002 is not only facilitating the maturation of B-cells but also promoting the proliferation and activation of these B-cells and hastening the transition of extrafollicular responses to germinal center responses. Follicular dendritic cells are critical in germinal center reactions and are indispensable in the immune system's ability to produce high-affinity binding, class-switched B-cells [141]. Garin et al., demonstrated that TLR4 ligation on follicular dendritic cells triggers maturation and activation of these cells [141]. Activated follicular dendritic cells will release IL-1β and IL-15, both which induce B-cell proliferation, and IL-6, which induces somatic hypermutation [141], [142]. In addition to directly impacting B-cell maturation and activation, which could lead to germinal center formation, INI-2002 could also enhance the quality of the germinal centers by activating and maturing follicular dendritic cells via TLR4. Ultimately, this could help explain why CoaB-specific B-cells are highly mutated and produce a higher magnitude of CoaB-specific antibodies, which is of importance because highly mutated BCRs are associated with increased affinity.

In this body of work, we optimized our Pf targeted adjuvanted vaccine for both intramuscular and intranasal administration. Although results of these initial studies demonstrate that adjuvants can increase

CoaB-specific sera and mucosal antibodies, we need to demonstrate if these antibodies are protective. Future challenge studies including both murine lung challenge and dorsal wound challenge would elucidate if antibodies from mice vaccinated with adjuvant can protect against a *P. aeruginosa* infection. Since we determined that CoaB-specific antibodies can disrupt the Pf bacteriophage replication cycle, the proposed future studies could search for indications of reduced Pf bacteriophage loads from infected lung and dorsal wound tissue between vaccinated mice and unvaccinated mice. Another readout from these proposed studies could be TNF cytokine levels, which is an important cytokine for phagocytosis [53]. Sweere et al., established that infections involving wild-type *P. aeruginosa* result in the reduction of TNF cytokine levels, and our studies demonstrate that INI-2002 boosts TNF cytokine production [53]. It is possible that vaccinating mice with INI-2002 could counteract Pf bacteriophage TNF reduction effects, thus enhancing phagocytic capabilities of the immune response.

We showed that mice vaccinated with conjugates containing peptides from acidic epitopes from both clades produce antibodies which can recognize peptides from both these clades. However, it is still unknown whether antibodies produced can recognize Pf bacteriophage that contain peptides from the CoaBb acidic epitopic clade. Additionally, protective qualities of these antibodies need to be tested in *P. aeruginosa* challenge studies. Naturally occurring biofilms, such as biofilms found on urinary catheters which can contain *P. aeruginosa* and *Escherichia coli* bacterial populations, tend to have multiple bacterial species [143]. Antibodies produced from mice vaccinated with our admixed vaccine could disrupt biofilm integrity, allowing for host immune response to penetrate multi-species biofilms and clear infections in future challenge studies.

We have demonstrated how INI-2002 and INI-4001 influence CoaB-specific B-cells at 14 days post- secondary intramuscular injections. However, monoclonal antibodies should be expressed and tested on Bio-layer interferometry in order to validate isolated CoaB-specific B-cells. In this study, we isolated CoaB-specific B-cells from draining lymph nodes, at 14 days post-secondary vaccination. Future studies should look at adjuvant effects of CoaB-specific B-cells in other tissues such as the bone-marrow and at

various other time points. Isolation of CoaB-specific B-cells from the bone-marrow at later time points could give insight into how adjuvants influence antigen-specific memory B-cells. Route of vaccination will also influence CoaB-specific B-cell populations. In Chapter 3 we demonstrated how intranasal vaccine administration resulted in mucosal IgA and sera IgG responses additional future studies can further characterize how INI-2004 could impact CoaB-specific B-cells.

To summarize, although we have demonstrated how adjuvants and specifically INI-2002 in combination with CoaB-CRM can broadly influence the cell-mediated and humoral response there are still many unknowns. Future studies can further characterize adjuvant influence on the immune response. The data presented in this dissertation broaden our understanding on how novel TLR adjuvants impact humoral responses and specifically antigen specific B-cells. Understanding how TLR agonists can impact B-cell development and activation can better inform decisions related to adjuvant selection for specific disease systems and development of next generation TLR agonists.

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