The Development of a New Pertussis Booster Formulation via the Implementation of New Adjuvants and Utilization of Alternate Routes of Administration

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The Development of a New Pertussis Booster Formulation via the Implementation of New Adjuvants and Utilization of Alternate Routes of Administration

Megan Ashley DeJong

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Doctor in Philosophy
in
Immunology and Microbial Pathogenesis

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Keywords: Vaccine, pertussis, adjuvant, intranasal vaccination, mucosal immunity, B. pertussis, humoral immunity, DTaP, Tdap, whooping cough

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Abstract

The Development of a New Pertussis Booster Formulation via the Implementation of New Adjuvants and Utilization of Alternate Routes of Administration

Megan Ashley DeJong

Pertussis (whooping cough) is a respiratory disease caused by airborne transmission of the Gram-negative bacterium, Bordetella pertussis. Prior to the development of the first pertussis vaccines (whole cell (wP) vaccines), the incidence of pertussis was in the hundreds of thousands of cases per year, which led to the death of many children, as the infection is most severe in younger populations. Thankfully, the wP formulation resulted in a dramatic decrease in the number of annual pertussis cases, nearly eradicating the disease. However, as wP contained the whole B. pertussis bacterium (and its lipooligosaccharide (LOS)), reactogenicity issues became apparent, leading to vaccine hesitancy amongst the public. Researchers in Japan had been testing the efficacy of an acellular pertussis (aP, DTaP, Tdap) vaccine, which contained select antigens rather than the whole bacterium, and found that it was capable of inducing high levels of antibodies against B. pertussis virulence factors. Further, the vaccine was much safer and elicited very minor side effects when compared to its wP counterpart. As a result, the wP vaccines were soon phased out in the United States and replaced with DTaP, the primary vaccine formulation. Yet, it soon became apparent that the duration of protection elicited by DTaP was short-lived, which prompted the implementation of an aP booster formulation, known as Tdap. The switch from wP to aP formulations coincides with a resurgence of pertussis cases in the United States, as well as in other countries that utilized aP vaccines. Research into the immune responses induced by both vaccine formulations has concluded that wPs induce a Th1 polarized immune response, which results in the activation of the cell-mediated immunity that is needed to clear B. pertussis from the respiratory tract and recover from infection. The aP vaccines, on the other hand, induce Th2 polarized immunity, which has been shown to provide neither long-lived immunity nor complete clearance of the pathogen from the respiratory tract, which allows for its transmission to another host. Because of this, there has been an ongoing effort to improve the immune responses induced by aP vaccines such that a more efficacious and durable immune response is elicited. In this dissertation, we aimed to evaluate the inclusion of new adjuvants into aP vaccine formulations as well as the utilization of intranasal vaccination. First, we determined the ability of the CpG 1018 adjuvant to improve the immunity afforded by the current booster, Tdap. In this study, mice were immunized with either Tdap or Tdap + CpG 1018 and then challenged with B. pertussis. We observed an increased clearance of B. pertussis from the respiratory tract, as well as an increase in serological responses to pertussis vaccine antigens. Further, in an effort to evaluate this formulation’s ability to protect against strains that have mutated, mice were challenged with either a strain of B. pertussis that expressed the virulence factor pertactin (PRN) or did not express PRN. Overall, the use of this adjuvant was able to protect against both strains, suggesting that the immunity provided
could withstand the variability seen with the evolution of *B. pertussis*. We then aimed to evaluate a lipid A mimic created via Bacterial Enzymatic Combinatorial Chemistry (BECC438b) as an adjuvant to include in DTaP. We determined the protection afforded by administering the DTaP + BECC438b formulation either intramuscularly or intranasally. The combination of the DTaP + BECC438b formulation and intranasal vaccination resulted in the most profound changes in the resulting immune response, as there was a decrease in bacterial burden within the respiratory tract as well as a robust induction of mucosal immune responses when compared to mock-vaccinated, challenged (MVC) mice. Further, the intranasally vaccinated mice had an increase in the expression of genes involved in both the activation and regulation of immune system processes. As a result of seeing such profound differences in the protective immune responses as a result of intranasal vaccination, we aimed to determine the effect of including an intranasally administered pertussis vaccine into the current vaccine schedule. All pertussis vaccines are administered intramuscularly; therefore, we felt it was essential to understand how these two routes and their respective immune responses would interact. We theorized that intramuscular priming would “push” immune responses out into the systemic circulation and establish robust adaptive immunity, while the intranasal booster would “pull” these responses to the site of vaccination or infection (in this case, the nasal cavity). The results of this study demonstrated the benefit of utilizing just one intranasal booster vaccine, as there was increase bacterial clearance throughout the respiratory tract as well as the induction of mucosal immune responses. The loss of an intramuscular booster had no effect on the induction of systemic immune responses. Further, when either a lipid A mimic (BECC438b) or β-glucan (IRI-1501) was added to the formulation, the effects of the intranasal booster were more pronounced. Taken together, the data in this dissertation build upon the established knowledge regarding the next generation of pertussis vaccines. Not only did we demonstrate efficacy as a result of implementing new adjuvants, but we also highlighted the importance of intranasal vaccination. The vaccines tested in this thesis serve as a stepping stone toward future studies that will help combat the resurgence of pertussis.
Dedication

To my mom and dad.

Thank you for everything.

Rose Nylund: *Can I ask a dumb question?*

Dorothy Zbornak (in response): *Better than anyone I know.*
Acknowledgements

First, thank you to Dr. Heath Damron for allowing me to join your lab. I joined without a rotation and out of the blue but you took me in, regardless. It isn’t easy to mentor an MD/PhD student, as there are many deadlines that need to be met. Nevertheless, you helped me meet all of them. I have grown into a stronger person over the last four years, and I will continue to grow stronger because of my time in your lab.

To the DB lab members, I cannot thank you enough. You have been with me for every experiment, every assay, every good day, and every bad day. I learned everything I know from you all. This dissertation and the works within it are not due to the effort of one person, but rather, a whole team. Without your help and guidance, this work would be null and void.

To my committee members, I am grateful for your guidance and reassurance throughout these past four years. You have helped me think more critically and create the best versions of my work. To Dr. Cooper: THANK YOU FOR EVERYTHING. I COULD NOT HAVE DONE THIS WITHOUT YOU. I AM SO GRATEFUL TO HAVE YOU IN MY CORNER.

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To my parents, Gary and Gloria, you get the biggest “thank you” of all. You have been there throughout this whole process. I have called to express joy (rarely) as well as sadness (mostly), and you have always helped me work things out. Without you, I couldn’t achieve anything. In reality, I feel that you should get an honorary doctorate, as you have been with me for the entire journey. There are not words to describe how much you mean to me, so all I can say is thank you, and I love you.

Last, but certainly not least, thank you to Beverly. I adopted you right before the pandemic hit, and we have had an unhealthy, co-dependent relationship ever since. She has heard all my presentations and has watched me work on all of my papers (including this dissertation). When days were rough and it felt like there was no end to the stress, she would come up beside me, purr her little heart out, and suddenly things would be okay. Bev, you can’t read this (because cat), but I love you to bits, and I’m grateful every day that you’re mine.
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<tbody>
<tr>
<td>AC</td>
<td>Adenylate cyclase domain</td>
</tr>
<tr>
<td>ACT</td>
<td>Adenylate cyclase toxin</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aP</td>
<td>Acellular pertussis</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AS04</td>
<td>Adjuvant System-04</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BECC</td>
<td>Bacterial Enzymatic Combinatorial Chemistry</td>
</tr>
<tr>
<td>Bp</td>
<td><em>Bordetella pertussis</em></td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DTap/Tdap</td>
<td>Diphtheria, tetanus, and acellular pertussis</td>
</tr>
<tr>
<td>DTP/DTwP</td>
<td>Diphtheria, tetanus, and whole cell pertussis</td>
</tr>
<tr>
<td>FHA</td>
<td>Filamentous hemagglutinin</td>
</tr>
<tr>
<td>FIM</td>
<td>Fimbriae</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissue</td>
</tr>
<tr>
<td>MVC</td>
<td>Mock vaccinated, challenged</td>
</tr>
<tr>
<td>MMR</td>
<td>Measles, mumps, rubella</td>
</tr>
<tr>
<td>MPL</td>
<td>Monophosphoryl lipid A</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal associated lymphoid tissue</td>
</tr>
<tr>
<td>NIBSC</td>
<td>National Institute of Biological Standards and Controls</td>
</tr>
<tr>
<td>NVNC</td>
<td>Non-vaccinated, non-challenged</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>OmvPV</td>
<td>Outer membrane vesicle of pertussis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PRN</td>
<td>Pertactin</td>
</tr>
<tr>
<td>RTX</td>
<td>Repeats-in-toxin domain</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>SII</td>
<td>Serum Institute of India</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>----------------------------</td>
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<tr>
<td>T_{CM}</td>
<td>T central memory cell</td>
</tr>
<tr>
<td>TCT</td>
<td>Tracheal cytotoxin</td>
</tr>
<tr>
<td>T_{EM}</td>
<td>T effector memory cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>T_{RM}</td>
<td>T resident memory cell</td>
</tr>
<tr>
<td>wP</td>
<td>Whole cell pertussis</td>
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Chapter 1

Introduction
1.1 Disease Manifestations of a Pertussis Infection

Within an ancient text written during the Sui dynasty, there is a description of a 100 day cough and that, if 100 children develop the cough, only a small fraction (10%) will recover from the illness (1). In 1578, French physician Guillaume de Baillou wrote of young children developing a cough so severe, that the lung becomes irritated, making both inspiration and expiration difficult (2). These cases are some of the earliest documentations that describe pertussis, or whooping cough, which is caused by the organism *Bordetella pertussis*, an exclusively human pathogen. There are three phases of a pertussis infection: 1) the catarrhal phase, 2) the paroxysmal phase, and 3) the convalescent phase (3). The catarrhal stage lasts 1-2 weeks and consists of symptoms that are akin to that of a common cold, such as a fever and a runny nose (4,5) (Figure 1). The similarity to other common illnesses makes it difficult to diagnose, and if confirmatory testing is not done, the risk of spreading *B. pertussis* to others increases. The disease then progresses into the paroxysmal phase, in which coughing becomes severe and develops the characteristic inspiratory “whoop” from which the disease gets its namesake (5). The whoop results from an inspiratory breath passing through a swollen glottis (6). During this stage, the intensity of the cough can result in vomiting, cyanosis, bradycardia, and apnea (5,7). As the coughing becomes less frequent, the patient enters into the final phase of the disease, the convalescent phase. The coughing can continue over the course of several months during the convalescent phase. The effects of a pertussis infection vary amongst each age group. While *B. pertussis* can infect people of all ages, it is rare to see pertussis develop in those over the age of 14 (4). However, when older individuals do develop the disease, it is common for them to develop a prolonged illness associated with a cough (8,9). Currently, the first-line treatment for those infected with *B. pertussis*, as well as those who have been exposed to an infected individual, is oral erythromycin (10). Overall, the best way
to prevent pertussis infections is through completing the vaccination series and following the recommended booster schedule (10).

**Figure 1. The three stages of a typical pertussis infection and associated symptoms.**

### 1.2 Pathogenesis of *B. pertussis*

*B. pertussis* is a Gram-negative pathogen that is transmitted via aerosolized droplets. Once transmitted, *B. pertussis* adheres to the ciliated epithelial cells of the respiratory tract (6). The expression of a myriad of virulence factors allows for a *B. pertussis* infection to occur. There is a two-component system, known as BvgAS, that regulates the expression of nearly all of the pathogen’s virulence factors (11,12). BvgAS is controls three distinct phenotypic phases: a fully active phase (Bvg\(^+\)), an inactive phase (Bvg\(^-\)), and an intermediate phase (Bvg\(^i\)) (11,13). During
the Bvg\textsuperscript{+} phase, BvgA will activate the transcription of \textit{vag} loci, which encodes for a number of virulence factors, including filamentous hemagglutinin (FHA), pertactin (PRN), pertussis toxin (PT), adenylate cyclase toxin (ACT), and fimbriae (FIM) (11,13). In the Bvg\textsuperscript{-} phase, there is no activation of the \textit{vag} loci, and the virulence factors are not produced (13). Partial activation of BvgA results in the Bvg\textsuperscript{i} phase, which may be important in transmission, though this has not yet been confirmed (14).

In the case when the virulence factors are expressed, the infectious process of \textit{B. pertussis} can begin. On the surface of \textit{B. pertussis} lies several molecules that play a role in the adhesion to host cells, including FHA. Studies have shown that FHA plays a role in the binding of the bacterium to host cells within the respiratory tract (15). Similarly, another surface protein, FIM, has a role in the adherence of \textit{B. pertussis} to the ciliated epithelium. \textit{B. pertussis} produces two types of FIM, known as Fim2 and Fim3, which both bind to glycosaminoglycans (16,17). PRN, an autotransporter located on the surface of \textit{B. pertussis}, is known to play a role in adhesion; however, its role in pathogenesis remains largely unknown (18). A major virulence factor of \textit{B. pertussis} is PT, which is an adenosine diphosphate (ADP)-ribosylating AB\textsubscript{5}-type toxin, which contains an active A subunit and five binding B subunits (14,19). PT is secreted across the bacterial membrane via a type 4 secretion system and then prevents the activation of G protein-coupled receptors (19,20). The downstream effects include some of the most notable symptoms associated with a pertussis infection, such as leukocytosis, histamine sensitivity, and hypoglycemia (20). In regard to fatal cases of pertussis, there has been a strong correlation between the development of severe leukocytosis and pulmonary hypertension (21–23). Post-mortem studies on the lungs of infants with fatal cases of pertussis reveal the development of a pulmonary infection with \textit{B. pertussis}, the
release of PT then causes leukocytosis, which increases the mass of the blood (24). The increased blood mass, combined with the hypoxia resulting from the whooping, leads to pulmonary hypertension, which ultimately results in cardiac failure (24) (Figure 2).

The aforementioned virulence factors, PT, FIM, PRN, FHA, have been included in acellular pertussis vaccine formulations. However, there are many other virulence factors expressed by B. pertussis that play a role in disease pathogenesis. ACT consists of two separate domains: the adenylate cyclase (AC) domain and the repeats-in-toxin (RTX) domain (20). When ACT is released from B. pertussis, it is activated by calmodulin, which results in an increase in cyclic adenosine monophosphate (cAMP) levels (25). Like PT, ACT is considered to be an important virulence factor of B. pertussis, as research has demonstrated its roles in inhibiting both neutrophil phagocytosis and the oxidative burst as well as causing apoptosis of macrophages (25,26). Therefore, it seems that ACT is critical in the survival of B. pertussis inside of the host. The function of tracheal cytotoxin (TCT), a component of the bacterial cell wall, is similar to that of ACT, in that is has been shown to inhibit various neutrophil functions, such as migration and complement activation, further allowing for the survival of B. pertussis (20,27). All of these virulence factors act in concert to cause tissue damage and the symptoms associated with a case of pertussis. It is interesting to note that there has been no toxin identified as the cause of the paroxysmal cough, and it may be that it results from a toxin that is yet to be identified (28).
1.3 The History of Vaccines

While many associate the word “vaccine” with a syringe injecting a solution into the deltoid muscle of the recipient, this methodology is the result of centuries of research. The earliest known documentation of inoculation dates back to a Chinese book published in 1549, which was discovered by Joseph Needham (29,30). In the text, Needham proports that, in order to vaccinate against smallpox, material from a smallpox wound would be collected and then blown up the nose as an inoculum (29,30). In a medical textbook published by physician Zhang Lu in 1695, he described a method of inoculation in which cotton would be dipped into a smallpox wound and
this cotton would be placed into the nostril of a healthy child, serving as a means of vaccination (31). While crude, these practices were effective. Perhaps a more famous case of early vaccination lies in the story of Edward Jenner, who hypothesized that inoculation with cowpox would protect against smallpox. In 1796, Sarah Nelmes, a milkmaid, became infected with cowpox and Jenner took exudate from Nelmes’ wound and used it to inoculate the arm of 8-year-old James Phipps (32,33). Within a week, Phipps developed wounds and felt slightly ill but made a full recovery, and when challenged with smallpox, Phipps had no resulting infection (32,33).

During the late 1800s, cases of chicken cholera were plaguing thousands of wild fowl annually. In an effort to absolve this issue, veterinarian Henry Toussaint sent Louis Pasteur a strain of Pasteurella multocida, the causative agent of chicken cholera, in the hopes that Pasteur could develop a solution to the infection (34). Pasteur began to grow the organism in chicken broth and discovered that the organism weakened over time, as when chickens were injected with a month old culture, they recovered and were protected from subsequent challenge (34,35). In a similar vein, cases of anthrax were killing large numbers of both sheep and cattle and Pasteur set out to find a solution. He learned that Toussaint had attempted to develop an anthrax vaccine by heating the bacterium at 55°C for 10 minutes (36). While not effective, Pasteur utilized the idea of weakening the pathogen and discovered that when heated at 42°C, Bacillus anthracis was unable to produce spores (35,36). When cows and sheep were inoculated with Pasteur’s vaccine, they all survived a challenge with B. anthracis, while animals not vaccinated, all died as a result of the infection (35). Up to this point, Pasteur’s vaccines had not been utilized in human recipients; however, when a young boy named Joseph Meister was suffering from a supposed rabid dog bite in a hospital, Pasteur got his chance to test one of his vaccines in a non-animal model (34–36).
Prior to administering a vaccine to Meister, Pasteur and colleague, Emile Roux, had been working to affect the virulence of rabies for several years. Eventually, the pair discovered that they could take the spinal cords of rabbits infected with rabies, leave them out in open air in combination with potassium hydroxide, and create a vaccine from the dried spinal cord material (35,36). Meister was given 13 inoculations of progressively weaker inoculum over 11 days and did not succumb to rabies, and the vaccine was garnered as a success (35). Pasteur’s vaccine was used to vaccinate over 300 people against rabies, and only one developed the disease (35).

The next major vaccine development occurred in 1921, when the first Bacillus Calmette-Guérin (BCG) vaccine was administered (37). In an effort to combat tuberculosis, Albert Calmette and Camille Guérin worked to subculture virulent strains of *Mycobacterium bovis* in an effort to attenuate the strain such that it could be used in a vaccine (37). Eventually, it was discovered that growing *M. bovis* every 21 days on a medium consisting of a mixture of glycerinated potato and beef bile for 3 hours at 75°C would modify the virulence of the original strain (38). Ultimately, after more than 230 passages over the course of 13 years, the strain had lost its virulence and, thus, the BCG strain came into existence (38). After the development of the BCG vaccine, the vast majority of vaccines – more than 40 – would be created by Maurice Hilleman and his team of researchers. Hilleman’s first vaccine was for Japanese B encephalitis, which was developed in 1944 to aid in the immunization of soldiers, and his last vaccine was for hepatitis A, which was licensed in 1995 (39).
1.4 Types of Vaccines

The ultimate goal of a vaccine is to induce a protective immune response against a pathogen without the individual having to develop the disease (40). The contents of the vaccine, itself, depend on the organism against which one is vaccinating. There are live, attenuated vaccines, which contain a weakened version of either a bacteria or virus and these vaccines create an immune response that is very similar to that of a natural infection (41). In order to create an attenuated – but not killed – version of the pathogen, it is repeatedly cultured in cells, which renders it too weak to cause an infection but also allows it to replicate and induce a proper immune response (42,43).

Examples of live, attenuated vaccines included measles, mumps, and rubella (MMR) and the influenza vaccine. Unlike attenuated vaccines, the pathogens included in inactivated vaccines are killed (via heat or chemicals) and, therefore, are unable to replicate (42). While these vaccines are unable to cause disease, even in an immunodeficient recipient, they elicit a weaker immune response with a shorter duration of protection when compared to live, attenuated vaccines (41,44). As a result of the lack of a robust immune response, recipients of inactivated vaccines often need multiple booster immunizations to induce a sufficient protective response (40). These vaccines rely on their structure and surface antigens to induce an immune response, as they are unable to replicate within the host (40). Examples of inactivated vaccines include whole-cell vaccines (such as pertussis and rabies vaccines), toxoid vaccines (such as diphtheria and tetanus vaccines), and subunit vaccines (such as pneumococcal vaccines) (42). A new category of vaccine, nucleic acid-based vaccines, was highlighted by the SARS-CoV-2 pandemic. These vaccines insert either DNA or RNA segments that utilize the host’s own cells to encode for proteins that will serve as antigens (40) (Figure 3).
1.5 Pertussis Vaccines: wP versus aP

The causative agent of whooping cough was identified in 1906 by Jules Bordet and Octave Gengou in 1906, when they were able to isolate colonies grown on their medium, which consisted of agar, glycerin extracted from a potato, and defibrinated blood (45). In the 1920s and 1930s, there were hundreds of thousands of pertussis cases each year, and of those cases, thousands of them claimed the lives of young children (46,47). In a large study conducted in the Faroe Islands, physician Thorvald Madsen utilized a whole cell (wP) vaccine made from recently cultured strains of *B. pertussis* that were immersed in a solution of sodium chloride and formaldehyde (48). The results of his studies were promising, as these vaccines prepared from cultures of *B. pertussis* seemed to offer some protection (48,49). Pearl Kendrick and Grace Eldering began working on whooping cough research when a virulent strain of *B. pertussis* passed through a Grand Rapids hospital in
1932 (50). Kendrick and Eldering began to perform controlled animal studies on potential vaccine candidates, and they eventually developed a formulation that was both safer and more potent than the experimental vaccines that were distributed to the public (51). To do this, the whole *B. pertussis* bacterium was inactivated with thiomersal while being stored at cold temperatures for at least one week, and once deemed safe, the vaccines were distributed to local physicians who would then administer doses to children (50,51). Further studies demonstrated efficacy when the killed *B. pertussis* bacterium was precipitated with alum and combined with diphtheria toxoid (52). Results from the studies of these vaccines demonstrated an 89% efficacy rate, and this wP vaccine was put into use in 1943 after the American Medical Association granted it approval (49,53). Kendrick and Eldering even went on to develop an intracerebral mouse challenge model in order to determine the potency of pertussis vaccines as a means of quality control for different manufacturers (54). Finally, in 1949, an approved formulation that included diphtheria toxoid, tetanus toxoid, as well as the whole *B. pertussis* bacterium – known as DTP – implemented and used globally.

As a result of the implementation of wP vaccines, the number of annual pertussis cases dropped dramatically, going from hundreds of thousands per year to less than 5,000 on average (55,56). Initially, the public was thrilled to see an almost absence of pertussis cases; however, it quickly became apparent that the wP formulation did have some potentially worrying side effects. Studies report that, within 48 hours after vaccine administration, some recipients ages 6 experienced typical symptoms such as swelling and pain at the site of injection, a fever, agitation, drowsiness, or crying (57). However, a small portion of vaccinated children would have convulsions or periods of hypotonic hyporesponsiveness (57). While the data did not demonstrate the ability of the wP vaccine to cause permanent damage or death, the public grew to fear the deleterious side effects
associated with the vaccine, prompting a reduction in vaccine compliance (58). Researchers knew that a new vaccine formulation was needed, and after work was performed by Sato and Sato in Japan, the first acellular formulation was introduced in 1999. Previous work demonstrated that antibodies against both PT and FHA protected animal models against *B. pertussis* challenge (59). As a result, the new vaccine formulation contained formalin-inactivated PT and formaldehyde-treated FHA, diphtheria toxoid, and tetanus toxoid and, when this formulation was administered in two doses to children ages 2 and younger, it afforded protection without the associated side effects (60,61). Other studies were performed with a five component acellular pertussis (aP) vaccine, which contained glutaraldehyde-inactivated PT, FHA, Fim2/3, PRN, diphtheria toxoid, and tetanus toxoid (62). After analyzing the data from nearly 100,000 vaccinated infants, it was determined that a five-component aP formulation would provide the most immunogenic response without compromising safety (62). The results of these data prompted the United States to allow children primed with DTP who were older than 15 months to continue the vaccine series with the diphtheria, tetanus, and acellular pertussis (DTaP) vaccine in 1992, and by 1997, only DTaP was used in the immunization schedule (63,64). While initial reports had promising data in regard to immunization with DTaP – better safety profile, similar serological responses to wP – this new formulation did not yet have data that addressed the long-term performance of these aP vaccines. Thus, several years would have to pass before the true efficacy of aP vaccines could be elucidated.

### 1.6 The Resurgence of Pertussis

As follow-up studies regarding the performance of aP vaccines began to emerge, it became clear that the initial three doses of DTaP resulted in waning immunity that began to appear in children around the ages of 6 or 7 (65). Further corroborating this hypothesis was the fact that, in children vaccinated with aP formulations, there was an increased incidence of pertussis cases among those
around the age of 7 (66). With multiple studies across the world providing evidence of waning immunity, the Tdap booster formulation containing reduced amounts of each antigen or toxoid included in the primary DTaP formulation was put into place in 2005 (67). The new booster formulation resulted in the following vaccine schedule: DTaP vaccination at 2, 4, and 6 months of age, DTaP vaccination at 15-18 months of age, DTaP at 4-6 years old, Tdap at 11-12 years of age, Tdap at 19 years old (if a previous Tdap vaccination was not administered), and Tdap during the third trimester of pregnancy (68). As time passed after the introduction of aP formulations, it became clear that cases of pertussis were again beginning to rise (Figure 4). Certainly, an increase in the number of cases can be attributed to an increase in disease surveillance as well as an increase in diagnostic methodologies (69). What cannot be denied, however, is that in the United States, many states experienced what could be considered epidemic levels of disease, with the year 2012 having the highest number of recorded cases since 1955 (46). Even more concerning is the fact that the highest incidence was seen in those under the age of 1, which is the demographic that is most likely to die as a result of a pertussis infection (46,70). In addition to seeing a rise in the number of cases in infants, there has also been a concomitant rise in adolescent and adult populations (55,61,71). In fact, even in individuals that have received the entire aP vaccination series, the odds of a pertussis infection occurring increases 1.3 times every year that passes after receiving DTaP (72). The rise in incidence of pertussis means that older individuals are serving as
“reservoirs” where they, as a result of aP’s waning immunity, once again become infected and can transmit the pathogen on to more susceptible populations (such as those under the age of 1).

![Figure 4. The number of annual pertussis cases dating back to 1920.](image)

1.7 Acellular Vaccines and Waning Immunity

As we have begun to realize that the immunity provided by aP vaccine formulations is waning, research has been focused to determine why exactly it is occurring. As previously mentioned, aP formulations can contain the following antigens: PT, FHA, PRN, and/or FIM2/3. Recently, many countries have reported cases of pertussis that have been caused by strains of *B. pertussis* that are deficient in PRN (18). In fact, the strains that lack PRN expression are increasing in prevalence such that they have become the predominate circulating strains in the countries that utilize only aP vaccines, such as the United States (85%), Australia (83%), Sweden (69%), and Italy (55%) (73–75). It has been hypothesized that the aP vaccine has resulted in “vaccine-driven evolution,” which has caused the rise in strains that lack PRN (18). While bacteria constantly evolve, the loss of PRN
expression has been achieved through deletions of the full gene, stop codons resulting from transversion mutations, deletions within the insertion sequence, etc. (75,76). Thus, the loss of PRN cannot be linked to one specific mutation, meaning that there have been numerous selection events resulting in a PRN mutant strain (18).

In addition to evolving strains of *B. pertussis*, the waning immunity can also be attributed to the immune response elicited by the aP formulations, themselves. Studies have shown that the predominate immune response provided by aP vaccines is Th2 polarized (77,78). Th2 cells are a subset of T helper cells and direct immune responses by means of cytokine production (79). Naïve T cells become Th2 cells upon stimulation with the cytokine interleukin (IL)-4 and these Th2 cells then go on to secrete a milieu of cytokines including IL-4, IL-10, and IL-13 (80). Th2 cytokines are involved in many facets of humoral-based immunity, such as B cell proliferation, antibody production, and class switching (80,81). While this type of immune response is great for extracellular pathogens that cannot be phagocytosed, studies have shown that it is a Th1-polarized immune response that allows for recovery from a *B. pertussis* infection (82,83). Upon polarization, Th1 cells secrete interferon (IFN)-γ, IL-2, and lymphotoxin-α, which then stimulate phagocytosis, the oxidative burst, and the killing of intracellular pathogens (80,84–86). In the case of *B. pertussis*, the cell-mediated immunity provided by Th1 cells is needed for not only clearance of the pathogen, but also in protection against subsequent infections (87,88).
1.8 Natural Infection and Vaccine-Mediated Immunity to \textit{B. pertussis}

Regardless of which pertussis vaccine is administered, neither wP nor aP formulations are able to offer a duration of protection as long as that of natural infection. Upon infection with \textit{B. pertussis}, cell-mediated immunity takes effect as macrophages and immature dendritic cells migrate to the respiratory tract (88). The innate response within the lungs involves the migration of macrophages, dendritic cells, neutrophils, and lymphocytes (89). From there, natural killer cells and T cells, most of which are CD4\(^+\), arrive at the lung to help clear the pathogen (89).

While \textit{B. pertussis} has the ability to enter and survive within macrophages, there is overwhelming evidence that supports their role in protective immunity (90–92). The secretion of both IFN-\(\gamma\) and IL-17 from T cells (specifically, Th1 and Th17 cells) promotes the killing of \textit{B. pertussis} within macrophages (93,94). As \textit{B. pertussis} is a Gram-negative pathogen, it has lipooligosaccharide (LOS) on its surface, which binds to Toll-like receptor 4 (TLR4). The binding of LOS to TLR4 receptors on immature dendritic cells promotes their maturation and the subsequent secretion of both IL-12 and IFN-\(\gamma\) (95). These cytokines stimulate the activation of Th1 cells and this polarization of the immune response, as previously mentioned, is crucial for clearance of the pathogen. When dendritic cells are stimulated with ACT, the result is the activation of the NLRP3 inflammasome, ultimately allowing for the production of IL-1\(\beta\), which is needed for the production of \textit{B. pertussis}-specific Th17 cells (96). Akin to macrophages, neutrophils also phagocytose \textit{B. pertussis} once they are recruited to the lungs by IL-6 (97). The ultimate result of these cellular responses is the activation of CD4\(^+\) T cells, specifically Th1 and Th17 cells. In regard to Th1 cells, serological studies performed on infants diagnosed with whooping cough demonstrated T cells that secreted Th1-assocaited cytokines, such as IL-2 and IFN-\(\gamma\) (82). Additionally, a seminal study by Mills \textit{et al.} illuminated the importance of T cells when they observed that, in the absence of
serum antibodies, mice that had antigen-specific T cells were able to clear a pertussis infection (87). Natural infection also induces the production of Th1 cells, and studies have shown that this subset of T cells is also important in clearing *B. pertussis* from the respiratory tract (96). Further, both IFN-γ and IL-17 induce the production of a subset of T cells, known as T resident memory cells (T\textsubscript{RMS}), which remain localized in mucosal tissues and play a key role long-term memory responses (98).

Upon administration of wP, similarly to natural infection, a Th1/Th17 polarized immune response is induced (99). As a result, wP is also able to induce a mostly cell-mediated immune response, allowing for clearance of the pathogen and the induction of long-lasting immunity. The main similarity between natural infection and wP immunization lies in the myriad of antigens that are able to be presented to immune cells, as both involve the entire *B. pertussis* bacterium. Thus, the antigens that are taken up and presented are comparable. However, wP is unable to replicate the downstream immunological processes associated with natural infection, such as bacterial cell replication and damage to the host mucosal epithelium (100). Further, human studies have demonstrated that natural infection induces the production of predominately the IgG1 subclass against PT, but ultimately, all IgG subclasses are produced with a *B. pertussis* infection (101,102). With wP vaccination, there is a shift in the proportion of IgG subclasses, in that the vast majority of anti-PT IgG becomes IgG2, along with a small fraction of IgG1 (102). This variation is important, as IgG1 is a strong activator of complement-mediated functions and the response to protein antigens, while IgG2 is a weak activator of those processes (103).
Unlike natural immunity to *B. pertussis* and wP immunization, aP vaccines result in an immune response that relies on humoral immunity, more so than the cell-mediated response (100). T cell studies on children vaccinated with aP formulations revealed that CD4+ T cells expressed high levels of IL-4, but not IL-17 or IFN-γ, which are critical in both clearance and the duration of protection (104,105). Indeed, immunization with aP results in the release of cytokines, such as IL-4 and IL-5, that induce the production of Th2 type cells, which result in the production of antibodies against antigens contained in the vaccine (82,106). While human studies suggest that there may be a protective role of antibodies against *B. pertussis* antigens, in that they reduced the severity of disease, there is little evidence that corroborates their ability to sufficiently protect against becoming infected with pertussis (88,107). The inability of aP vaccines to induce immune processes on par with that of natural infection or wP lies in their formulation. To start, aP vaccines do not contain the whole *B. pertussis* bacterium, but rather, a select few antigens (ranging from 3 to 5 antigens). This, in conjunction with the utilization of the alum adjuvant, makes for a less robust immune response and a lack of induction of cell-mediated immunity.

1.9 Aluminum Salts as Adjuvants

An adjuvant is a substance or compound that is included in a vaccine in order to enhance the resulting immune response to the antigens included in the formulation (108). While there are many adjuvants available today, early in vaccine development, the only adjuvants used were aluminum salts (alum) (109). Alexander Glenny included potassium aluminum sulfate in a diphtheria toxoid vaccine and found that its inclusion resulted in the induction of greater antibody responses when compared to the toxoid alone when tested in guinea pigs (109,110). In subsequent vaccine formulations, the vaccine components were adsorbed to aluminum hydroxide or alhydrogel and aluminum phosphate (111). While the mechanism of action by which alum works as an adjuvant
remains elusive, there are a few leading theories. The earliest theory was that of “depot formation,” in which alum retains vaccine antigens at the immunization site and allows for a slow release of the vaccine components (112). This theory emerged when skin from the injection site of guinea pigs immunized against diphtheria and tetanus was removed, homogenized, and injected into naïve guinea pigs. Naïve guinea pigs immunized with the injection site containing alum had titers against both diphtheria and tetanus toxoids, while guinea pigs that received skin that did not contain alum, did not (112). It was speculated that the ability of alum to maintain vaccine components at the site of injection enhanced the uptake of antigen by antigen presenting cells (APCs) (113).

Another theory of alum’s mechanism of action is that of the “antigen targeting” theory, which consists of three phases: 1) Cell accumulation at the injection site, 2) Presentation of antigen to APCs, and 3) Migration of activated APCs to draining lymph nodes (114). Evidence supporting the first phase involved the observation that immunization with alum resulted in leukocyte recruitment at the injection site (115, 116). As a result of this recruitment, mRNA expression of cytokines, complement proteins, and adhesion molecules are increased (115). Data supporting phase two includes the utilization of Ealpha green fluorescent protein (EαGFP) to assess both antigen uptake and degradation as well as the YAe antibody to assess antigen presentation (117). In this model, EαGFP is taken up by dendritic cells (DCs), the Eα peptide is presented on MHC class II, and the Eα-MHC class II complex can be detected by the YAe antibody (117). This study demonstrated that the adsorption of antigen to alum increased antigen uptake by DCs and increased the duration of presentation time by DCs in vitro (117). Further, studies also illustrated the ability of alum to trigger the differentiation of monocytes into myeloid DCs and increase the expression of both CD86 and CD40 on human peripheral blood mononuclear cells (PBMC)-derived
macrophages (118). The final phase has been supported by studies that observed that, after human PBMCs differentiate into DCs, these DCs have a slowed solubilization of alum, which allows them to gain inflammatory signals such as IL-1β, TNF-α, and IL-6 (116,118,119). These inflammatory signals induce the trafficking of DCs to the draining lymph nodes and, from there, can present to lymph node-based APCs, which can activate the innate immune system in distant organs like the spleen (120,121).

Regardless of the theory by which alum exerts its effects, it has long been realized that alum induces strong Th2 polarized immune responses. A study in mice illustrated that alum adjuvants stimulated the release of both IL-1β and IL-18 from DCs via the activation of caspase-1 and that these DCs go onto to activate CD4+ T cells, which will secrete IL-4 and IL-5 (akin to the Th2 cell phenotype) (122). The secretion of IL-4 is of particular importance, as research has supported its role in the down-regulation of Th1-polarized responses (123). As previously mentioned, this is quite problematic, as protective immunity against B. pertussis is largely dependent upon the induction of cell-mediated immunity (Th1 polarized immunity) (88). Further, studies on IL-4 −/− mice demonstrate that vaccination with aP was protective, suggesting that Th2 immune responses are not needed to combat B. pertussis infections (105). As a result, there has been an ongoing effort to improve aP vaccine formulations via the inclusion of new adjuvants that either replace alum or work in conjunction with alum to improve vaccine immune responses.

1.10 Utilization of Alternative Adjuvants in Pertussis Vaccines

Given that the robust immune response elicited by both natural infection and wP immunization is, in part, driven by the activation of the TLR4 pathway by LOS, many researchers have thought that taking advantage of an adjuvant that elicits similar responses could be beneficial. One example of
such an adjuvant is that of monophosphoryl lipid A (MPL), which is a derivative of lipid A from *Salmonella minnesota* (124). The chemical detoxification of lipid A results in a molecule that still exhibits immunostimulatory properties but does not harbor the deleterious inflammation and toxicity associated with lipopolysaccharide (LPS) or LOS. MPL, like LPS, activates APCs and leads to the release of a number of pro-inflammatory cytokines such as IL-12 and TNF-α (125). MPL also stimulates the production of Th1 cells via IL-2 and IFN-γ release, which prompts cell-mediated immune responses and activation of complement (125). In regard to pertussis vaccines, a study demonstrated that when alum was replaced with MPL in a deconstructed DTaP vaccine, mice challenged with *B. pertussis* exhibited reduced bacterial burden and higher anti-PT IgG antibodies when compared to the standard DTaP adjuvanted with alum (126). Building on this concept is Adjuvant System 04 (AS04), which combines MPL with alum. AS04 is currently used in two licensed vaccines: Cervarix (vaccinates against human papilloma virus (HPV)-16 and HPV-18) and FENDrix (vaccinates against Hepatitis B) (127–129). While studies have shown that the inclusion alum did not enhance MPL’s activation of the innate immune response, it has been found that the alum component prolongs cytokine responses at the site of immunization (130). While AS04 has not been tested solely as an adjuvant in aP vaccines, its inclusion in an HPV-16/18 vaccine adjuvanted with AS04 elicited an acceptable safety profile and the novel adjuvant did not interfere with the baseline immune response of Tdap (131). One final example of a TLR4 agonist is that of lipid A mimetics manufactured via bacterial enzymatic combinatorial chemistry (BECC) (132). BECC is a process by which a mutant strain of *Yersinia pestis* is grown at varying temperatures, resulting in the deletion or addition of enzymes involved in the lipid A synthesis pathway (133). The result is the generation of a modified lipid A structure that has immunostimulatory properties without the deleterious effects typically associated with LPS. A
particular BECC variant, BECC438b, was tested in a pre-clinical mouse model, as this molecule was shown to elicit a balanced Th1/Th2 immune response (133,134). Data from this study illustrated the ability of DTaP + BECC438b to induce both humoral and cell-mediated immune responses when the vaccine was administered either intramuscularly or intranasally (DeJong et al., 2022; manuscript in submission). Further, this novel formulation was able to reduce bacterial burden in the respiratory tract after both an intranasally deposited inoculum as well as an aerosol challenge with *B. pertussis* (DeJong et al., 2022; manuscript in submission).

In addition to utilizing adjuvants that bind to TLR4, there has also been research into the efficacy of other TLR agonists. In mice, it has been found that certain lipoprotein ligands of *B. pertussis* are able to bind to TLR2, resulting in a potent immunostimulatory effect that induces DC maturation and the release of pro-inflammatory cytokines (135). Further, the activation of TLR2 stimulates both a Th1 and Th17 polarized immune response and conferred increased protection against *B. pertussis* than a standard aP formulation containing alum (135). Additionally, agonists of TLR9 have been of interest. Bacterial DNA acts as a pathogen associated molecular pattern (PAMP) and the CpG motifs within bacterial DNA are able to bind TLR9 (136). TLR9 is expressed on plasmacytoid DCs and B cells in humans, while mice express TLR9 on cells of myeloid lineage (137). As a result of binding TLR9, type 1 interferons are released, inducing a potent Th1 type response (138). Data supporting the use of TLR9 agonists in pertussis vaccines includes the observation that, when CpG oligodeoxynucleotides (ODNs) were combined with both alum and aP vaccine antigens, the serological responses to those antigens were greater than utilizing CpG ODNs alone (139). This suggests that the effect of CpG ODNs are increased as a result of the alum adjuvant. When a class B CpG ODN, CpG 1018, was incorporated into a Tdap formulation, it was
shown that CpG 1018 increased serological responses against pertussis antigens, reduced bacterial burden in the respiratory tract, and was able to redirect the typical Th2-polarized response of aP vaccines into a Th1 response, when compared to vaccination with Tdap alone in a murine model (136).

Another category of potential pertussis vaccine adjuvants is that of β-glucans, which are polysaccharides that are found within the cell walls of yeast, fungi, and bacteria (140). β-glucans bind to receptors on innate immune cells and trigger cytokine release, immune cell migration and maturation, and the induction of a Th1 polarized immune response (141). β-glucans are recognized by a number of receptors including Dectin-1 (expressed on a number of innate immune cells and ultimately leads to the activation of Th1/Th17 cells) and complement receptor 3 (CR3) (expressed on myeloid cells and clears pathogens opsonized by iC3b) (141–143). Further, murine studies have illustrated the potential of β-glucans to elicit an amplified immune response upon secondary exposure to a stimulus as a result of a process known as trained innate immunity (140). In murine pertussis studies, when curdlan – a 1,3 β-glucan – was added to a deconstructed, intranasally administered DTaP vaccine, it resulted in the production of serum IgG and mucosal IgA antibodies as well as the production of IL-17 (144). Further, in another study that utilized a β-glucan, IRI-1501, adjuvant in an intranasally administered DTaP vaccine, it was observed that this adjuvant increased humoral immune responses and offered a long duration of protection after vaccination (145) (Figure 5).
1.11 Mucosal Immunity

As *B. pertussis* is a respiratory pathogen, it stands to reason that localized immunity within the nasal cavity and lower respiratory tract are an important aspect of protection. In nonhuman primate and murine studies, there is evidence that aP vaccines protective against severe disease but are unable to prevent both colonization and transmission of the pathogen (146,147). The lack of clearance from the nasal cavity is hypothesized to be a factor contributing to the resurgence of pertussis cases (146,148). In order to combat this issue, there has been an ongoing effort to develop intranasally-administered pertussis vaccines, as this would both vaccinate at the site of infection and induce mucosal immune responses. The body has a breadth of mucosal tissues, including the gastrointestinal tract, the respiratory tract, and the urogenital tract, which are sites that are often the first point of contact for a pathogen (149). The mucosa-associated lymphoid tissue is comprised
of epithelial cells and lymphoid cells, and when a pathogen encounters this surface, microfold (M) cells take up antigen from the mucosal lumen and present it to APCs beneath the surface (150). From there, the APCs are able to activate T cells, which will further differentiate and migrate to tissues, and B cells, which will act as APCs and produce secretory IgA (sIgA) (151,152). The production of antigen-specific sIgA is paramount, as its main function is the prevention of pathogen adherence to the mucosal epithelium (152). In the case of *B. pertussis*, the presence of sIgA prevents the bacterium from binding to the ciliated respiratory epithelium and the subsequent release of virulence factors and toxins. While the main mediator of humoral immunity within the mucosal tissues is sIgA, it is important to note that mucosal vaccination is also able to induce the production of systemic IgG antibodies, as well (150). As for cell-mediated immune responses, a particular subset of T cells – T resident memory cells (T_{RMS}) – reside at the mucosal surface and are the first line of defense against infection (98). Central memory T cells (T_{CMS}) can migrate to effector organs and differentiate into effector memory T cells (T_{EMS}) upon antigen stimulation, and the T_{EMS} can then migrate to peripheral tissues (98). T_{RMS} reside permanently within peripheral tissues and, therefore, do not require the migration of T cells to the site of infection in order to initiate effector functions (153). Further, murine studies have observed that these cells are able to persist for long periods of time in the peripheral tissues (154,155).

There is currently a live-attenuated, intranasally administered pertussis vaccine – BPZE1 – that is currently in phase 2 clinical trials (ClinicalTrials.gov, NCT03541499). Pre-clinical studies in mice demonstrated that one intranasal dose of BPZE1 was able to protect against *B. pertussis* challenge, induce Th1 polarized immune responses, and generate antigen-specific IgG antibodies (156). Further, one dose of BPZE1 was able to protect against both disease and nasopharyngeal
colonization of the pathogen in baboons (157). Thus far in human trials, BPZE1 has been able to elicit both IgG and IgA antibody responses against *B. pertussis* antigens and activate a Th1 immune response (158).

There are a multitude of pre-clinical models evaluating the efficacy of other intranasally administered aP vaccine formulations. For example, intranasal administration of outer membrane vesicles of pertussis (omvPV) demonstrated in a mouse model as the vaccine was able to prevent colonization of the lungs and nares, increased IgA antibody levels, and elicited Th1/Th17 type cytokine responses (159). Another study, as previously mentioned, evaluated intranasal administration of DTaP as well as aP adjuvanted with curdlan and found that intranasal immunization induced greater mucosal immune responses and increased IL-17A production (144). Additionally, the evaluation of aP formulations adjuvanted with IRI-1501 demonstrated, not only the induction of mucosal immune responses, but also increased the generation of *B. pertussis*-specific plasma cells in the bone marrow, suggesting a longer duration of protection (145). A study utilizing a TLR4 agonist, Bacterial Enzymatic Combinatory Chemistry 438b (BECC438b), in combination with the DTaP vaccine demonstrated that intranasal vaccination of this formulation induced superior mucosal immune responses when compared to intramuscular administration in a mouse model (DeJong et al., 2022; manuscript in submission). Further, intranasal vaccination also increased activation of genes involved in regulation of the immune system and responses to stimuli (such as a pathogen) (DeJong et al., 2022; manuscript in submission). Overall, these data strongly encourage the use of an intranasally administered pertussis vaccine as well as novel formulations that could take advantage of the new vaccination route.
1.12 Overall Objectives

The objective of this thesis is to contribute to the development of novel pertussis booster formulations. As previously discussed, the immunity afforded by the aP formulations is waning, resulting in vaccinated individuals getting infected with *B. pertussis*. The result is older demographic serving as reservoirs that are able to transmit the pathogen onto more susceptible populations. There is a need to improve pertussis vaccines, which can be done via the inclusion of new adjuvants into the current formulation and/or the utilization of different routes of vaccine administration. In chapter 2, we evaluated the CpG 1018 adjuvant and its potential to improve the current Tdap formulation. In this study, we titrated Tdap in order to determine the sub-optimal dose that would allow us to properly evaluate the effects of the CpG 1018 adjuvant. From there, the titrated dose and CpG 1018 were utilized in subsequent experiments that evaluated bacterial burden, serological responses, and T cell responses at 1, 3, and 7 days post *B. pertussis* challenge. Further, we challenged with two strains of *B. pertussis* – UT25 (expresses PRN) and H762 (does not express PRN) – to determine the ability of the Tdap + CpG 1018 formulation to protect against strains that have undergone vaccine-driven evolution. In chapter 3, the goal was to evaluate the BECC438b adjuvant in both intramuscular and intranasal DTaP vaccines. We compared bacterial burden, serological responses, mucosal immune responses, T cell responses, and gene expression profiles between both vaccination route and vaccine formulation. Further, we also performed histological scoring of the lung to determine the extent of inflammation resulting from vaccination and challenge. In chapter 4, we aimed to evaluate the effect of utilizing an intramuscularly administered primary vaccine followed by an intranasal booster. Currently, all pertussis vaccines are administered intramuscularly; therefore, any new intranasal formulation would be building off of the already established systemic immune response. We used two adjuvants, IRI-1501 and BECC438b, and compared their resulting protection after two intramuscular vaccines or an
intramuscular prime followed by an intranasal boost. We used bacterial burden in the respiratory tract, systemic antibody production, mucosal antibody production, and ELISpot data to evaluate protection against *B. pertussis* challenge. In chapter 5, the major conclusions from this work and how they have contributed to the field are discussed. While there are many potential candidates to evaluate as the next generation of pertussis vaccines, this thesis evaluates both adjuvants and vaccine routes and how these new formulations could be implemented into the current vaccine schedule.
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CpG 1018 Adjuvant\textsuperscript{®} enhances Tdap immune responses against \emph{Bordetella pertussis} in mice
CpG 1018 Adjuvant® enhances Tdap immune responses against *Bordetella pertussis* in mice

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2.1 Abstract

*Bordetella pertussis* is the causative agent of whooping cough (pertussis), a severe respiratory disease that can be fatal, particularly in infants. Despite high vaccine coverage, pertussis remains a problem because the currently used DTaP and Tdap vaccines do not completely prevent infection or transmission. It is well established that the alum adjuvant is a potential weakness of the acellular vaccines because the immunity provided by them is short-term. We aimed to evaluate the potential of CpG 1018® adjuvant to improve antibody responses and enhance protection against *B. pertussis* challenge in a murine model. A titrated range of Tdap vaccine doses were evaluated in order to best identify the adjuvant capability of CpG 1018. Antibody responses to pertussis toxin (PT), filamentous hemagglutinin (FHA), or the whole bacterium were increased due to the inclusion of CpG 1018. In *B. pertussis* intranasal challenge studies, we observed improved protection and bacterial clearance from the lower respiratory tract due to adding CpG 1018 to 1/20th the human dose of Tdap. Further, we determined that Tdap and Tdap 1018 were both capable of facilitating clearance of strains that do not express pertactin (PRN−), which are rising in prevalence. Functional phenotyping of antibodies revealed that the inclusion CpG 1018 induced more bacterial opsonization and antibodies of the Th1 phenotype (IgG2a and IgG2b). This study demonstrates the potential of adding CpG 1018 to Tdap to improve immunogenicity and protection against *B. pertussis* compared to the conventional, alum-only adjuvanted Tdap vaccine.
2.2 Introduction

Pertussis, also known as whooping cough, is a respiratory disease caused by the Gram-negative bacterium, *Bordetella pertussis*. Before vaccines were developed, pertussis killed 450 infants out of every 100,000 births per year (1). After the development of the whole cell vaccine (DTP) in the 1940s, there was a dramatic decrease in pertussis incidence worldwide; however, reactogenicity issues prompted the switch to an acellular pertussis (aP) vaccine formulation, which occurred in 1996 in the US (2). While the aP vaccine was able to reduce the risk of potentially dangerous side effects, it was later realized that this benefit came with a compromise of faster waning immunity (3). Due to a shorter duration of protection provided by aP, children, adolescents, and adults, are still being infected with *B. pertussis* and are capable of transmitting the pathogen to younger, more susceptible populations, such as infants (4). As a result, there has been an increased effort to develop next generation vaccines that can provide a longer duration of protection. In the past decade, the field has accumulated a large amount of evidence which demonstrates that aP vaccines (DTaP and Tdap) are effective at preventing death in infants but that the immunity they provide is short-lived and they do not prevent transmission (5–8).

One aspect of aP vaccines that is thought to limit the protection provided is the adjuvant, alum (9). DTP vaccines required alum to induce antibodies to diphtheria and tetanus toxins (10). Neutralization of diphtheria and tetanus toxins is sufficient to prevent disease; however, *Bordetella pertussis* is a more complex pathogen armed with numerous toxins and virulence factors (11). While DTaP and Tdap vaccines with alum lead to the production of antibodies, the field generally agrees that the Th2-response induced by aP vaccines is not sufficient to provide long-term protection and protect against transmission (9,12,13). Further, aP vaccines are provided by intramuscular administration, and it is appreciated that the chemically detoxified pertussis toxin
(PT) in the aP formulation is not optimal compared to genetically detoxified PT (14). Both natural 
*B. pertussis* infection and the DTP vaccine elicit a Th1/Th17 immune response, which is important 
for both bacterial clearance as well as the induction of long-lasting protection (9,15). In the case 
of natural pertussis infection, the activation of toll-like receptors (TLRs) plays an important role 
in establishing an immune response against the pathogen; therefore, adjuvants that can activate 
these pathways are of particular interest (16). Bacterial DNA is a pathogen-associated molecular 
pattern which, unlike human DNA, contains high amounts of unmethylated cytidine 
phosphoguanosine (CpG) dinucleotides (17). The CpG motif within the bacterial DNA binds to 
TLR9 receptors, which are expressed in and activate plasmacytoid dendritic cells (pDCs) and B 
cells in humans, and are also expressed on myeloid-lineage cells in mice (18). Once CpG motifs 
bind the receptor, type 1 interferon (IFN) gene expression is induced, which helps to stimulate a 
potent immune response (19). As previously mentioned, alum alone skews toward a Th2 immune 
response, but interestingly, CpG motif-containing oligodeoxynucleotide adjuvants (CpG ODN), 
when combined with alum, are reported to improve upon vaccine-mediated immunity (20). A 
previous study by Asokanathan *et al.* has shown that the inclusion of CpG ODN with alum 
enhanced serological responses to pertussis antigens (20).

Dynavax Technologies has developed and clinically evaluated CpG 1018® adjuvant, a B class 
CpG, which are short, synthetic, single-stranded CpG ODNs containing a phosphorothioate 
backbone modification. CpG ODN stimulation has been shown to induce IFN-α production from 
pDCs and additional cytokines such as IL-12 and IFN-γ – promoting development of Th1-
mediated immunity (21,22). In safety and immunogenicity trials, HEPLISAV-B® (Hepatitis B 
surface antigen [HBsAg] +CpG 1018) vaccine was shown to improve upon the rapidity of
induction, magnitude and longevity of anti-HBsAg antibody responses, while having a similar safety profile as compared to the alum-adjuvanted HBsAg vaccine, ENGERIX-B® (23,24). Here, we hypothesize that CpG 1018 is a logical solution to improve aP vaccines, as a CpG ODN adjuvant has already demonstrated the ability to improve humoral responses and shift the T cell response to Th1 in response to aP vaccination (20). Furthermore, in a study that combined CpG ODN adjuvant with an intranasally administered aP vaccine consisting of PT, FHA, and PRN, it was found that CpG ODN, in combination with alum, was able to increase titers to pertussis antigens, including pertussis toxin (PT) (20).

Since the introduction of the aP vaccine formulation, there has been a concomitant rise in the number of circulating B. pertussis strains that lack the virulence factor, PRN. The rise in the number of PRN deficient strains has become a worldwide issue, and in the United States, 85% of circulating strains do not express PRN (25). Further, the longer a country has utilized the aP vaccine, the higher the prevalence of PRN- strains; therefore, unless a novel formulation that addresses this issue is implemented, we will continue to see a rise in PRN deficient strains (26). With that, studies that aim to create a novel vaccine formulation must ensure that protection is still provided regardless of whether or not a strain of B. pertussis expresses PRN.

In our study, we aimed to evaluate the inclusion of clinical grade CpG 1018 into a Tdap formulation. We used a murine intranasal challenge model to determine: immunogenicity, bacterial clearance within the lung/trachea and NALT (nasal associated lymphoid tissue), innate responses, and the isotypes of antibodies produced. We titrated the human dose of Tdap and determined that the addition of CpG 1018 to 1/20th Tdap improved bacterial clearance and
increased antibody responses against *B. pertussis* antigens. Additional studies showed that the addition of CpG 1018 to Tdap improved the production of antibodies against pertussis vaccine antigens, created a more balanced Th1/Th2 T cell response, and increased the number of antibodies that bound to the *B. pertussis* bacterium. Additionally, we also demonstrated that CpG 1018 reduces bacterial burden within the lung and trachea regardless of whether a strain of *B. pertussis* expresses pertactin (PRN). The data presented detail the value of the CpG 1018 adjuvant in improving Tdap’s humoral response and protection against *B. pertussis* challenge.

### 2.3 Results

CpG 1018 improved IgG antibody responses to the whole pertussis bacterium as well as FHA, PT, and PRN antigens.

In this study, we evaluated the effect of combining CpG 1018 with a Tdap vaccine formulation. Tdap is first given at ages 11-12 years with subsequent boosts being given every 10 years or to pregnant women after 20 weeks gestation in the US (27,28). To evaluate CpG 1018 enhancement of antibody responses to Tdap antigens, CD-1 outbred mice were immunized with Tdap at decreasing doses (1/10th, 1/20th, 1/40th, and 1/80th human dose) or Tdap at decreasing doses supplemented with 10 µg of CpG 1018. Control vaccines included two whole cell formulations: Serum Institute of India’s (SII) DTP vaccine (1/20th human dose) and the National Institute of Biological Safety and Control’s (NIBSC) wP (1/20th human dose). Additionally, there were vehicle controls in which the mice were vaccinated with 0.9% saline. All Tdap vaccines, including those containing CpG 1018, as well as the SII DTP vaccine contained the alum adjuvant (Table 1).
Sera was collected from mice at three days post challenge and was used to determine IgG titers to PT, filamentous hemagglutinin (FHA), PRN, and the whole pertussis bacterium (Bp) strain used for the challenge. Mice immunized with 1/20<sup>th</sup> Tdap + CpG 1018 had significantly increased serum titers against PT (6.6-fold increase) and Bp (3.25-fold increase) over mice vaccinated with 1/20<sup>th</sup> Tdap alone. (Fig. 1A, D). The effect on anti-PT antibodies is of particular interest, because PT is considered to be an essential virulence factor for B. pertussis and is included in all pertussis vaccines (29). Additionally, PT is responsible for leukocytosis which, in infants, can aggregate in blood vessels, leading to pulmonary hypertension and death; therefore anti-PT antibodies are of great importance when studying the immune responses that result from pertussis vaccinations (30).

Serum titers against FHA were elevated for mice that received 1/10<sup>th</sup> and 1/20<sup>th</sup> doses of Tdap + CpG 1018 when compared to each dose of Tdap alone, with a 2.7-fold and 4.2-fold increase in titers, respectively (Fig. 1B). In regard to PRN, the addition of CpG 1018 to any tested dilution of Tdap did not result in a significant increase versus Tdap alone, but the only dose that approached

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Table 1. Vaccine doses and components for each dilution tested in mice.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Human Tdap Dose (HD)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1/10&lt;sup&gt;th&lt;/sup&gt; HD</th>
<th>1/20&lt;sup&gt;th&lt;/sup&gt; HD</th>
<th>1/40&lt;sup&gt;th&lt;/sup&gt; HD</th>
<th>1/80&lt;sup&gt;th&lt;/sup&gt; HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis Toxoid (PT)</td>
<td>8 mcg</td>
<td>0.8 mcg</td>
<td>0.4 mcg</td>
<td>0.2 mcg</td>
<td>0.1 mcg</td>
</tr>
<tr>
<td>Filamentous Hemagglutinin (FHA)</td>
<td>8 mcg</td>
<td>0.8 mcg</td>
<td>0.4 mcg</td>
<td>0.2 mcg</td>
<td>0.1 mcg</td>
</tr>
<tr>
<td>Pertactin (PRN)</td>
<td>2.5 mcg</td>
<td>0.25 mcg</td>
<td>0.125 mcg</td>
<td>0.063 mcg</td>
<td>0.031 mcg</td>
</tr>
<tr>
<td>Tetanus Toxoid (TT)</td>
<td>5 Lf&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5 Lf</td>
<td>0.25 Lf</td>
<td>0.125 Lf</td>
<td>0.062 Lf</td>
</tr>
<tr>
<td>Diphtheria Toxoid (DT)</td>
<td>2.5 Lf</td>
<td>0.25 Lf</td>
<td>0.125 Lf</td>
<td>0.062 Lf</td>
<td>0.031 Lf</td>
</tr>
<tr>
<td>Aluminum Hydroxide (hydrated)</td>
<td>0.39 mg</td>
<td>0.039 mg</td>
<td>0.02 mg</td>
<td>0.01 mg</td>
<td>0.005 mcg</td>
</tr>
<tr>
<td>CpG 1018</td>
<td>3 mg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 mcg</td>
<td>10 mcg</td>
<td>10 mcg</td>
<td>10 mcg</td>
</tr>
</tbody>
</table>

<sup>a</sup>The Tdap vaccine utilized was provided by Serum Institute of India.

<sup>b</sup>The nominal human dose of CpG 1018.

<sup>c</sup>Lf represents limit of flocculation.
significance was 1/20\textsuperscript{th} Tdap (Fig. 1C). Overall, it was interesting to see that most improvements in antibody responses could be seen when CpG 1018 was added to the 1/20\textsuperscript{th} Tdap dose. There were no statistically significant increases in antibodies at 1/40\textsuperscript{th} or 1/80\textsuperscript{th} Tdap, and the 1/10\textsuperscript{th} dilution of Tdap + CpG 1018 only gave a significant increase in anti-FHA antibodies.

![Image of graphs]

**Figure 1. Serum IgG antibody titers to (A) PT, (B) FHA, (C) PRN, and (D) Bp were determined for all groups at 3 days post challenge.** Data presented as geometric means ± SD, n = 5 per treatment group. The exceptions to this are the MVC group (n =10), the Tdap 1/10 group (n=6), and the Tdap 1/80 + CpG 1018 group (n=4). Data was log transformed. Yellow data points indicate samples that were at the lowest limit of detection. A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination for each dilution tested. *p<0.05, **p<0.01. NVNC = non-vaccinated, non-challenged group. MVC= mock-vaccinated and challenged; SII = Serum Institute of India; NIBSC = National Institute for Biological Standards and Control.
Vaccination with Tdap adjuvanted with CpG 1018 decreased bacterial burden in the respiratory tract.

The clearance of bacteria from the respiratory tract is paramount in preventing transmission from one individual to another; therefore, an effective vaccine must offer the ability to clear bacterial burden (31). We hypothesized that the inclusion of CpG 1018 to the Tdap vaccine would enhance protection against *B. pertussis* in this murine model by increasing the clearance of the pathogen due to adaptive and innate responses. To study this, we evaluated bacterial burden within the respiratory tract at three days post-*B. pertussis* challenge. Within the lung and trachea, the addition of CpG 1018 to Tdap afforded a significant decrease in bacterial burden for only the 1/20th dilution, with an 83-fold reduction, when compared to Tdap alone (Fig. 2A). We also evaluated the bacterial burden within the NALT (nasal associated lymphoid tissue) and found that there was a statistically significant decrease in bacterial burden due to the addition of CpG 1018 at the 1/80th dose of Tdap, which had a 3-fold reduction in bacteria, when compared to this dose of Tdap alone (Fig. 2B). Additionally, while not significant, CpG 1018 added to the 1/20th Tdap dose did result in a 2.8-fold decrease in bacterial burden in the NALT. The large decrease in bacterial burden in the lung and trachea at the 1/20th dose was of particular importance, as the mice are intranasally challenged with *B. pertussis*; therefore, we expect that a large portion of the bacteria will deposit into the lungs due to the challenge volume of 20 µl.
The inclusion of CpG 1018 to the Tdap vaccine formulation decreased bacterial burden in the respiratory tract out to 7 days post challenge

CpG 1018 was able to increase antibody responses to PT, FHA, and the whole bacterium (Fig. 1) and improved bacterial clearance when combined with 1/20th the human dose of Tdap (Fig. 2). Based on these data, we selected the 1/20th human dose to evaluate bacterial clearance over a 7-day time-course experiment. Mice were euthanized at days 1, 3, and 7 post-challenge with *B. pertussis* strain UT25 and CFUs from the lung and trachea as well as the NALT were enumerated. Within the lung and trachea, the mice vaccinated with Tdap + CpG 1018 had the lowest bacterial burden out to 7 days post challenge.

**Figure 2.** *B. pertussis* within the respiratory tract was quantified by counting serially diluted CFUs at 3 days post challenge. CFU counts were determined from the lung/trachea homogenates (A) and the NALT homogenate (B). Presented as geometric means ± SD, n=5 per treatment group for 3-post challenge. The exceptions to this are the MVC group (n=10), the Tdap 1/10 group (n=6), and the Tdap 1/80 + CpG 1018 group (n=4). Data was log transformed. Yellow data points indicate samples that were at the lowest limit of detection. A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination for each dilution tested. *p*<0.05, **p**<0.01. MVC= mock-vaccinated and challenged; SII = Serum Institute of India; NIBSC = National Institute for Biological Standards and Control.
burden across all tested days when compared to all other vaccine groups (Fig. 3A). While not significant, at days 1 and 3 post challenge, when compared to vaccination with Tdap alone, mice vaccinated with Tdap + CpG 1018 saw a 9.2-fold and 41.5-fold reduction in bacterial burden, respectively (Fig. 3A). Additionally, there was a significant reduction in the lung and trachea bacterial burden at day 7 post challenge for mice vaccinated with CpG 1018 when compared to those vaccinated with Tdap only (40-fold reduction) (Fig. 3A). At day 7 post challenge within the NALT, mice that were vaccinated with Tdap + CpG 1018 had a significant reduction in bacterial burden when compared to the Tdap vaccinated group (Fig. 3B). Across all time points post-challenge, the clearance of bacterial burden resulting from including the CpG 1018 adjuvant was equal to that of the SII DTP formulation within the NALT. Overall, these data demonstrate the capacity of the CpG 1018 adjuvant to offer an increased reduction in respiratory tract bacterial burden.

To address the ability of the CpG 1018 adjuvant to curtail the migration of inflammatory cells to the lung and trachea after infection, we performed flow cytometry at days 1, 3, and 7 post-challenge. We utilized the panel previously described (32,33). While there were no significant differences between mice vaccinated with Tdap and those vaccinated with CpG 1018, at day 3 post challenge, the CpG 1018 adjuvant was able to reduce the average number of inflammatory cells in the lung and trachea tissues (Fig. S1). At day 3, there was a 36% reduction in macrophage recruitment to the lung/trachea as well as a 47% reduction in neutrophils in mice that received Tdap + CpG 1018 versus those that only received Tdap (Fig. S1A,B). The addition of CpG 1018 was also able to reduce lung/trachea monocyte and dendritic cell counts at day three by 72% and
48%, respectively, when compared to mice that were vaccinated only with Tdap (Fig. S1C,D). The gating strategy utilized is described in Fig. S2.

**Figure 3. B. pertussis within the respiratory tract was quantified by counting serially diluted CFUs at 1, 3, and 7 days after challenge.** CFU counts were determined from the lung and trachea homogenates (A) and the NALT homogenate (B). Shaded regions represent the SD for each group as denoted by the legend. Data shown has been log transformed. Treatment groups are n=5 for 1 day, 3 days, and 7 days post-challenge. The exceptions to this are the day 3 MVC group (n=4) and the day 7 MVC group (n=4). Dotted lines along the Y-axis indicate the lowest limit of detection. A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination for each time point. *p<0.05, **p<0.01. MVC= mock-vaccinated and challenged; SII = Serum Institute of India.
Tdap and Tdap CpG 1018 protect mice against challenge with either PRN$^+$ or PRN$^-$ strains of *B. pertussis*.

Recently, PRN negative strains have begun to emerge, and it is widely believed that vaccine pressure is contributing to the rapid divergence of circulating *B. pertussis* strains away from vaccine reference strains (34–36). Here, our study utilized *B. pertussis* strain UT25, a PRN$^+$ strain that was isolated in the 1970s (37). In order to determine the capacity of CpG 1018 adjuvant to provide protection against vaccine escape strains when included in the Tdap vaccine, we performed a study in which mice were challenged with *B. pertussis* strain H762, a PRN$^-$ strain (35). Strain H762 is representative of the most prevalent CDC237 clade affecting the US (38). We observed that the infection kinetics of both PRN$^+$ and PRN$^-$ strains were similar in the lung/trachea (Fig. 4A-D). It is important to note that, while not statistically different, for mice vaccinated with Tdap + CpG 1018, the bacterial burden within the lung and trachea did demonstrate differences between challenge strains ($p = 0.0674$ for day 3 post-challenge and $p = 0.0926$ or 7 days post-challenge) (Fig. 4D). We also utilized area under the curve analysis (AUC) to assess the total lung/trachea bacterial burden over all time points studied and, again, found no significant differences between the two strains (Fig. 4E). Overall, the two strains showed no significant differences across all vaccines and time points studied, supporting the ability of the vaccines and CpG 1018 to protect regardless of PRN expression status.
Figure 4. Bacterial burden within the lung/trachea across all timepoints and between mice challenged with different *B. pertussis* strains, UT25 or H762, which were both administered intranasally at 2 x 10^7 CFU/mL. CFU counts were determined from the lung and trachea homogenates of MVC mice (A), SII DTP vaccinated mice (B), Tdap vaccinated mice (C), and Tdap + CpG 1018 vaccinated mice (D). Area under the curve analysis was performed comparing strains for all vaccines across all days studied (E). Data shown has been log transformed. Treatment groups are n=5 for 1 day, 3 days, and 7 days post-challenge. Shaded regions represent the SEM for each group as denoted by the legend. Dotted lines along the Y-axis indicate the lowest limit of detection. A Student’s t-test was used to determine statistically significant differences between strains. MVC= mock-vaccinated and challenged; SII = Serum Institute of India; AUC = area under the curve.
The addition of CpG 1018 to Tdap elevated serum IgG titers against *B. pertussis* antigens out to 7 days post challenge.

We hypothesized that the addition of CpG 1018 to Tdap would elicit increased production of IgG antibodies that recognize antigens included in Tdap, and that these antibody levels would continue to increase after *B. pertussis* post-challenge due to recall. Anti-*Bp* IgG titers in mice vaccinated with Tdap + CpG 1018 maintained the highest level of antibody production over the duration of the study, with significant elevations at days 3 and 7 post-challenge when compared to the Tdap only vaccinated mice (9.6-fold and 4.7-fold increase, respectively) (Fig. 5A). As for anti-PT IgG antibodies, for both days 1 and 3 post challenge, the mice that received Tdap + CpG 1018 that had significantly increased antibody production when compared to Tdap vaccinated mice (3-fold and 2.8-fold increase, respectively) (Fig. 5B). At day 3 post challenge, the Tdap + CpG 1018 vaccine gave a significant increase in anti-FHA antibodies over mice that only received Tdap, with a 16-fold increase (Fig. 5C). Serum IgG antibodies against PRN were only significantly elevated at day 1 post-challenge (Fig. 5D). This could be due, in part, to the already low amount of PRN antigen in Tdap that has then been diluted to the 1/20th human dose. Interestingly, for most antigens, the antibodies resulting from vaccination with Tdap + CpG 1018 exceed those afforded by DTP vaccination, indicating that protection is established without the induction of deleterious inflammatory responses, as DTP resulted in a large influx of immune cells to the lung and trachea, while Tdap + CpG 1018 did not (Fig. S1). These data demonstrate that Tdap adjuvanted with CpG 1018 elicits strong humoral responses to pertussis antigens and that these responses are capable of exceeding those produced by DTP.
CpG 1018 adjuvanted vaccines increased the proportion of antibodies that bind to *B. pertussis*.

We aimed to determine the adjuvant’s ability to enhance production of high affinity antibodies that recognize the whole *B. pertussis* bacterium via an IgG binding assay with flow cytometric analysis. This is of importance, as Tdap only has two antigens (FHA and PRN) that are involved in the

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**Figure 5.** Serum IgG antibody titers to (A) *Bp*, (B) PT, (C) FHA, and (D) PRN were determined for all groups at days 1, 3, and 7 post challenge. Presented as geometric means ± SD with n=5 per treatment group for 1 day, 3 days, and 7 days post-challenge. The exceptions to this are the day 3 MVC group (n =4) and the day 7 MVC group (n=4). Data has been log transformed. A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination for each time point. *p<0.05, ***p<0.001, ****p<0.0001. MVC= mock-vaccinated and challenged; SII = Serum Institute of India.
binding of pertussis to the respiratory epithelium. Unlike a standard ELISA, the flow-based IgG binding assay allows one to visualize an individual bacterium binding to IgG in a non-static system. The flow output can then be used to compare numbers and the percent of \( Bp \) bound to IgG instead of determining titers based on a colorimetric assay (Fig. S3). An increased proportion of antibodies that can bind to the pertussis bacterium suggests that there could be a concomitant increase in clearance. Overall, both the percentage and counts of \( B. \) pertussis that were bound by IgG antibodies was higher for Tdap + CpG 1018 when compared to MVC mice or Tdap vaccination alone (Fig. 6A,B). This corroborates previous ELISA data in which Tdap + CpG 1018 increased IgG titers to pertussis antigens (Figs. 1 and 5). Interestingly, the antibody binding between the Tdap + CpG 1018 group and the SII DTP control group were similar, despite Tdap + CpG 1018 containing a reduced number of \( B. \) pertussis antigens than the DTP formulation (Fig. 6).

In addition to measuring serum antibody levels, we also utilized ELISpot (Enzyme Linked ImmunoSpot) assays to determine the number of antigen specific plasma cells in the bone marrow. Upon stimulation with either a pathogen or antigen, B cells migrate from lymphatic tissues to the bone marrow, where they are exposed to factors that allow for their differentiation into long-lived plasma cells, which are responsible for long-term and sustained antibody production (39,40). We were able to enumerate the number of antibody producing cells within the bone marrow that were secreting \( B. \) pertussis-specific IgG. While not significant, the addition of CpG to Tdap did result in a 2-fold increase in antigen specific B cells versus what was observed after vaccination with Tdap alone (Fig. S4).
Tdap + CpG 1018 elicited a more Th1-type immune response when compared to Tdap vaccination alone based on antibody isotype analysis.

As mentioned previously, the alum-adjuvanted aP vaccines tend to induce Th2, or humoral based, immune response (41,42). The reduced induction of cell-mediated responses as well as the lack of a proper immunostimulatory effect is proposed as one weakness of the aP vaccines. Previous studies on CpG ODNs have demonstrated their ability to shift the resulting T cell response; therefore, we aimed to determine if the addition to CpG 1018 to Tdap was able to alter the resulting polarization of T helper cells (18,20). To do this, sera from mice that were primed and boosted, but not challenged, were evaluated for the presence of various IgG isotypes: IgG1, IgG2a, and...
IgG2b. As expected, we saw that vaccination with SII DTP elicited a strong Th1-based immune response (high IgG2b antibodies), while Tdap vaccinated mice produced a Th2-skewed response (high IgG1 antibodies) (Fig. 7). Interestingly, the addition of CpG 1018 to the Tdap booster formulation was able to create a more balanced immune response with similar IgG1 and IgG2b titers. These data indicate that CpG 1018 is able to redirect the resulting immune response to vaccination, potentially without inducing as much inflammation as observed with DTP whole cell vaccination (43,44).

Figure 7. Pre-challenge anti-Bp IgG antibody isotypes in the sera between vaccine groups were quantified to determine the induction of Th1 versus Th2 immune responses. Serum was collected 2 weeks post-boost immunization. A visual part of a whole analysis was created for each vaccine group (A). Titers for each isotype were also enumerated (B). The part of a whole graphs take each antibody isotype titer (numerator) and divide that into the total of all antibody titers (denominator). This is done for each vaccine group. Data presented as geometric means ± SD with n= 5 per group. The exception to this is the SII DTP groups (n=4). Data has been log transformed. The dotted line along the Y-axis represents the serum IgG titer values for the MVC mice. A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination. *p<0.05.
2.4 Discussion
With the observation that the immunity afforded by aP vaccines wanes over time, there has been an overall objective in the field to determine the best way to improve the current formulations (45). The DTP vaccine, which appears to have longer lived protection, contains the highly immunogenic molecule, LOS (lipooligosaccharide) that is thought to serve as a potent adjuvant (16). LOS binds to TLR4 and initiates a downstream signaling cascade which plays a role in the clearance of the pathogen, skews toward a Th1 immune response, and enhances bacterial killing via macrophages (16,46). However, the immune responses elicited by DTP were highly reactogenic; therefore, a new adjuvant needs to be able to induce a potent memory response while minimizing reactogenicity. TLR9 ligands are one such potential solution, as they have been shown to provide more robust and rapid antibody responses against *B. pertussis* challenge (versus TLR4 agonists), and enhance bacterial clearance (47). Additionally, Auderset *et al.* demonstrated that, after priming with DTaP, it was only the TLR9 agonists that were able to enhance Th1 (IgG2a) antibody responses; thus shifting the typical Th2 immune response induced by the current aP vaccines (47).

CpG 1018® adjuvant is a TLR9 agonist that is included in the FDA approved HEPLISAV-B® vaccine. In contrast to other CpG ODN molecules, CpG 1018 contains both human and rodent-optimized CpG motifs, allowing evaluation of the same molecule in preclinical and clinical studies. Preclinical work on HEPLISAV-B® demonstrated its superior ability to increase the anti-HBsAg antibodies when compared to administration of HBsAg alone (21). Similarly, in this study we observed enhanced antibody production against both the whole *Bp* bacterium and the aP antigens upon the addition of CpG 1018 to Tdap (Fig. 5). In regard to utilizing CpG ODN in a vaccination-challenge model, a study by Asokanathan *et al.* demonstrated the ability of the mouse-specific CpG 1826 and alum to enhance serological responses, Th1-skewed responses, and macrophage...
Another study by Al-Mariri et al, also using CpG 1826, demonstrated that the CpG ODN adjuvant was able to enhance both IFN-γ production and T cell responses upon vaccination with Brucella abortus antigens and subsequent challenge (48).

In our study, we aimed to study the pre-clinical efficacy of a TLR9 agonist, CpG 1018, specifically its capacity to provide improved protection against B. pertussis challenge when added to a vaccine formulation already including alum. We first performed a titration of Tdap to determine a “sub-optimal dose;” that is, the dose at which the benefits afforded by CpG 1018 could be observed. A 1/20\textsuperscript{th} dose of Tdap was selected, as it allowed for an increase in antibody titers against pertussis antigens and reduced bacterial burden in the lung and trachea (Fig. 1 and Fig.2). The 1/20\textsuperscript{th} dose of Tdap did not result in sterilizing immunity, which would mask the effect of adding any new vaccine component. It could be that 1/10\textsuperscript{th} Tdap contained antigen levels that were too high, thus eliciting a protective response that prevented us from observing any benefits from adding CpG 1018 to the formulation. At the opposite end of the spectrum, the 1/40\textsuperscript{th} and 1/80\textsuperscript{th} doses of Tdap might have had antigen concentrations that were too low to create a substantial immune response, and the CpG 1018 adjuvant could not improve such a weak response. Once the 1/20\textsuperscript{th} dose was selected, we evaluated the performance of Tdap + CpG 1018 across multiple time points post-challenge and found that, when compared to Tdap alone, Tdap + CpG 1018 enhanced serological responses to pertussis antigens over time. We also observed decreased respiratory bacterial burden and the ability of Tdap + CpG 1018 to induce a more Th1-based immune response when compared to Tdap only, which elicited a more Th2-based immune response, via quantifying IgG isotypes from pre-challenge sera.
Most of the data within this study was obtained utilizing the PRN\(^+\) strain, UT25. However, as circulating \textit{B. pertussis} strains continue to genetically diverge from the original vaccine reference strains, we thought it was imperative to determine if Tdap + CpG 1018 was still able to elicit a protective immune response when challenged with a PRN\(^-\) strain (H762) (35). Since the introduction of aP vaccines, strains of \textit{B. pertussis} have undergone genetic changes, the most prominent of which is a deficiency of PRN (26). In the US, 85\% of circulating strains are PRN deficient; therefore, the ability of new pertussis vaccines to afford protection despite PRN expression status is of the utmost importance (36). From this study, we determined that when mice were challenged with either UT25 or H762, there was no significant difference in the lung/trachea bacterial burden across all timepoints studied (Fig. 4). This finding is crucial, as it demonstrates that this adjuvant would still afford protection in the face of a genetically divergent strain lacking one of the 3 antigens contained in the vaccine, negating the fears that a PRN\(^-\) mutant would be able to withstand the vaccine-mediated immune responses. However, for mice vaccinated with Tdap + CpG 1018, there was a notable difference in bacterial burden between challenge at days 3 and 7 post-challenge (Fig. 4D). The biggest difference in bacterial burden within the lung and trachea is at 3 days post-challenge but by 7 days post-challenge, the difference between strains begins to diminish. We believe that if we were to study timepoints further out after challenge, such as 14 days, the difference in bacterial burden would be marginal; therefore, this is an objective as we move forward with the utilization of difference challenge strains. Human studies are needed to determine the full capability of Tdap + CpG 1018 to protect against various \textit{B. pertussis} strains, but our results are certainly promising.
While our study did demonstrate the ability of CpG 1018 to improve immune responses to Tdap, there were some limitations that need to be considered. To start, our challenge model utilizes a large challenge dose (2×10^7 CFU/mL); therefore, it could be that the quantity of bacteria placed into the nasal cavity may limit the clearance capacity of our vaccines in the upper respiratory tract. However, we appreciate that the field utilizes the nasal lavage as a parameter to examine protection. We believe that it may be beneficial in the future to enumerate CFUs from either the nasal septum or the entire nasal cavity (49,50). Further, the utilization of an aerosolization challenge model may provide a more natural localization and quantity of bacteria. Additionally, our results were obtained from a mouse model, which only provides a glimpse at the breadth of responses that could be observed in other animal models, such as rats or baboons.

In summary, this study evaluated the ability of CpG 1018 + alum adjuvant system to improve the current alum-adjuvanted Tdap vaccine, both in immunogenicity and protection against airway bacterial challenge. Compared to the alum-adjuvanted Tdap vaccine, Tdap adjuvanted with both CpG 1018 and alum enhanced the production of pertussis-specific antibodies, and offered protection against the airway colonization of both PRN^+ and PRN^- B. pertussis strains. As this vaccine candidate has similar antigen composition (PT, FHA, and PRN) and alum content to a currently used Tdap booster vaccine, it represents a potentially attractive development pathway. Currently, Tdap + CpG 1018 is being further studied in challenge studies, and safety and immunogenicity is being evaluated in a Phase 1 clinical study. Our present study supports the potential application of the CpG 1018 + alum adjuvant system to address unmet needs associated with presently licensed Tdap vaccines utilizing alum alone as adjuvant.
Supplemental Figure 1. Immune cell populations were detected in the lung and trachea homogenates from all groups at 1, 3, and 7 days post-challenge. The number of macrophages (A), neutrophils (B), monocytes (C), and dendritic cells (D) are shown. Data presented as minimum and maximum values along with means, n = 5 per treatment group. The exceptions to this are the day 3 MVC group (n =4) and the day 7 MVC group (n=4). A Student’s t-test performed between Tdap vaccination only and Tdap + CpG 1018 vaccination. MVC= mock-vaccinated and challenged; SII = Serum Institute of India.
Supplemental Figure 2. The gating strategy used to quantify immune cells in the lung and trachea. Lungs/tracheas were prepared as described in the Methods section. Live cells were selected and cell populations were gated from singlets. Neutrophils were defined as being CD11b+Ly6G+. Monocytes were gated as CD11b+Ly6C+. Dendritic-like cells were gated as CD11b+CD11c+. Alveolar macrophages were defined as CD11b+F4/80+. Antibodies used within the panel are detailed in Table 2.

Supplemental Table 1. Immune cell panel used to identify cell types via flow

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Manufacturer</th>
<th>Catalogue Number</th>
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<td>Fisher Scientific</td>
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<tr>
<td>CD11c</td>
<td>APC-CyTM7</td>
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<td>PerCP eFluor</td>
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<tr>
<td>F4/80</td>
<td>FITC</td>
<td>Thermo Fisher Scientific</td>
<td>11-4801-82</td>
</tr>
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Supplemental Figure 3. The gating strategy and histogram utilized in the IgG binding assay. Mice were primed and boosted and, two weeks post boost, sera was collected and utilized in the assay. The *B. pertussis* bacterium as well as counting beads were selected as the first gate (A). From there, the Green *Bp* was selected as events that were positive for Baclight Green but negative for APC-Cy7. Events that were positive for both Baclight Green and APC-Cy7 were defined as *Bp* bound with IgG (A). Panel B demonstrates the histograms that were used to select samples positive for Baclight Green (*Bp*, shown in the histogram as GRN-B-ALog) as well as the IgG binding to *Bp* (APC-Cy7, shown in the histogram as NIR-V-ALog).
Supplemental Figure 4. *B. pertussis*-specific IgG producing cells isolated from the bone marrow at 1 day post challenge determined via ELISpot. Data presented as means ± SD, n = 5 per treatment group. The exception to this is the NVNC mice (n=4). NVNC = non-vaccinated, non-challenged; MVC= mock-vaccinated and challenged; SII = Serum Institute of India.
2.5 Materials and Methods

**Vaccine and adjuvant composition.** The vaccines administered in the study were prepared no longer than 1 h before administration. All vaccines were diluted using 0.9% saline. Vaccine formulations are described in Table 1. The Tdap vaccine used was provided by the Serum Institute of India (Batch #: 3729H001) as was their DTP formulation (Batch #: 2829X019A). The Serum Institute of India DTP contains the following in each 0.5 mL human dose: diphtheria toxoid ≤ 25 Lf (≥ 30 IU), tetanus toxoid ≥ 5 Lf (≥ 40 IU), *B. pertussis* ≤ 16 OU (≥ 4 IU), adsorbed on aluminium phosphate, Al+++ ≤ 1.25 mg, and a preservative: 0.005% Thiomersal. The National Institute for Biological Standards and Control also provided a wP control ( #94/532 batch 41S).

*B. pertussis strains and growth conditions.* *B. pertussis* strains UT25Sm1 and H762 were used for murine challenge (37,38). Both *B. pertussis* strains have been fully genome sequenced (UT25Sm1 NCBI Reference Sequence: NZ_CP015771.1; H762 NCBI Reference Sequence: NZ_CP011696.1). UT25 was originally isolated in Texas in 1977. UT25Sm1 was cultured on Bordet Gengou agar (Difco) plus 15% defibrinated sheep’s blood (Hemostat Laboratories) with streptomycin 100 µg/mL. *B. pertussis* was incubated at 36°C for 48 h, then transferred to modified Stainer-Scholte liquid medium (51). H762 was cultured in Connecticut in 2011 and is a part of the clade CDC237 (38). H762 was cultured similarly to UT25Sm1, however, no antibiotics were added to the agar plate. SSM liquid cultures were incubated for 24 h at 36°C, with shaking at 180 rpm until reaching an OD₆₀₀ of ~0.6, at which time cultures were diluted for the challenge dose.

**Vaccine administration.** CD-1 (outbred; strain code 022) mice aged four to five weeks were obtained from Charles River Laboratories. Mice were administered 50 µL of vaccine or vehicle
control intramuscularly (IM). Mice were boosted with the same vaccine formulations 21 days after priming. All murine infection experiments were performed according to protocols approved by the West Virginia University Animal Care and Use Committee (protocol numbers:1602000797 and 1602000797_R1).

**Vaccine challenge model.** At thirty-five days post prime, mice were challenged with IN $2 \times 10^7$ CFUs of *B. pertussis* (10 µL per nostril). At days 1, 3, and 7 post-challenge, mice were euthanized, and blood was collected by cardiac puncture. Complete blood cell counts were performed using a ProCyte IDEXX and serum was separated by centrifugation through a BD Microtainer blood collector and stored at $-80^\circ$C until analysis. The trachea and lungs were removed and homogenized together. Lungs and trachea were suspended in 2 mL of sterile PBS in gentleMACS C tubes (Miltenyi; Cat. Number: 130-096-334) using a GentleMACS Octo Dissociator with Heaters (Miltenyi) using the m_lung_02 setting. A sample of lung/trachea homogenate was used for flow cytometry and the remaining sample was centrifuged at 14,000 x g for 4 mins and supernatants were stored at $-80^\circ$C until cytokine and antibody analyses were performed. For the NALT, a scalpel was used to cut out the hard palate. The hard palate was placed in a culture tube, homogenized with a polytron, and then filtered through a 70µM filter. Bacterial burden was determined in the lung/trachea and NALT by colony forming units (CFUs) using serial dilutions. Serial dilutions were done in PBS and then plated on BG containing streptomycin (100 µg/mL) to ensure that only UT25 *B. pertussis* was cultured. For enumerating CFUs within H762-challenged mice, serial dilutions were plated on BG plates containing no antibiotics.
**IgG binding assay.** Anti-*B. pertussis* IgG in serum was quantified using a flow-based IgG binding assay. To obtain the sera used for the assay, a cardiac puncture was performed on each mouse. The mice had been primed and boosted and were processed at 2 weeks post-boost. The blood was placed into serum separator tubes which contained a separating gel in order to help isolate the sera. The samples were centrifuged for 2 min at 14,000 x g. Serum was collected and stored at −80°C until analyses were performed. *B. pertussis* strain UT25Sm1 was grown as described above. After, a 24 hr culture was diluted 1:20 in PBS and an aliquot was pulled off to determine CFUs before *B. pertussis* was heat-killed (HK) at 56°C for 30 mins. 500µL aliquots of HK *B. pertussis* were frozen in -20°C for the assay. Prior to starting the assay, *B. pertussis* was centrifuged at 8000 rpm for 5 mins, the supernatant was decanted, and the pellet was resuspended in 100µL PBS. 10µL of serum was added to *B. pertussis* and incubated at 36°C while shaking at 200 rpm for 1 hr. Samples were then washed with 300uL PBS (x2) by centrifuging samples at 7000 x g for 5 min. Samples were brought up to 100uL with PBS and incubated with Fc block (clone 2.4G2, Thermo Fisher Scientific, Cat. Number: 553142) for 15 mins at 4°C. Then, 0.25 ug of secondary goat anti-mouse IgG APC-Cy7 (minimal x-reactivity) was added to samples for 30 min at 4°C. Samples were then washed with 300uL PBS (x2) by centrifuging samples at 7000 x g for 5 min, and the pellet was resuspended in 1mL PBS. *B. pertussis* was stained with Baclight Green Bacterial Stain (Invitrogen, Cat. Number: B35000), which was prepared according to the manufacturer’s instructions. Controls included: *Bp* unstained, *Bp* with Baclight Green only, *Bp* stained with secondary only (IgG-APC-Cy7), unstained beads, beads with secondary only (IgG-APC-Cy7). Samples were loaded into a U-bottom 96 well plate (Fisher Scientific, Cat. Number: 08-772-5) and 10,000 events per sample were analyzed on a Guava® easyCyte 12HT flow cytometer (Luminex). Data were analyzed with Guava® InCyte™ Software (Version 4.0).
Serological analysis of *B. pertussis* specific antibodies. On the day of processing, a cardiac puncture was performed on each mouse. Approximately 1 mL of blood was removed from each mouse. The samples were centrifuged for 2 min at 14,000 x g. Serum was collected and stored at −80°C until analyses were performed. Serological responses specific to *B. pertussis* antigens were quantified by ELISA. High-binding microtiter plates were coated with PT (50 ng/well) (PT#180, LIST Biologicals), FHA (50 ng/well) (Enzo Life Sciences), or PRN (50 ng/well) (PRN#187, LIST Biologicals) as described in Boehm et al (52). For serological responses to *B. pertussis*, UT25Sm1 was cultured to an OD$_{600}$ of ~0.6 and diluted down to an OD$_{600}$ of 0.245 and microtiter plates were coated with 50 µL of bacteria per well. After coating, the plates were washed with PBS + 0.05% v/v Tween 20 (Cat. Number: P1379-1L, Sigma-Aldrich) (PBS-T) and blocked with 5% non-fat dry (NFD) milk in PBS-T overnight at 4°C. Blocked plates were washed with PBS-T and then serum (1:50) or lung supernatant (1:4) samples were prepared in 5% NFD milk in PBS-T. All samples were serially diluted (1:2). After 2 h incubation at 37°C, plates were washed and incubated with goat anti-mouse-IgG alkaline-phosphatase conjugated antibodies (IgG:1030-04, SouthernBiotech) (1:2,000) for 1 h at 37°C. For IgG isotyping, plates were washed and incubated with either goat anti-mouse IgG2a alkaline-phosphatase conjugated antibody (Southern Biotech, Cat. #1081-04), goat anti-mouse IgG2b alkaline-phosphatase conjugated antibody (Southern Biotech, Cat. #1091-04), or goat anti-mouse IgG1 (Southern Biotech, Cat.#1071-04) for 1 h at 37°C (all antibodies diluted 1:2000 in 5% NFD milk in PBS-T). Plates were then washed and incubated with Pierce *p*-Nitrophenyl Phosphate (PNPP) (Cat. Number: 37620; Thermo Fisher Scientific) following the manufacturer’s instructions. The absorbance of the plates was read at
OD$_{405}$ using a Synergy H1 plate reader (BioTek). Positive antibody titers were determined as any values above the baseline (set at two times the average of blanks).

**Statistical analysis.** Statistical analyses were performed using Prism version 10 software (GraphPad). Comparisons between two variables were made utilizing an unpaired student’s t-test.

**Ethics statement.** Animal work in this study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (53). The West Virginia University Institutional Animal Care and Use Committee (IACUC) approved the protocols and this research under the IACUC protocol 1602000797. All lab work with *Bordetella pertussis*, including *in vitro* work, infection, *in vivo* studies, and pathogen culture, were completed under an approved IBC (Institutional Biosafety Committee) protocol (#17-01-11).

**Data availability.** Data for all figures are available upon reasonable request to the corresponding author.

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F.H.D, J.R.B, M.A.D, and J.D.C. developed the experimental design. Dynavax Technologies supplied the CpG 1018 adjuvant and Serum Institute of India provided DTP and Tdap vaccines. M.A.D. and M.A.W. performed vaccine administration as well as bacterial challenge. All authors participated in the pre-clinical mouse challenge experiments. E.S.K. contributed to flow cytometry panel design and analysis. M.A.D., M.A.W., and E.S.K. prepared and ran flow cytometry samples.
M.A.W. performed cytokine analysis. M.A.W., M.A.D., and J.R.B. performed serological analysis. M.A.D. performed ELISpots, analyzed all data, prepared figures, and composed the manuscript with critical revisions from all authors. All authors composed or reviewed the manuscript. F.H.D and J.R.B managed the project and provided expertise. We thank Kathleen Brundage for her support at the WVU Flow Cytometry and Single Cell Core Facility. The WVU Core Facility is supported by TME CoBRE GM121322 grant and by the NIH S10OD016165 grant. We thank Paula Traquina, Robert Coffman, Gage Pyles, and Kelly Weaver for critical review of the manuscript.

**DISCLOSURES/CONFLICTS OF INTEREST**

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2.6 References


28. Centers for Disease Control and Prevention (CDC). Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine (Tdap) in pregnant women and persons who have or anticipate having close contact with an infant


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[Accessed June 11, 2019]


Chapter 3

BECC438b TLR4 agonist improves responses to nasal and muscular DTaP pertussis vaccines in murine challenge models
BECC438b TLR4 agonist improves responses to nasal and muscular DTaP pertussis vaccines in murine challenge models

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3.1 Abstract
The protection afforded by acellular pertussis vaccines wanes over time and there is a need to develop improved vaccine formulations. Options to improve the vaccines involve the inclusion of different antigens, the utilization of different adjuvants, and immunization via different routes. While intramuscular (IM) vaccination provides a robust systemic immune response, intranasal (IN) vaccination hypothetically induces a localized immune response within the nasal cavity. In the case of a *Bordetella pertussis* infection, IN vaccination results in an immune response that is similar to natural infection, which provides the longest known duration of protection. Current acellular formulations utilize alum adjuvant however antibody levels wane over time. To overcome the current limitations with the acellular vaccines, we included a novel TLR4 agonist, BECC438b into both IM and IN acellular formulations to determine its ability to protect against infection in mouse airway challenge models. Following immunization and challenge, we observed that DTaP + BECC438b reduced bacterial burden within the lung and trachea for both administration routes when compared to mock vaccinated and challenged mice. Interestingly, IN administration of DTaP + BECC438b induced a Th1 polarized immune response, while IM vaccination, regardless of formulation, polarized toward a Th2 immune response. RNAseq analysis of the lung demonstrated that DTaP + BECC438b activated biological pathways similar to natural infection. Additionally, IN administration of DTaP + BECC438b activated the expression of genes involved in a multitude of pathways associated with the immune system including immunoglobulins. Overall, these data support the continued evaluation of the BECC438b adjuvant and the IN vaccination route to improve the efficacy of pertussis vaccines.
3.2 Introduction

While pertussis, colloquially known as whooping cough, was nearly eradicated in the United States in the 1950s, several cyclic increases in the number of cases over the past 20 years have been observed (1,2). This resurgence can be correlated to the use of acellular pertussis (aP) vaccines. aP vaccines (DTaP/TdaP) have several distinctions from whole cell pertussis vaccines DTP including: 1.) The induction of a Th2 dominant immune response due to the alum adjuvant (3,4) and 2.) the loss of pertactin (PRN) antigen from many strains of B. pertussis (5,6), and 3) waning immunity. Unlike the aP vaccine, the whole cell pertussis (DTP/wP) formulations offered long-lasting protection via the induction of a Th1-polarized immune response and the inclusion of the entire B. pertussis bacterium, which provided more antigens against which the immune system could mount a response (4,7,8). However, the robust immune response elicited by the wP vaccine came at a cost of occasional deleterious side effects, which prompted vaccine hesitancy and lawsuits; hence, a protein subunit acellular vaccine was developed that replaced wP in the United States (9,10). Initially, the data from aP vaccine studies demonstrated that the acellular formulation induced antibodies to B. pertussis antigens at similar levels to what was seen with the wP formulation (10–12). However, as time went on, the issue of waning vaccine immunity became apparent, as the number of pertussis cases began to rise in the years after the implementation of the aP formulation (9,13). One consequence of waning immunity is the issue of asymptomatic nasal carriage, which is when an individual harbors the bacteria within their nasal mucosa but do not show signs of infection (14,15). The pathogen can then be passed on to highly susceptible populations, specifically those less than 1 year of age, resulting in illness, hospitalization, and even death (2). As a result, there is an ongoing effort in the field to improve aP vaccine formulations such that their resulting immune responses are more similar to what is seen with a natural infection, as a natural infection provides the longest known duration of protection against B. pertussis (7,16,17).
There are many ways in which pertussis vaccines could be improved: different routes of vaccine administration could be utilized (17–21), more immunogenic antigens could be implemented (22–24), and improved adjuvants could either replace or be included with alum (25–27).

Currently, pertussis vaccines are only administered intramuscularly (IM). IM vaccines deliver the antigens to the draining lymph nodes. From there, the antigens are presented to immune cells, which allows for the induction of a systemic immune response and the production of antibodies that will bind the pathogen upon re-encounter (28). While IM vaccination offers protection from disease, it does not completely address the issue of nasal carriage; that is, upon exposure to *B. pertussis*, the bacterium can remain in the nasal cavity and infect susceptible hosts (29). Additionally, many studies have shown the value of resident memory T cell responses and their importance in clearance and protection (15,30). One alternative route of vaccine administration that has shown to be quite promising in regard to pertussis infections is that of intranasal (IN) vaccination (26,31). With IN vaccination, the antigens are also delivered to the draining lymph nodes, which results in a systemic immune response and the induction of antibodies. However, there is also the activation of the nasal mucosa, which results in the induction of a mucosal immune response (32). As a result, there is the production of antigen specific IgA antibodies, as well as the localization of immune cells to the site of colonization. Multiple studies have demonstrated the effectiveness of IN vaccines in protecting both animal models and people against pertussis infections (21,26,31,33,34).

aP vaccines utilize the adjuvant alum, to which the antigens included in the formulation are adsorbed, allowing for their presentation to immune cells (35). While safe, alum elicits a Th2-
polarized immune response, a response that leads to the downstream activation of humoral immunity via antibodies (36,37). Both the wP vaccine and natural infection activate a Th1-polarized immune response, which induces more inflammation and is characterized by the activation of cell-mediated immunity (7,38). Current research supports the importance of cell-mediated immunity in the clearance of _B. pertussis_ (7). To ameliorate this issue, a number of different Th1-polarizing adjuvants could be utilized in the aP vaccine, such as beta glucans, TLR9 ligands, and TLR4 agonists (25,26,39,40).

One category of adjuvant of particular interest is that of lipid A mimetics, which have been shown previously to induce a Th1-polarized immune response by binding to Toll-like receptor 4 (TLR4) (40,41). Lipopolysaccharide (LPS), a membrane component of Gram negative bacteria, binds canonically to TLR4 and its co-receptor, MD-2 (42,43). LPS consists of the O antigen, a core, and lipid A, which varies between bacterial species and is responsible for most of the deleterious side effects that stem from a Gram negative bacterial infection (43,44). The structure of lipid A determines the downstream immune response; therefore, in the case of vaccination, the ability to generate novel lipid A structures that activate specific pathways would make for an interesting adjuvant (45–47). It has been appreciated that the potent immune response of the wP formulation results from both the number of antigens available, as well as the immunostimulatory effect of _B. pertussis_’ lipooligosaccharide (LOS) molecule (48,49). We hypothesize that the use of an engineered lipid A mimetic would offer the best aspects of both types of pertussis vaccines: the protective immunity of wP and potentially the safety of aP. For these studies, we utilized a novel TLR4 agonist (BECC438b) that was engineered using Bacterial Enzymatic Combinatorial Chemistry (BECC) technology. BECC is a process by which novel lipid A structures are generated.
that will induce the desired downstream TLR4-responses (50). An attenuated strain of *Yersinia pestis* is grown at different temperatures – either 26°C or 37°C – resulting in the synthesis of lipid A structures (39,47,50,51). The end result is a breadth of lipid A molecules which have been shown to induce a strongly biased Th1 response, a Th2 response, or a balanced Th1/Th2 response (52). In particular, BECC438b, a bisphosphorylated lipid A molecule derived from a *Yp* strain that lacks the C$_{12:0}$ acyltransferase (*MsbB*) but expresses a functional C$_{16:0}$ acyltransferase (*PagP*), resulting in a molecule that elicits a more balanced Th1/Th2 immune response when compared to standard lipid A structures (47,51). BECC has been used to create adjuvants that have been used in efficacious pre-clinical vaccines for a number of pathogens, including *Yersinia pestis*, SARS-CoV-2, *Pseudomonas aeruginosa*, and influenza type A (39,51,53–55).

Here, we detail the effects of adding the BECC438b adjuvant to the current DTaP formulation, which contains alum. Further, the efficacy of the DTaP + BECC438b formulation in regard to both IM and IN vaccine administration was evaluated. Female CD-1 mice were vaccinated and challenged in order to determine protection via the enumeration of colony forming units (CFUs) and the quantification of antibodies against various pertussis vaccine antigens. Additionally, lung histology was performed and cytokines within the lung and trachea were quantified such that we could determine the extent of inflammation resulting from vaccination and challenge. Finally, RNAseq analysis was performed to further differentiate the downstream immune processes that resulted from vaccination with BECC438b and subsequent *B. pertussis* challenge (Sup. Fig.1). Overall, the addition of BECC438b to DTaP administered IM was able to improve upon serological immune responses as well as reduce bacterial burden within the lower respiratory tract. Furthermore, when DTaP + BECC438b was administered IN, there was an increase in the
downstream activation of immune response pathways (such as those involved in both activation and regulation of the immune system) as well as an increase in IgA production, an indicator of mucosal immunity.

3.3 Materials and Methods

Vaccine and adjuvant composition. The GSK Infanrix formulation of DTaP was used and diluted to 1/40th the human dose (12.5μl vaccine diluted to 50μl total volume administered in 0.9% saline) and were administered no longer than 1 h after preparation. BECC438b was reconstituted using endotoxin free water. After reconstitution, BECC438b (50 µg per dose) was combined with Infanrix DTaP, and the mixture was turned end-over-end for 2 hours. All vaccines administered to mice contained the alum adjuvant at the dose included in the base Infanrix formulation.

Vaccine administration. Female CD-1 (outbred; strain code 022) mice aged four to five weeks were obtained from Charles River Laboratories. Mice were administered 50 μL of vaccine or vehicle control intramuscularly (IM) in the right thigh. For intranasal vaccines, mice were anesthetized with ketamine/xylazine (77 mg/kg of body weight of ketamine and 7.7 mg/kg of xylazine) and 25 μL of the vaccine was administered into each nostril. Mice were boosted with the same vaccine formulations 21 days after priming. All murine infection experiments were performed according to protocols approved by the West Virginia University Animal Care and Use Committee (protocol numbers:1602000797 and 1602000797_R1).

B. pertussis strains and growth conditions. B. pertussis strain UT25Sm1 was used for murine challenge. The B. pertussis strain has been fully genome sequenced (UT25Sm1 NCBI Reference
UT25 was originally isolated in Texas in 1977 (56). UT25Sm1 was cultured on Bordet Gengou (BG) agar (Difco) supplemented with 15% defibrinated sheep’s blood (Hemostat Laboratories) and streptomycin 100 µg/mL. *B. pertussis* was incubated at 36°C for 48 h, then transferred to modified Stainer-Scholte liquid medium. SSM liquid cultures were incubated for 24 h at 36°C, with shaking at 180 rpm until reaching an OD$_{600}$ of ~0.6, at which time cultures were diluted for the challenge dose. For challenge with a liquid inoculum *B. pertussis*, the mice were anesthetized with 77 mg/kg of body weight of ketamine and 7.7 mg/kg of xylazine and inoculated with 20 µL (10 µL per nostril) of the challenge dose ($2 \times 10^7$ CFU). For mice challenged with aerosolized *B. pertussis*, the mice were placed into a chamber and 20 mL of the challenge dose ($10^9$ CFU/mL) was nebulized for 10 minutes (57).

**Vaccine challenge model.** At thirty-five days post prime, mice were challenged with IN $2 \times 10^7$ CFUs of *B. pertussis* (10 µL per nostril or nebulized challenge). At day 3 post-challenge, mice were euthanized, and blood was collected by cardiac puncture. Three days post-challenge was selected as our end-point based on our previous studies (25,26). No mice were excluded from analysis. A complete blood cell count with differential was performed using a ProCyte Dx hematology analyzer (IDEXX) and serum was separated by centrifugation through a BD Microtainer blood collector and stored at -80°C until analysis. The trachea and lungs were removed and homogenized together. Lungs and trachea were suspended in 2 mL of sterile PBS in gentleMACS C tubes (Miltenyi; Cat. Number: 130-096-334) using a GentleMACS Octo Dissociator with Heaters (Miltenyi) using the m_lung_02 setting. The lung/trachea homogenates were centrifuged at 14,000 x g for 4 mins and supernatants were stored at -80°C until cytokine and antibody analyses were performed. The nasal lavage was collected by flushing 1mL of 1x PBS
through the nasal cavity and collecting the wash in an Eppendorf tube. A scalpel was used to cut out the hard palate and NALT tissue. The hard palate was placed in a culture tube with 1 mL of 1x PBS, homogenized with a polytron, and then filtered through a 70μM filter. To isolate the septum, the nasal lavage was collected, and the hard palate was removed for NALT collection. Then, the skin of the skull was removed, and a cut was made anterior to the eyes. Bacterial burden was determined in the lung/trachea, nasal lavage, NALT, and septum by colony forming units (CFUs) using serial dilutions. Serial dilutions were done in PBS and then plated on BG agar containing streptomycin (100 μg/mL) to ensure that only UT25 *B. pertussis* was cultured.

**Serological analysis of *B. pertussis* specific antibodies.** On the day of mouse processing (euthanasia and dissection), a cardiac puncture was performed on each mouse. Approximately 1 mL of blood was removed from each mouse. The samples were placed into serum separator tubes (Cat. Number: 365967; BD) centrifuged for 2 min at 14,000 *x* g. Serum was collected and stored at −80°C until analyses were performed. Serological responses specific to *B. pertussis* antigens were quantified by ELISA. High-binding microtiter plates were coated with PT (50 ng/well) (PT#180, LIST Biologicals), FHA (50 ng/well) (Enzo Life Sciences), or PRN (50 ng/well) (PRN#187, LIST Biologicals) as described in Boehm *et al* (31). For serological responses to *B. pertussis*, UT25Sm1 was cultured to an OD$_{600}$ of ~0.6 and diluted down to an OD$_{600}$ of 0.245 and microtiter plates were coated with 50 μL of bacteria per well overnight. After coating, the plates were washed with PBS + 0.05% v/v Tween 20 (Cat. Number: P1379-1L, Sigma-Aldrich) (PBS-T) and blocked with 5% non-fat dry (NFD) milk in PBS-T overnight at 4°C. Blocked plates were washed with PBS-T and then serum (1:50) from vaccinated and challenged mice were prepared in 5% NFD milk in PBS-T. All samples were serially diluted (1:2). After 2 h incubation at 37°C,
plates were washed and incubated with goat anti-mouse-IgG alkaline-phosphatase conjugated antibodies (IgG:1030-04, Southern Biotech) (1:2,000) for 1 h at 37°C. For IgG isotyping, plates were washed and incubated with either goat anti-mouse IgG2a alkaline-phosphatase conjugated antibody (Southern Biotech, Cat. #1081-04), goat anti-mouse IgG2b alkaline-phosphatase conjugated antibody (Southern Biotech, Cat. #1091-04), or goat anti-mouse IgG1 (Southern Biotech, Cat.#1071-04) for 1 h at 37°C (all antibodies diluted 1:2000 in 5% NFD milk in PBS-T). Plates using the alkaline phosphatase-conjugated secondary antibody were then washed and incubated with Pierce p-Nitrophenyl Phosphate (PNPP) (Cat. Number: 37620; Thermo Fisher Scientific) following the manufacturer’s instructions. The absorbance of the plates was read at OD$_{405}$ using a Synergy H1 plate reader (BioTek). Positive antibody titers were determined as any values above the baseline as determined by the final dilution at which the values were two times the average of blanks. To determine the proportion of each IgG isotype present in the serum, the end-point titer value for one isotype was divided into the total summation of all end-point titer values across all isotypes measured (25,26,58–60). Sera obtained from mice vaccinated with either 1/5th the human dose of wP or DTaP were used in the assay as technical positive controls of the assay.

**Analysis of mucosal IgA antibodies.** On the day of processing, the nasal lavage and lung/trachea supernatants were collected. The nasal lavage was stored at -80°C until analysis and the lung/trachea homogenates were centrifuged at 15,000 $g$ x 3 minutes. Upon completion of centrifugation, the supernatants of the lung and trachea were collected and transferred to new Eppendorf tubes and stored at -80°C until analysis. For analysis of IgA antibodies, UT25Sm1 was cultured to an OD$_{600}$ of ~0.6 and diluted down to an OD$_{600}$ of 0.245 and microtiter plates were
coated with 50 µL of bacteria per well and incubated overnight. After coating, the plates were washed with PBS + 0.05% v/v Tween 20 (Cat. Number: P1379-1L, Sigma-Aldrich) (PBS-T) and blocked with 5% non-fat dry (NFD) milk in PBS-T overnight at 4°C. Blocked plates were washed with PBS-T and then nasal lavage (no initial dilution) or lung supernatant (1:4) samples were prepared in 5% NFD milk in PBS-T. All samples were serially diluted (1:2) down the plate. After 2 h incubation at 37°C, plates were washed and incubated with goat anti-mouse IgA heavy chain secondary antibody conjugated to horseradish peroxidase (HRP) (Cat. #: NB7504, Novus Biologicals) (1:4,000) for 1 h at 37°C. The plates that utilized the HRP enzyme were then washed and incubated with TMB substrate (Cat. #421101, BioLegend). The absorbance of the plates were read at OD_{605} using the Synergy H1 plate reader mentioned above. Positive antibody titers were determined as any values above the baseline (set at two times the average of blanks).

**Analysis of cytokines within the lung and trachea supernatant.** To quantify inflammatory cytokines at the site of infection, lung/trachea homogenate supernatants were prepared, as suggested by the kit manufacturer, and diluted 1:2. Quantitative analysis of cytokines was performed using a custom kit from R&D Systems which analyzed CXCL-13, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, TNF-α, and IFN-γ according to the manufacturer’s instructions. Samples were run on a Magpix (Luminex) instrument. Bead counts below 35 were invalidated and not used in the final quantification of cytokine levels.

**Histological analysis of lung tissue.** Upon euthanasia, the lungs were removed and the right lobe was placed into 10% formalin for 48 hrs at 37°C. The right lobe was then embedded in paraffin and stained with H&E (hematoxylin and eosin) by the West Virginia University Department of
Pathology. H&E stained sections were sent to iHisto for blinded analysis and histological scoring by a board-certified pathologist. The H&E-stained slides were reviewed for both acute and chronic lung inflammation present at parenchymal, perivascular and peribronchial regions. Inflammation and damage to the epithelial cells in bronchi and bronchioles were also evaluated. Semiquantitative scores were made following standard toxicologic scoring criteria (0- none, 1- minimal, 2- mild, 3- moderate, 4- marked, 5- severe). Individual scores of the evaluated parameters were recorded. Acute inflammation score for acute or chronic inflammation score for chronic inflammation was calculated by adding the scores of individual parameters. Total inflammation score was calculated by adding acute inflammation score, chronic inflammation score, and the inflammation score for epithelia in bronchi or bronchioles.

**RNAseq analysis of the lung.** Transcriptomic analysis was performed as described previously (53). Lungs of mice were excised, and the left lobe of the lung was placed into TRIzol (Cat. Number: 15596026; Thermo Fisher) at a ratio of 1:3 volumes of sample to TRIzol. RNA was purified using the Direct-zol RNA miniprep kit (Zymo Research; R2053) following the manufacturer protocol. RNA quantity was measured with Qubit 3.0 Fluormeter using the RNA high sensitivity (Cat. Number: Q33216; Life Technologies) and RNA integrity was assessed on an Agilent 4200 TapeStation System. RNA was treated with DNase (Qiagen), per manufacture’s protocol, before library preparation. Illumina sequencing libraries were prepared with TruSeq Stranded with RiboZero Plus depletion. Resulting libraries passed standard Illumina quality control PCR and were sequenced on an Illumina NovaSeq platform at Admera Health (South Plainfield, NJ). A total of ~200 million 2 x 150 bp reads were acquired per sample. The reads were trimmed for quality and mapped to the *Mus musculus* reference genome using CLC
Genomics Version 21.0.5. An exported gene expression browser table is provided at https://data.mendeley.com/. Statistical analysis was performed with the Differential Gene Expression tool and genes were annotated with the reference mouse gene ontology terms. Genes with a Bonferroni corrected p value of <0.05 were considered differentially regulated. Genes of interest were plotted in a heat map that was generated in GraphPad Prism version 9.0. Genes that were differentially regulated were further analyzed via the online WEB-based GEne SeT AnaLysis Toolkit using over-representation analysis using the mouse enrichment category gene ontology and biological process (61). Heat maps were generated using Morpheus (62). The bubble plot of GO biological processes was created using RStudio and the ggplot2 package as described in the protocol by Bonnot et al. (63). Venn diagrams of common activated genes were created using the VennDiagram package of RStudio. Chord diagrams were created via the circlize package of RStudio.

**Statistical analysis.** Statistical analyses were performed using Prism version 9.0 software (GraphPad). Comparisons between three or more groups were performed via a one-way ANOVA. Comparisons between two variables were made utilizing an unpaired Student’s t-test.

**Ethics statement.** Animal work in this study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (64). The West Virginia University Institutional Animal Care and Use Committee (IACUC) approved the protocols and this research under the IACUC protocol 1602000797_R1. The West Virginia University Office of Lab Animal Resources is an AAALAC International accredited facility, which is a voluntary accrediting organization. All lab work with
*Bordetella pertussis*, including *in vitro* work, infection, *in vivo* studies, and pathogen culture, were completed under an approved IBC (Institutional Biosafety Committee) protocol (#17-01-11).

**Data availability.** Data for all figures are available upon reasonable request to the corresponding author. Fastq raw read data is currently deposited at SRA and a new SRA identifier is being processed (SUB12268173). The analyzed RNAseq data table in CLC file format and Excel file format are deposited at Mendeley Data (DOI: 10.17632/gdtx97tj3t.1).

### 3.4 Results

**The BECC438b adjuvant reduces bacterial burden in the respiratory tract for both intramuscular and intranasal DTaP immunizations.**

Previous studies in our lab have shown that pertussis vaccines can be protective in mice when administered intranasally (IN) (26,31). Here in this study, we utilized the BECC438b adjuvant in vaccines administered either IM or IN in order to evaluate its ability to induce adaptive immune responses in the respiratory tract to protect against *B. pertussis* challenge (39,52). To do this, female CD-1 mice were vaccinated with either 1/40th the human dose of DTaP alone or 1/40th DTaP with BECC438b. Alum was included as DTaP antigens are already adsorbed to alum. At three days post *B. pertussis* challenge, mice were processed and samples were collected for analysis. To determine bacterial burden, colony forming units (CFUs) were enumerated from the lung and trachea, the nasal associated lymphoid tissue (NALT), and the nasal lavage. Within the NALT, only mice vaccinated IM with DTaP showed a statistically significant reduction in bacterial burden when compared to the mock-vaccinated, challenged (MVC) mice (Fig. 1A). There were no statistically significant reductions in bacterial burden within the NALT between mice vaccinated IN with DTaP and IN with DTaP + BECC438b. However, the reduction did approach significance
\( p = 0.08 \) for mice that were IM vaccinated with DTaP + BECC438b when compared to their counterparts that were only vaccinated with IM DTaP (Fig. 1A). Within the nasal lavage, the addition of BECC438b to DTaP showed a slight increase the number of bacteria that are unattached to the nasal mucosa, and this is true regardless of the vaccine route (Fig. 1B). In the nasal lavage, DTaP vaccinated mice showed a significant decrease in bacterial burden (Fig. 1B). It is likely that our large intranasal challenge dose results in data that is not reflective of what is seen with a natural pertussis infection; that is, the inhalation of respiratory droplets that contain the pathogen. In this murine model, \textit{B. pertussis} manifests in the lungs of the mouse. Thus, we determined the number of bacteria within the lung and the trachea after vaccination and challenge. Within the lung and the trachea, all vaccine groups showed a statistically significant reduction in bacterial burden when compared to the MVC control mice (Fig. 1C). Additionally, while not statistically significant, the addition of BECC438b to DTaP resulted in a 352.7-fold and 44.3-fold reduction in bacterial burden for IM and IN vaccination, respectively (Fig. 1C).
Intramuscular administration of DTaP + BECC438b elicits an increase in serological responses to *B. pertussis* antigens.

Current pertussis vaccines (DTaP and Tdap) are adjuvanted with alum which induces humoral responses (Th2) (7,9,65–67). In *B. pertussis* infections, the function of antibodies is paramount for preventing adhesion to the respiratory epithelium and neutralizing its virulence factors (68–71). Numerous adjuvants have been evaluated for their ability to improve responses to pertussis antigens included in the current aP formulations (25,26,33,72,73). The production of antibodies to
the antigens included in DTaP, such as the adhesion proteins filamentous hemagglutinin (FHA) and pertactin (PRN) inhibits the adherence of the bacterium to the respiratory epithelium (71) and others that neutralize pertussis toxin. Pertussis toxin (PT) is considered to be an essential virulence factor for *B. pertussis*, resulting in leukocytosis, heart abnormalities, and disruption of the integrity of the vasculature (74–77). Additionally, it is well established that antibodies against PT serve as an unofficial correlate of protection and can reduce the extent of disease manifestation (78,79). Therefore, we determined the extent to which BECC438b could improve serological responses against *B. pertussis* infection. Serum IgG antibodies against the whole *B. pertussis* bacterium, PT, FHA, and PRN were quantified by ELISA. All vaccine formulations that were administered IM showed a statistically significant elevation in antibodies specific to the whole *B. pertussis* bacterium (UT25), while this was not the case for vaccines given IN (Fig. 2A). Additionally, the inclusion of BECC438b to the IM DTaP significantly increased anti-*Bp* antibodies over IM DTaP vaccination alone (Fig. 2A). There was also a statistically significant increase in antibody titer levels when BECC438b was administered with DTaP IM as opposed to IN, resulting in a 3-fold increase in anti-*Bp* antibodies (Fig. 2A). In regard to anti-FHA antibodies, again we observed a statistically significant increase in titer levels in both IM vaccinated groups when compared to MVC mice, and no significant increases for mice vaccinated IN (Fig. 2B). Surprisingly, only the IN DTaP + BECC438b formulation showed statistically significant increase in anti-PT antibody levels when compared to MVC mice (Fig. 2C). While not significant, the addition of BECC438b to DTaP did result in a slight increase in titer levels for both IM and IN administered vaccines, with a 0.47- and 0.70- fold increase, respectively (Fig. 2C). Despite the small induction of PRN specific antibodies, we observed a statistically significant increase in titer levels with IM administration of the DTaP + BECC438b formulation when compared to MVC mice (Fig. 2D).
Overall, these data suggest that it is the combination of DTaP administration both IM and in conjunction with the BECC438b adjuvant that accounts for increases in serological responses against *B. pertussis* antigens.

Figure 2. Quantification of serum IgG antibody titers specific to the whole pertussis bacterium and pertussis vaccine antigens at 3 days post *B. pertussis* challenge. IgG antibody titers against Bp (A), FHA (B), PT(C), and PRN(D) were determined. Data presented as geometric means ± SD, n = 10 per treatment group. A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment group. #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. A Student’s t-test was performed between each experimental group in order to determine statistically significant differences between each vaccine route and formulation. *p < 0.05, **p < 0.01. *P*-values that approach statistical significance are indicated. NVNC = non-vaccinated, non-challenged, MVC = mock-vaccinated (with 1X PBS), challenged. IM = intramuscular. IN = intranasal.
Intranasal administration of DTaP + BECC438b is able to induce a Th1-polarized immune response.

One reason for the switch from the wP to the aP formulation lies in the strong adverse immunostimulatory effect resulting from wP vaccines (10). While the aP formulations decreased the occurrence of post-vaccination side effects, it created another challenge, waning immunity (80). Similar to a natural pertussis infection, wP vaccination elicits a Th1-polarized immune response, with CD4+ T cells playing a major role in the clearance of *B. pertussis* from the respiratory tract (38). In contrast, aP vaccines induce a mixed Th1 and Th2 immune responses and the resulting antibodies are able to prevent severe disease but are unable to completely clear the pathogen from the respiratory tract, resulting in its transmission (81). Our objective was to determine the T helper cell polarization that resulted from the inclusion of BECC438b into both IM and IN DTaP formulations via antibody isotype analysis. Pre-challenge sera from mice was analyzed for the presence of IgG isotypes: IgG1 (indicative of a Th2-polarized response) and IgG2a/IgG2b (indicative of a Th1-polarized response). Both IM administered vaccines triggered similar immune responses, with DTaP having 73% of the total IgG isotypes being IgG1 and DTaP + BECC438b having 76% of the total IgG isotypes being IgG1 (Fig. 3A and 3C). Upon IN vaccination of DTaP alone, the breakdown of IgG isotypes is similar to what was seen with the IM results, with IgG1 making up 67% of the IgG antibodies (Fig.3B). Interestingly, it is the addition of BECC438b to IN DTaP changes the proportion of IgG1 antibodies to around 33% and the proportion of IgG2 antibodies to around 67% (Fig. 3D). This suggests that BECC438b is likely inducing increased Th1 cell-mediated response, perhaps within the NALT and lungs, which is releasing the IFN-γ needed to produce the IgG2 antibodies.
The inclusion of BECC438b to DTaP increases the number of circulating immune cells in the blood but does not alter the inflammatory cytokine profile of the lungs.

To further characterize the inclusion of the BECC438b adjuvant to DTaP on cellular responses, we performed a complete blood count with differential on blood taken from the mice at 3 days post-challenge. It is appreciated that the release of PT results in many of the symptoms associated
with a pertussis infection, including leukocytosis (82). Similarly to histological analysis, we observed a statistically significant increase in both white blood cells and lymphocytes when the BECC438b adjuvant was added to DTaP versus what was seen with DTaP vaccination alone (Sup. Fig. 2A and 2C). Quantification of neutrophils within the blood revealed that all vaccines resulted in statistically significant increase when compared to MVC mice (Sup. Fig. 2B). Monocyte quantification revealed no significant differences across both the control and vaccine groups (Sup. Fig. 2D). Further, enumeration of cytokines within the pulmonary supernatant revealed that, when compared to MVC mice, all vaccination groups had a significant reduction in a number of cytokines typically associated with inflammation, such as TNF-α, IL-6, and IFN-γ (Sup. Fig. 3).

Overall, the addition of BECC438b stimulates a strong initial immune response, as evidenced by the increased number of circulating immune cells; however, the release of proinflammatory cytokines within the lung remain similar among all vaccines, regardless of the presence of the BECC438b adjuvant.

**The addition of BECC438b to DTaP increases the total inflammation found in the lungs for both IM and IN immunization.**

BECC adjuvants are TLR4 agonists; as such, it is expected that some degree of inflammation will occur with their use. Specifically, BECC438b is known to induce a balanced Th1/Th2 immune response (39). To determine the extent of inflammation caused by vaccination with the BECC438b adjuvant, lungs were fixed, and paraffin embedded to allow for H&E staining. A board-certified pathologist blinded to the group designations evaluated the extent of acute, chronic, and total inflammation within the parenchymal, perivascular, and peribronchial regions of the lung. In regard to acute inflammation scores, which are characterized by the infiltration of neutrophils, only
IM DTaP + BECC438b vaccinated mice did not have a statistically significant reduction when compared to MVC mice (Fig. 4A). Interestingly, chronic inflammation scores, characterized by the infiltration of lymphocytes and plasma cells, were only statistically different between MVC and NVNC mice (Fig. 4B). When compared to MVC mice, the NVNC, IM DTaP- vaccinated, and the IN DTaP-vaccinated mice showed a statistically significant reduction in total inflammation scores, which considers both acute and chronic scores (Fig. 4C). However, levels of total inflammation were not reduced in BECC438b adjuvanted vaccine groups (administered IM or IN) did not afford a significant reduction in inflammation when compared to MVC mice (Fig. 4C). These data suggest higher recruitment of neutrophils, lymphocyte, and plasma cells to the lung tissue, which is consistent with a Th1- driven immune response. It is important to note that while inflammation levels in the BECC438b adjuvated groups were more similar to MVC mice, the groups adjuvanted with BECC438b had the lowest bacterial burden in the lungs following *B. pertussis* challenge (Fig. 1C). These data warranted studies to more deeply characterize the lung environment following BECC438b administration, in order to understand the unique responses of IN vaccination to subsequent *B. pertussis* challenge.
The addition of BECC438b increases the number of activated genes when compared to administration of DTaP alone.

While we observed both serological and bacterial burden differences between routes and formulations, that histopathological analysis also revealed a unique inflammatory profile resulting from vaccination and subsequent challenge (Fig. 4). Changes in antibody responses against the pertussis vaccine antigens were similar between vaccine groups; therefore, we hypothesized that administration of the vaccine to the nasal mucosa could drive a different immune response in the
tissue. In order to characterize how each vaccinated cohort responded to challenge, we used total bulk RNAseq analysis to discover differences in downstream biological pathways occurring within the lung as a result of changes in gene expression profiles at day 3 post-challenge. RNA reads were mapped to the mouse genome and statistical analysis was performed to identify differentially expressed genes. To identify genes that were activated or repressed by challenge, we compared all gene expression profiles to non-vaccinated, non-challenged (NVNC) mice as baseline. Challenge with *B. pertussis* only (MVC mice) resulted in 295 genes activated and 458 genes repressed (Fig. 5A). On a surface level, regardless of the route of vaccine administration, the BECC438b adjuvant was able to increase the number of genes that were activated in the lung when compared to vaccination with DTaP alone (Fig. 5A). While IM vaccination with DTaP resulted in the activation of 149 genes, the addition of BECC438b to the formulation increased the number of activated genes to 197 genes (Fig. 5A). Similarly, IN DTaP alone activated 83 genes, while IN DTaP + BECC438b activated 174 genes (Fig. 5A). Additionally, the BECC438b adjuvant increased the number of activated genes that were shared by both MVC and vaccinated mice over mice that were vaccinated with DTaP alone (Fig. 5B and Fig. 5C). In terms of repressed genes, IM administration of DTaP + BECC438b increased the number of repressed genes from 26 to 38, when compared to IM DTaP vaccination alone (Fig. 5D and 5E). IN vaccination with DTaP only resulted in the repression of 47 unique genes, while the addition of BECC438b to the formulation slightly decreased uniquely repressed genes down to 40 (Fig. 5D and 5E). When comparing to mice only vaccinated with DTaP, IM administration of the vaccine containing BECC438b increased the number of repressed genes that are shared with MVC mice, while IN administration of DTaP + BECC438b had the opposite effect and decreased the number of genes shared with MVC mice (Fig. 5E). The overlap with genes activated in MVC mice is important, as convalescent mice are
known to have the longest duration of protection; therefore, if a vaccine is able to activate or repress similar genes to what is seen with natural infection, then there is potential that the vaccine will offer an increased duration of protection (16).

Figure 5. RNAseq analysis was performed to elucidate changes in genetic profiles between each experimental group. RNAseq analysis revealed the number of both activated (red) and repressed (blue) genes per experimental group. A bar chart representation of activated and repressed genes is shown in panel A. Venn diagrams which illustrate common activated genes between MVC mice and those vaccinated with DTaP (B) and MVC mice and those vaccinated with DTaP + BECC438b. All activated and repressed genes were deemed to be statistically different from NVNC mice via the Bonferonni $p$-value ($p < 0.05$). MVC = mock-vaccinated, challenged. NVNC = non-vaccinated, not-challenged, IM = intramuscular, IN = intranasal.
The BECC438b adjuvant alters the downstream biological processes induced by vaccination.

After determining the extent of gene activation afforded by the BECC438b adjuvant, we then evaluated the function of the activated genes. GO term analysis was used to determine the biological processes associated with the top 40 most highly activated genes (Fig. 6A). The top 40 genes were then used to construct chord diagrams in order to determine changes in biological pathway activation between groups. As expected, MVC mice induced many pathways that correspond to responses to a pathogen as well as immune system activation (Fig. 6B). DTaP administered IM resulted in the activation of pathways similar to that of MVC mice (Fig. 6C). The addition of BECC438b to the IM vaccine induced an increased number of immune system-involved pathways when compared to either MVC mice or mice vaccinated only with IM DTaP (Fig. 6D). Upon IN administration of DTaP, there was a marked activation of pathways involved in apoptosis or the regulation of apoptosis (Fig. 6E). However, it is of note, that while the genes that were activated were all significantly different from NVNC per Bonferroni analysis, none of the GO biological pathways were considered statistically significant for IN DTaP vaccinated mice, as determined by the FDR p-value (Fig. 6H). With mice were vaccinated with IN DTaP + BECC438b, there was a shift back to the activation of pathways that tied into the immune system, whether that be via activation of regulation (Fig. 6F). Additionally, unlike IN DTaP vaccination, the addition of BECC438b resulted in the activation of pathways that were considered significant per the FDR p-value (Fig. 6H). Overall, it seems that the BECC438b is able to increase immune system activation when added to the base DTaP vaccine, regardless of the route of vaccine administration.
Figure 6. Transcriptomic and systems analysis of mouse lungs at day 3 post *B. pertussis* challenge. Data from RNAseq analysis was used to determine the most common activated genes amongst all experimental groups and the downstream GO biological processes activated by those genes. Panel A depicts the top 40 activated genes shared by all experimental groups with blue boxes corresponding to minimum values, while red boxes correspond to maximum values. GO chord diagrams illustrated the GO biological processes activated by top activated genes were created for MVC (B), IM DTaP (C), IM DTaP + BECC438b (D), IN DTaP (E), and IN DTaP + BECC438b (F) vaccinated mice were created. A key showing which colors correspond to each GO biological process is shown in panel G. A bubble plot was created to show the biological processes activated by MVC mice as well as each vaccine group (H). The bubble plot shows the enrichment score, number of genes, and the FDR p-value of each pathway. Activated genes were determined to be statistically different from NVNC mice per the Bonferoni p-value (p < 0.05). The online tool, WEB-based GEne SeT AnaLysis, was used to determine the downstream biological processes associated with each activated gene.
Intranasal administration of DTaP and BECC438b increased diversity and expression of immunoglobulin genes in the lung.

The transcriptomic analysis illustrated the ability of IN vaccination of DTaP alone or with the BECC438b adjuvant to activate immune system pathways (Fig. 6). However, one observation that was surprising to us is that gene ontology analysis does not include immunoglobulin genes. Therefore, we performed specific analysis on genes that encode the variable or constant regions of immunoglobulins. To do this we examined total gene expression of immunoglobulin genes in the lungs of mice after challenge. This type of analysis can only indicate the presence of RNA that encodes immunoglobulin therefore we are not able to fully assemble antibody sequences or determine their antigen specificity. There are two ways that immunoglobulin genes could change in expression when evaluating an organ’s overall RNA transcriptome: 1) cells are recruited that express immunoglobulin genes or 2) immunoglobulin genes of existing cells are increased. We hypothesized that the detection of immunoglobulin genes reflects the presence of B cells, potentially plasmablasts or plasma cells. Specifically, we wanted to determine expression changes of the variable regions of the heavy chain (Igh) and the two variable light chains (Igk or Igl). With that, RNA reads corresponding to *Igh*, *Igk* and *Igl* variable genes were analyzed to determine the breadth and diversity of immunoglobulin genes being expressed. On the other hand, when we observe a specific immunoglobulin variable gene that is overrepresented, it suggests that the specific B cell clone is present in higher amounts likely due to immunization. For the total counts of Ig genes, we observed a marked increase in the number of both *Igh* and *Igk* genes for IN vaccination with IN DTaP + BECC438b having the highest overall counts (Fig. 7A and 7B). Interestingly, while the counts of *Igh* and *Igk* genes were negligible for IM vaccination, the total count of *Igl* genes for IM vaccinated mice increased to the level seen with IN vaccination (Fig.
7C). Of the genes that had the highest overall counts, a vast majority were immunoglobulin constant genes; therefore, we enumerated the number of gene counts of each Ig constant within each vaccine group. As seen in previously discussed data, immunoglobulin constant gene counts are almost negligible until vaccines were administered IN (Fig. 7G). Within the IN vaccinated mice, the gene counts were highest for Igha, Ighg1, and Ighg2b, while, in mice vaccinated IN with DTaP adjuvanted with BECC438b, there is a large increase in the number of Ighm genes (Fig. 7G). To further corroborate these findings, we performed ELISAs on the pulmonary supernatant to quantify the level of IgA, IgG, and IgM within these respiratory tissues. On par with the gene count data seen in Figure 7, there was an unsurprising increase in IgA in mice that received IN vaccination, and the antibody response was greater as a result of the BECC438b adjuvant (Fig. 7H). Interestingly, the effect of BECC438b on IgG antibodies was more pronounced with IM vaccination, as IM vaccination of DTaP + BECC438b resulted in a robust increase in IgG antibodies when compared to all other vaccine groups (Fig. 7I). Further, it is important to note that we did not detect significant amounts of IgG genes (Ighg1, Ighg2b, Ighg3) (Fig. 7G); however, IgG antibodies were present in the serum (Fig. 2) and pulmonary supernatant (Fig. 7I). Surprisingly, when IgM antibodies within the pulmonary supernatant were quantified, we determined that the presence of anti-Bp IgM was negligible across all experimental groups, which is in contrast to what was expected given the high number of Ighm reads seen in mice vaccinated IN with DTaP + BECC438b (data not shown). Overall, we were able to delve into serological data and highlight distinct antibody responses that were unique to each vaccine formulation and route. Specifically, it seems that induction of mucosal immune responses is largely dependent upon, not only IN immunization, but also the inclusion of the BECC438b adjuvant. The presence of B. pertussis specific IgA and gene reads for IgA constant, suggests that IN immunization with and
without BECC438a results in antibody producing B cells in the lungs. Further characterization of this antibody producing immune cell population would be necessary to assign them a proper identifier of antigen specificity. With these data we cannot conclude if these cells are resident, due to immunization, or if they were recruited due to challenge.

Figure 7. Analysis of immunoglobulin genes expressed in mouse lungs at day 3 post *B. pertussis* challenge. Total gene counts were determined for *Igh* (A), *Igk* (B), and *Igl* (C) immunoglobulins. Additionally, we looked at the number of unique genes within the lung tissue for *Igh* (D), *Igk* (E), and *Igl* (F) immunoglobulin genes. To evaluate the number of constant genes within each vaccine groups, we measured the number of gene counts for each heavy chain (G). To corroborate the heavy chain gene count data, we performed ELISA assays to enumerate the amount of IgA antibody within the pulmonary supernatant (H) as well as the amount of IgG antibodies within the pulmonary supernatant (I). The data represents the total number of genes for all heavy and light chain portions of the immunoglobulins, including all variants. Data presented as geometric means ± SD, n = 5 per treatment group. The exception to this is ELISA data, in which n = 10 per treatment group. A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment group. ##p < 0.01, ###p < 0.001. A Student’s t-test was performed between each experimental group in order to determine statistically significant differences between each vaccine route and formulation. *p < 0.05, **p < 0.01. MVC = mock-vaccinated, challenged. NVNC = non-vaccinated, not-challenged, IM = intramuscular, IN = intranasal.
Aerosol challenge with *B. pertussis* demonstrated protection in the respiratory tract across all tested vaccines.

In pre-clinical models that evaluate protection against *B. pertussis*, it is appreciated that there is a lack of a standard protocol for challenge. A majority of studies involve growing *B. pertussis*, diluting the bacteria to obtain a specific dose for infection, then delivering the pathogen as a liquid inoculum via intranasal administration. While mice are infected via this method, there is a large deposition of the bacteria within the nasal cavity, which exceeds what would occur with any natural infection. As a result, it is difficult to determine the ability of a vaccine to reduce the nasal colonization, as the vaccine must clear an unnaturally large deposition of *B. pertussis*. To resolve this issue, we opted to perform a study in which mice were vaccinated and then given an aerosol challenge of *B. pertussis*. With aerosolization of the bacteria, the result is a deposition of *B. pertussis* within the respiratory tract that more closely resembles what is seen in a natural infection. For this study, mice were vaccinated as shown above; however, upon challenge, mice were placed into a chamber and were exposed to an aerosolized dose of *B. pertussis*. Mice were then processed 3 days post-challenge. In the NALT, all vaccine groups had a sizable reduction in bacterial burden when compared to MVC mice (Fig. 8A). For IM vaccinated mice, the addition of BECC438b to DTaP resulted in an increase in bacteria, but this difference was not statistically significant; however, for IN vaccination, the addition of BECC438b did result in a statistically significant increase in NALT bacterial burden versus IN administration of DTaP alone (Fig. 8A). With IM administration, none of the mice were at the lower limit of CFU detection; however, with IN DTaP-vaccinated mice, two were near or at the lower limit of detection. Further, with IN DTaP immunization, we can see the maximal number of bacteria is lower than both IM administered vaccines (Fig. 8A). We next enumerated the number of viable bacteria on the nasal septum. Within
the nasal septum, mice vaccinated with DTaP + BECC438b showed a reduction in bacterial burden for both IM and IN vaccines, with a 0.45- and 0.35-fold reduction in bacterial burden, respectively (Fig. 8B). Similarly, while not statistically significant, within the nasal lavage, the addition of BECC438b to DTaP did result in a slight reduction in bacterial burden, with a 0.15- and 0.35-fold reduction for IM and IN vaccinated mice, respectively (Fig. 8C). Finally, within the lung and the trachea, the aerosolization of *B. pertussis* resulted in the largest quantity of bacteria seen within MVC mice when compared to all other organs. For mice vaccinated IM, the use of the BECC438b adjuvant resulted in a 0.48-fold reduction in bacterial burden versus DTaP mice alone, although this change was not statistically significant (Fig. 8D). While the statistical analysis illustrated that all four vaccine groups had reduced bacterial burden in the lung and trachea, it is important to point out that in the BECC438b-immunized groups, some mice demonstrated a complete clearance of the pathogen. This suggests that IN immunization was able to induce robust bacterial clearance in the lower respiratory tract of some mice. This is a promising finding, as large shortcoming of aP vaccines lies in their inability to clear the pathogen from the respiratory tract, which then results in the transmission of *B. pertussis* to a new host (38,83).
Figure 8. Respiratory *B. pertussis* bacterial burden in naïve and vaccinated mice challenged by aerosol delivery. Mice were aerosolized with *B. pertussis* to provide a more natural localization of bacteria within the respiratory tract and organs were processed to determine bacterial burden at 3 days post-challenge. CFU counts were enumerated within the NALT (A), Septum (B), Nasal Lavage (C), and the Lung/Trachea (D). Data presented as geometric means ± SD, n = 5 per treatment group. A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment group. ###p < 0.001. A Student’s t-test was performed between each experimental group in order to determine statistically significant differences between each vaccine route and formulation. Red data points indicate CFU values at the lowest limit of detection. CFU = colony-forming units. MVC = mock-vaccinated, challenged. IM = intramuscular. IN = intranasal.
3.5 Discussion

In this study, we determined the effect of including the BECC438b adjuvant in DTaP vaccine after either IM or IN administration. To do this, female CD-1 mice were primed and boosted with either 1/40th the human dose of DTaP alone or 1/40th DTaP + BECC438b. Mice were then challenged with *B. pertussis* and processed at 3 days post-challenge. We first observed that the addition of BECC438b to DTaP reduced the bacterial burden in the respiratory tract, most notably, in the lung and trachea. This was the case regardless of the route of vaccine administration; however, a larger reduction in bacterial burden was noted with IN vaccination (Fig. 1). Serological analysis determined that the DTaP + BECC438b formulation was able to offer an increase in serum IgG antibodies against pertussis antigens when compared to DTaP alone (Fig. 2). Most notably, we observed a large increase in anti-PRN antibodies when mice were vaccinated IM with DTaP containing BECC438b. This data shines light on the immunostimulatory effect of the BECC438b adjuvant, as PRN is present in the lowest amount in the DTaP formulation.

When IgG isotype analysis was performed on pre-challenge sera, IM-administered vaccines elicited a Th2-polarized response; whereas when DTaP + BECC438b was given IN, the response was more Th1-polarized (Fig. 3). In the case of a pertussis infection, it has been established that it is cell-mediated immunity that plays a large role in the clearance of the pathogen (7,38). It is interesting that, only after IN BECC438b-containing vaccine, did we observe a change from the typical Th2-polarized immune response to that which is mostly Th1-based. It could be that the induction of mucosal immune responses and the local uptake of antigen allowed for a more inflammatory based reaction to occur, thus causing the activation of Th1 cells and the production of IgG2b. Additionally, it has been reported that Th1 cells of the nasal mucosa are capable of producing IFN-γ, which could lend to the predominance of IN vaccination in eliciting a Th1-
polarized response (84). Further, as this vaccine formulation contains both alum and BECC438b, it could be that the BECC438b adjuvant takes advantage of the depot effect of alum, allowing the TLR4 agonist to remain localized at the site of vaccination for a prolonged period of time. As a result of this prolonged adjuvant presentation to the NALT and subsequent trafficking to multiple sites throughout the body via IN vaccination, there was an increase in Th1-polarized responses when compared to standard IM immunization.

When RNAseq analysis was performed on the lungs of the mice, some interesting trends occurred between both the routes and the vaccine formulations. In terms of the activation of downstream pathways, GO term analysis revealed that mice vaccinated either IM with DTaP or IN with DTaP + BECC438b were similar to that of MVC mice (Fig. 6). It was interesting to observe that the addition of BECC438b to the formulation was able to result in robust changes in gene expression. While IM DTaP resulted in robust responses to other organisms, stimuli, and cytokines, the inclusion of BECC438b shifted the response to one that had major involvement of immune system pathways (Fig. 6). This was expected, as the TLR4 pathway not only activates the release of pro-inflammatory cytokines, but also the activation of both innate and adaptive immune cells (85). Additionally, while IN administration of DTaP had no pathways activated that were deemed to be statistically significant, the addition of BECC438b to IN DTaP increased the activation of biological pathways associated with responses to stimuli and immune system activation,

While the antibody titer data did illustrate increases in serum antibodies upon the use of BECC438b, the RNAseq gene count data was able to illustrate even more differences between vaccine groups. Both IN vaccination groups had elevated counts of Igha, Ighg1, and Ighg2b (Fig. 
7). The increased level of \textit{Igha} is expected with mucosal vaccination, and the \textit{Ighg1} and \textit{Ighg2b} are indicative of a mixture of both Th1 and Th2-polarized immune response, which is expected with both aP vaccine formulations, as well as with BECC438b. Of interest is the ability of IN DTaP + BECC438b to cause such a large increase in \textit{Ighm} gene counts. IgM is the first antibody produced due to an encounter with a foreign pathogen and is an activator of the complement cascade. Additionally, IgM contains a J-chain, which allows the antibody to interact with pIgR, meaning it can access the mucosal lumen (86). Previous studies suggests that immunity within the lung is dependent upon a subset of B cells known as B-1 cells, which secrete large amounts of secretory IgM (87). With this knowledge, the increased gene counts of \textit{Ighm} are interesting to see as a result of IN vaccination with DTaP + BECC438b, as it may result in a more robust mucosal immune response, as potentially not only IgA is playing a role in protection. However, though we observed high gene counts, when we performed ELISA assays to corroborate this data, we did not see any appreciable levels of IgM antibody in the pulmonary supernatant (data not shown). While we looked for antibody that was specific to the whole \textit{B. pertussis} bacterium, it could be that the high counts of \textit{Ighm} could be antibody that is specific to other antigens included in DTaP such as diphtheria toxoid, or tetanus toxoid.

Based on our studies presented here, we hypothesize that intranasal DTaP with BECC438b potentially induces memory lymphocyte clusters that participate in bacterial detection and clearance. RNAseq analysis of gene counts within each vaccination group revealed a marked increase in \textit{Cd69} gene expression in mice IN vaccinated with DTaP + BECC438b (426-fold activated over NVNC; \textit{p}-value = 0.002). CD69 is expressed on cells that are classified as “tissue resident cells” and it is considered to be the marker of T resident memory cells (TRMS) (88). TRMS
remain localized at a particular location (such as the lung, the skin, or genital mucosa) and serve as the first line of defense against a pathogen (89). Previous studies support that the clustering of TrM and antigen presenting cells may play a role in protecting against subsequent infections with a pathogen (90,91). It may be that IN administration of the BECC438b adjuvant allowed for the development of memory lymphocyte clusters in the lung tissue, which explains why these mice, when compared to all other vaccine groups, had greater induction of downstream immune response pathways and such a striking number of Ighm reads. Further, while the immunoglobulin gene expression data obtained from RNAseq cannot be assigned as specific for B. pertussis antigens, these data suggest that additional immune pathways are being activated and that B cells are likely in the lungs. It is possible that nonspecific immune activation is occurring, as was seen with BPZE1, an intranasal pertussis vaccine candidate found to be cross-protective against lethal challenge with Streptococcus pneumoniae (92).

When we initially performed these studies, we challenged mice with a high dose liquid inoculum of B. pertussis. While this method of challenge is able to induce disease, it results in a large deposit of bacteria into the nasal cavity – much more than what would be seen with natural infection. As a result, we performed an aerosol challenge, in which mice were nebulized with B. pertussis, resulting in the deposition of the pathogen in a more realistic manner. All tested vaccines resulted were able to reduce the number of bacteria within the respiratory tract when compared to MVC mice, but for some organs the effect of BECC438b remained unclear. For example, in the NALT, the addition of BECC438b to the vaccine formulation appears to have resulted in an increase in bacterial burden (Fig. 8). This is also the case for IN immunization and bacterial burden in the lung and trachea (Fig. 8). We believe this is due to both the aerosol challenge, which results in a smaller
deposit of bacteria into the respiratory tract, and sufficient protection afforded by the base vaccine, DTaP. While there was plenty of reason to use the 1/40th dilution dose, we could see where lower vaccine doses could possibly illuminate the values of IN and BECC438b adjuvant inclusion.

Additionally, we understand that mice and humans vary greatly in the anatomy and structural architecture of their respiratory tracts, as well as the timing of immune responses. This provides a caveat for challenge models, regardless of the means by which the mice are challenged, which impacts the clearance that we can observe from vaccination. There is also an impact on bacterial deposition within the respiratory tract, which is affected by the challenge method and dose of *B. pertussis* used (57). Also, we only utilized one time point post challenge: 3 days. In humans, antibody levels against pertussis vaccine antigens wane each year after receiving a booster vaccination (93). In mice, however, the antibody levels within the sera are maintained and do not seem to wane long after vaccination and our previous studies have utilized this particular data collection point (25,26,94). Further, we know that beyond this time point, the bacterial burden begins to decrease, as innate responses take effect and clear the pathogen even without prior vaccination. Therefore, we cannot attest to the long-term protection afforded by these particular formulations.

Overall, in this work, we observed that the novel BECC438b adjuvant was capable of improving immune responses in both IM and IN immunization with DTaP. Upon the addition of BECC438b to DTaP, there was a substantial reduction in bacterial burden within the lung and trachea as well as an increase in serological responses against *B. pertussis* antigens. IN vaccination with DTaP + BECC438b resulted in a Th1-polarized immune response, while IM vaccination maintained the
Th2 response associated with aP vaccine formulations. Further, IN vaccination allowed for the increased induction of mucosal immune responses, as illustrated by the increased levels of IgA in the pulmonary supernatant and nasal lavage. Finally, IN vaccination with DTaP + BECC438b was able to change the downstream activation of biological pathways, shifting aP responses to become similar to what is seen with natural infection. To conclude, the BECC438b adjuvant shows promise in terms of vaccination against *B. pertussis*, as we have observed its benefits with two different vaccination routes. While this is only the first step in evaluating BECC438b against *B. pertussis* challenge, it is beneficial to know that BECC438b works in conjunction with alum, meaning that there is no need to deconstruct the existing formulation. Further, as we continue to work to develop the next generation of pertussis vaccines, we will continue to evaluate IN immunization. As a result of this study, we have insight into the protective capacity of a new adjuvant, which could be utilized in the development of the next generation of pertussis vaccines. As more studies are completed on BECC438b, in terms of immunogenicity, safety, and performance in non-human primate models, it could become a likely candidate for the inclusion into future pertussis vaccines.

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**Author Contributions**

M.A.D. and F.H.D. developed the experimental design. M.A.D. and M.A.W. performed vaccine administration as well as bacterial challenge. All authors participated in mouse processing experiments with additional help from Annalisa Huckaby, Sarah Jo Miller, Joshua Chapman, Dr. Dylan Boehm, and Olivia Miller. M.A.D. enumerated CFUs, performed ELISA assays, performed cytokine assays, and analyzed RNAseq data. M.A.D. comprised the manuscript and figures with revisions from all authors. RNAseq analysis was performed by Admera Health. Histological scoring and analysis of the lung tissue was performed by Dr. Michelle X. Yang, M.D.,Ph.D. at iHisto.
Supplemental Figure 1. Graphic explaining the experimental design, including the vaccination schedule, as well as the assays that were performed at the conclusion of the study.
Supplemental Figure 2. A complete blood count was performed on whole blood collected via cardiac puncture at 3 days post-challenge. A ProCyte IDEXX was used to enumerate the number of circulating WBCs (A), neutrophils (B), lymphocytes (C), and monocytes (D). Data presented as geometric means ± SD, n = 10 per treatment group. A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment group. #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. A Student’s t-test was performed between each experimental group in order to determine statistically significant differences between each vaccine route and formulation. *p < 0.05, **p < 0.01. MVC = mock-vaccinated, challenged. NVNC = non-vaccinated, not-challenged, IM = intramuscular, IN = intranasal.
Supplemental Figure 3. Cytokines and chemokines were quantified in the lung supernatant at 3 days post-challenge. Levels of cytokines are illustrated in pg/mL for TNF-α (A), IL-12p70 (B), IL-1b (C), CXCL-13 (D), IL-4 (E), IL-6 (F), IL-10 (G), IL-17A (H), and IFN-γ (I). Data presented as geometric means ± SD, n = 10 per treatment group. The exception to this is ELISA data, in which n = 10 per treatment group. A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment group. #p < 0.05, ##p < 0.01. A Student’s t-test was performed between each experimental group in order to determine statistically significant differences between each vaccine route and formulation. *p < 0.05, ***p < 0.001. P-values that approach statistical significance are indicated. MVC = mock-vaccinated, challenged. NVNC = non-vaccinated, not-challenged, IM = intramuscular, IN = intranasal.
Supplemental Figure 4. OD405 values obtained from spectrometer readings used to determine IgG antibody end-point titers. At the completion of the ELISA assay, the OD$_{405}$ values were used to calculate the end-point titer by finding the dilution at which the absorbance reading was less than or equal to two times the blank row. Sera was diluted down a plate at a 1:50 ratio for each antigen tested. Readings were done at OD$_{405}$, as the secondary antibody was conjugated to the enzyme alkaline phosphatase (AP), and the p-Nitrophenol phosphate (PNPP) substrate was used.
Supplemental Figure 5. Raw OD405 values and end-point titer values obtained from IgG isotype analysis were determined. At the completion of the ELISA assay, the OD\textsubscript{405} values were used to calculate the end-point titer by finding the dilution at which the absorbance reading was less than or equal to two times the blank row. Sera was diluted down a plate at a 1:50 ratio for each antigen tested. Readings were done at OD\textsubscript{405}, as the secondary antibody was conjugated to the enzyme alkaline phosphatase (AP), and the p-Nitrophenol phosphate (PNPP) substrate was used. Raw OD405 values are given for IgG1 (A), IgG2a (B), and IgG2b (C). Further, antibody titer data for each vaccine is presented for each IgG isotype (D). In panel D, the dotted line along the Y-axis represents the limit of detection for the assay.
Supplemental Figure 6. OD605 values obtained from spectrometer readings used to determine IgA antibody end-point titers. At the completion of the ELISA assay, the OD\textsubscript{605} values were used to calculate the end-point titer by finding the dilution at which the absorbance reading was less than or equal to two times the blank row. Samples were diluted down a plate at a 1:1 ratio for the nasal lavage (A) and at a 1:4 ratio for the pulmonary supernatant (B). Readings were done at OD\textsubscript{605}, as the secondary antibody was conjugated to the enzyme horseradish peroxidase (HRP) and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was used.
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Chapter 4

Utilizing two vaccine administration routes to provide increased protection against *B. pertussis* challenge in a murine model
Utilizing two vaccine administration routes to provide increased protection against *B. pertussis* challenge in a murine model

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4.1 Abstract
Cases of pertussis (whooping cough) have begun to increase since the introduction of the acellular (aP) formulation. Current aP vaccines do not provide a long duration of protection nor are they able to completely clear the causative agent, *Bordetella pertussis*, from the respiratory tract. Given that *B. pertussis* is a mucosal pathogen, the implementation of an intranasally (IN) administered booster vaccine could prove beneficial, as it would induce mucosal immune responses at the sight of infection. Currently, all pertussis vaccines are administered intramuscularly (IM); therefore, it is important to understand how the two vaccine routes would interact. We hypothesized that a systemic-mucosal (IM/IN) “push-pull” strategy would improve immune responses against *B. pertussis*, as the IM prime would induce strong systemic responses, while the IN booster would bring these responses to the nares. In this study, we determined the efficacy of combining two different vaccine routes – IM prime followed by an IN boost – and evaluated numerous endpoints to evaluate protection. Additionally, we also incorporated two novel adjuvants (BECC438b and IRI-1501) into the push-pull strategy in order to determine if they improved immune responses. We observed that the IM/IN strategy improved upon the reduction of bacterial burden in the lower respiratory tract when compared to IM vaccination alone. Further, the utilization of two different vaccination routes improved the induction of both systemic and mucosal antibodies specific to pertussis antigens. Overall, the results of this study illuminate the benefit that just one IN booster vaccine can have in regard to bacterial clearance and humoral responses.
4.2 Introduction

*Bordetella pertussis* is the causative agent of whooping cough, a respiratory illness that can result in hospitalization and death, most notably in infants (1–3). The first type of vaccine used to combat *B. pertussis* infections was a whole cell (DTP, wP) vaccine, which contained a killed form of the whole bacterium (4,5). As a result of DTP implementation in the 1940s, cases of pertussis dropped by around 99%; however, side effects such as febrile seizures and hypotonic-hyporesponsive episodes caused vaccine hesitancy (6,7). The deleterious side effects were attributed to the lipooligosaccharide (LOS) that was still bound to the outer membrane of the killed bacterium in the vaccine (8,9). Because of this, when an acellular (aP, DTaP, TdaP) formulation – which contained select antigens rather than the whole bacterium – became available, the United States phased out wP immunizations and adopted a vaccine schedule that utilized only aP formulations (10–12). While the occurrence of severe side effects decreased as a result of the switch, surveillance studies have revealed that the number of pertussis cases have begun to increase, which prompted many to believe that the aP vaccines have a shorter duration of protection than anticipated (6,13–15).

There has been an ongoing effort to address the issue of the aP formulations’ waning duration of protection. One proposed solution has been the utilization of alternative adjuvants, as alum, the current adjuvant in aP formulations, is known to induce a Th2-polarized immune response (16,17). While humoral immunity is important in protection against pertussis infections, studies have shown that it is the cell-mediated immunity (Th1-polarized immunity) that is important in the clearance of *B. pertussis* as well as the longer duration of protection (18,19). With that, numerous pre-clinical studies have evaluated a number of different adjuvants, including CpG ODNs, TLR4
agonists, TLR2 agonists, and β-glucans, in order to see how they improve upon existing vaccine-induced immune responses (20–24). Indeed, some of these adjuvants have demonstrated the ability to increase serological responses against *B. pertussis* antigens and decrease bacterial burden; however, when these novel formulations were administered intranasally, the induction of mucosal immune responses became apparent (21,22).

Currently, all pertussis vaccines are administered intramuscularly (IM), which is known to induce strong systemic immune responses (25). Yet, *B. pertussis* is a respiratory pathogen and its pathogenesis depends upon its initial adhesion to the ciliated respiratory epithelium (26–28). As such, it stands to reason that the implementation of a vaccine that is able to activate the mucosal immune responses within the respiratory tract would be of great benefit in regard to pertussis prevention. To do this, an intranasal (IN) vaccine could be utilized, as both the nasal cavity and lung tissue contain mucosa-associated lymphoid tissues (MALTs) (29–31). When antigens are presented to the nasal mucosa, specialized epithelial cells, known as microfold (M) cells, take up the antigens and present them to T cells and B cells (30). The result is the induction of both mucosal immune responses, such as antigen-specific secretory IgA (sIgA) and the activation of effector T cells as well as systemic immune responses, such as serum IgG production (30). In the case of a pertussis infection, the generation of sIgA would prevent the adhesion factors, such as filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae from being able to attach to the ciliated respiratory epithelium (32). The utilization of IN pertussis vaccines has proved to be beneficial in generating efficacious immune responses in both pre-clinical models and humans (21,22,33,34). As such, administering aP vaccines IN may address some issues of waning immunity via the induction of localized immune responses.
As previously mentioned, all currently approved aP formulations are administered IM. With that, if an IN vaccine were to be implemented into the current vaccine schedule, that vaccine would be building off of previously established immunity. Therefore, the utilization of an IN vaccine would create a systemic-mucosal “push-pull” strategy: the IM prime pushes out systemic immunoglobulin and T/B cell responses, and the IN boost pulls these induces responses to the site of vaccination (Figure 1). Push-pull strategies have proved beneficial in the study of SARS-CoV-2, as a study by Li et al. demonstrated that mice primed IM then boosted IN had higher levels of IgG and IgA antibodies as well as higher T cell responses when compared to mice only vaccinated IM with the same formulation (35). Additionally, in minipigs challenged with C. trachomatis, those vaccinated with the IM-IN push-pull strategy induced a strong mucosal IgA response, which corresponded to increased bacterial clearance, when compared to minipigs only vaccinated IM (36). With this in mind, we hypothesized that mice primed IM and boosted IN would have increased mucosal immune responses, increased bacterial clearance, and increased serological responses after B. pertussis challenge when compared to mice only vaccinated IM.

In this study, we aimed to determine the effect of combining two different vaccine aP vaccine routes in protecting against a B. pertussis infection. To do this, female CD-1 mice were primed IM with either DTaP alone or DTaP combined with a novel adjuvant (BECC438b or IRI-1501). BECC438b is a TLR4 agonist, while IRI-1501 is a β-glucan that can induce a Th1/Th17 immune response (21,37). The mice were then boosted with the same formulation IN, and the resulting immune responses were determined after B. pertussis challenge. We determined that the combination of two different vaccination routes improved upon bacterial clearance in the lower
respiratory tract. Further, we observed an increase in both systemic and mucosal antibody production as a result of the push-pull strategy, particularly when the IRI-1501 adjuvant was used. Finally, the push-pull strategy combined with the BECC438b adjuvant increased the production of a biomarker, CXCL-13, involved in germinal center formation. This study builds upon previous work that demonstrated the efficacy of an IN pertussis vaccine and how the introduction of an IN booster would fit into the current vaccine schedule.
Figure 1. The immune response resulting from either intramuscular or intranasal immunization. Intramuscular vaccination deposits vaccine contents into the muscle, which are then taken up by resident antigen presenting cells. These APCs then migrate to the draining lymph nodes where they present antigen to naïve T cells. The T cells then present antigen to B cell receptors, which results in the formation of both memory B cells and antigen specific plasma cells. The plasma cells produce antibody (IgG, IgM), which goes into the general circulation. Plasma cells migrate to the bone marrow and, upon cytokine stimulation, become long-lived plasma cells. Intranasal vaccination deposits vaccine contents into the nares, which contains mucosal associated lymphoid tissues (MALT). The MALT contains M cells which take up vaccine antigen and present it to APCs within the local immune follicle. Within the follicle, APCs present antigen to T cells, which go on to activate B cells. The B cells can become either memory B cells or plasma cells. The plasma cells produce IgA which will be transferred to the surface of the respiratory epithelium to provide localized protection. Antigen specific T and B cells will travel to draining lymph nodes, allowing for antibody responses to enter the systemic circulation. T cells, upon stimulation with cytokines, undergo a change in surface molecules and become T resident memory cells (TRMs). TRMs will remain localized at the site of antigen deposit (in this case, the upper and lower respiratory tracts). The result is both local and systemic immunity.
4.3 Results

Combining two vaccination routes improves bacterial clearance within the lower respiratory tract

One issue surrounding current aP formulations is their inability to provide sterilizing immunity within the nasal cavity (38,39). As a result, bacteria remains within the nares after one is infected with *B. pertussis*, allowing for transmission of the pathogen (40). With this in mind, we aimed to evaluate the ability of the IM prime/IN boost vaccination strategy to reduce bacterial burden within the respiratory tract. Female CD-1 mice were primed IM then boosted IN 21 days later with the formulations detailed in Supplementary Figure 1. Then, mice were challenged with *B. pertussis*, and three days after challenge, the mice were processed. Within the nasal lavage, the bacterial burden for all vaccine groups was significantly reduced when compared to the MVC mice (Fig. 2A). There is 0.44-fold increase in the number of viable bacteria within the IM DTaP/IN DTaP vaccinated mice when compared to the IM DTaP/IM DTaP group; however, when the BECC438b and IRI-1501 adjuvants were added to the push-pull formulations, the number of bacteria within the nasal lavage was reduced when compared to IM DTaP/IM DTaP mice by 0.3- and 0.65-fold, respectively (Fig. 2A). Given that our model of *B. pertussis* infection induces pneumonia, we aimed to enumerate the bacterial burden within the lungs and trachea in order to assess the ability of this novel vaccination strategy to protect in the lower respiratory tract. As was seen in the nasal lavage, all vaccinated mice had a significant reduction in bacterial burden when compared to MVC mice (Fig.2B). While the IM DTaP/IM DTaP vaccination strategy resulted in an 80-fold reduction in bacterial burden when compared to MVC mice, all IM/IN vaccinated mice had a 100-fold reduction in bacterial burden (Fig. 2B). Further, when compared to IM DTaP/IM DTaP vaccinated mice, the IM/IN DTaP (95-fold reduction), IM/IN DTaP + BECC438b (100-fold reduction), and
IM/IN DTaP + IRI-1501 (100-fold reduction) groups all had increased bacterial clearance (Fig. 2B).

**Figure 2. Enumeration of bacterial burden within both the upper and lower respiratory tract at 3 days post *B. pertussis* challenge.** CFUs were quantified within the nasal lavage (A) and lung/trachea (B). Data presented as geometric means ± SD, n = 10 per treatment group. Exceptions to this are the IM/IN DTaP + IRI-1501 vaccinated mice (n =5). A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment groups. #p < 0.05, ##p < 0.01, ####p < 0.001. A one-way ANOVA was also performed to determine statistically significant differences between IM/IM DTaP vaccinated mice and all other treatment groups. **p < 0.01. CFUs = colony forming units, MVC = mock vaccinated, challenged. IM = intramuscular. IN = intranasal.

**Utilization of a β-glucan adjuvant improves serological responses generated by the push-pull vaccination strategy**

We next evaluated the ability of the push-pull vaccination strategy to induce serological responses to antigens included in the current aP formulation. While cell-mediated immunity is important in
the clearance of \textit{B. pertussis}, humoral immune responses are also necessary for tagging the pathogen for phagocytosis as well as preventing adherence to the respiratory epithelium (27,41,42). IgG antibody levels were determined in the sera for both pertussis toxin (PT) (a crucial virulence factor for \textit{B. pertussis}) as well as the whole \textit{B. pertussis} bacterium (captures antigens located on the surface of the pathogen). When compared to MVC mice, only the IM/IN IRI-1501 vaccinated mice had a statistically significant increase (4197-fold) in serum anti-\textit{Bp} IgG antibody levels (Fig. 3A). While not a significant increase when compared to MVC mice, the IM/IM DTaP group, the IM/IN DTaP group, and the IM/IN DTaP +BECC438b group did have increases in anti-\textit{Bp} IgG with a 972-, 1074- and 966-fold increase in titers, respectively (Fig. 3A). Further, when compared to IM/IM DTaP-vaccinated mice, excluding the mice vaccinated with IRI-1501, the other push-pull groups had very similar average levels of anti-\textit{Bp} IgG in the sera (Fig. 3A). When observing levels of anti-PT IgG in the sera, the groups of mice that were vaccinated either with IM/IM DTaP (543-fold) or IM/IN DTaP + IRI-1501 (1689-fold) had a statistically significant increase when compared to the MVC group (Fig. 3B). Again, it was the IM/IN DTaP + IRI-1501 vaccinated mice that had a statistically significant increase in anti-PT IgG levels when compared to mice vaccinated with IM/IM DTaP (Fig. 3B). Overall, serological responses were similar between mice vaccinated only IM or mice that were vaccinated with the push-pull strategy; however, the utilization of the push-pull strategy, in combination with the IRI-1501 adjuvant, allowed for a more robust serological response to \textit{B. pertussis} antigens.
Mucosal immune responses can be induced by only one intranasal vaccination but not intramuscular immunization alone

A major benefit of intranasal vaccination is its ability to induce mucosal immune responses, which includes the generation of sIgA (29,43). The IgA can bind to the *B. pertussis* bacterium, preventing its adhesion and neutralizing released toxins (43). IgA levels were measured in both the nasal lavage and lung/trachea in order to determine the capacity of the push-pull strategy to induce...
mucosal immune responses. Similar to what was seen with IgG antibody responses, it was only the IM/IN DTaP + IRI-1501 vaccinated mice that had a significant increase (41-fold) in anti-\textit{Bp} IgA antibody levels in the nasal lavage when compared to MVC mice and IM/IM DTaP vaccinated mice (Fig. 4A). While not statistically significant, the IM/IN DTaP and IM/IN DTaP + BECC438b groups did have an increase in nasal lavage IgA antibodies when compared to either MVC mice or IM/IM DTaP vaccinated mice with 3.6- and 4.7-fold increases, respectively (Fig. 4A). When IgA titers were quantified in the pulmonary supernatant, the IM/IN DTaP + BECC438b push-pull group had a statistically significant increase when compared to both MVC mice and IM/IM DTaP mice (Fig. 4B). The other push-pull strategy formulations afforded negligible, if any, increases in anti-\textit{Bp} IgA titers within the pulmonary supernatant (Fig. 4B). Unsurprisingly, the mice vaccinated only IM had no quantifiable IgA antibodies, but some push-pull strategies were able to induce robust mucosal responses. Thus, it seems that just one IN vaccine could be sufficient in inducing localized protection within the respiratory tract.
Mucosal vaccination reduces blood immune cell counts to levels lower or on par with intramuscular vaccination only.

Once *B. pertussis* enters into the respiratory tract, it releases a number of virulence factors, including PT. Upon the release of PT, the recruitment of neutrophils to the airways is prevented as is extravasation of lymphocytes across the endothelium, which causes lymphocytosis (44–46).
Extreme lymphocytosis is considered to be an underlying mechanism behind cases of fatal pertussis (1,47). As such, we wanted to evaluate the extent to which the push-pull vaccination strategy could prevent the accumulation of lymphocytes in the blood. When compared to MVC mice, both IM/IM DTaP vaccinated mice as well as mice vaccinated with IM/IN DTaP + IRI-1501 had a statistically significant reduction in the number of neutrophils in the blood (Fig. 5A). None of the push-pull groups had a significant reduction in blood neutrophils when compared to IM/IM DTaP vaccinated mice, but it is important to note that they did not result in a significant increase in this cell population either (Fig. 5A). When quantifying white blood cells (WBCs) in the blood, no vaccination group was statistically different from MVC mice (Fig. 5B). However, when comparing to IM/IM DTaP vaccinated mice, one push-pull group – IM/IN DTaP + BECC438b – had a statistically significant increase in WBCs in the blood (Fig. 5B). This is expected, as BECC438b is a TLR4 agonist and, therefore, causes some extent of inflammation. Analysis of blood lymphocytes revealed that when compared to either the MVC mice or IM/IM DTaP vaccinated mice, only the mice vaccinated with IM/IN DTaP + BECC438b had a significant increase in lymphocytes (Fig. 5C). For blood monocyte levels, no statistical differences were observed between the push-pull strategies and either MVC mice or IM/IM DTaP vaccinated mice (Fig. 5D). To summarize, most of the push-pull strategies resulted in either a reduction in immune cells in the blood when compared to MVC mice or were not significantly different from IM/IM DTaP vaccinated mice. The exception to this is the vaccine that contained the lipid A mimetic, BECC438b, which induced additional inflammation per its effects as an adjuvant (37).
Figure 5. A complete blood cell with differential was performed on whole blood at 3 days post B. pertussis challenge. The number of neutrophils (A), white blood cells (B), lymphocytes (C), and monocytes (D) was determined from whole blood samples. Data presented as geometric means ± SD, n = 10 per treatment group. Exceptions to this are the IM/IN DTaP + IRI-1501 vaccinated mice (n=5). A one-way ANOVA was performed to determine statistically significant differences MVC mice and all other treatment groups. \#p < 0.05, \##p < 0.01, \###p < 0.001. A one-way ANOVA was also performed to determine statistically significant differences between IM/IM DTaP vaccinated mice and all other treatment groups. *p < 0.05, **p < 0.01. WBC = white blood cells. NVNC = not vaccinated, not challenged. MVC = mock vaccinated, challenged. IM = intramuscular. IN = intranasal.
Combining the push-pull vaccination strategy with a TLR4 agonist increases the production of indicators of long-term immunity

As the main reason for the recent resurgence in pertussis cases is the short duration of protection afforded by aP formulations, it is important to assess the longevity of immunity induced by our novel push-pull strategy (14,48). In lieu of a long-term study, we quantified plasma cells in the bone marrow that produced *B. pertussis*-specific IgG via an ELISpot assay, as these are likely long-lived plasma cells. Further, we also enumerated the amount of the chemokine, CXCL-13, in the lung, as this molecule is involved in the germinal center reaction, which is important in the production of highly specific, long-lived antibodies. To start, all vaccine groups, except for the IM/IN DTaP mice, had a significant increase in spots when compared to MVC mice (Fig. 6A). The largest increase in spots was seen in the IM/IM DTaP vaccinated mice; however, the IM/IN DTaP + BEC438b and IM/IN DTaP + IRI-1501 groups were not statistically different from the mice vaccinated only via IM (Fig. 6A). When CXCL-13 was quantified in the pulmonary supernatant, it was only the IM/IN DTaP + BECC438b push-pull group that had a statistically significant increase in the chemokine when compared to MVC and IM/IM DTaP mice (Fig. 6B). In terms of averages, all of the other groups were similar in terms of the amount of CXCL-13 in the lower respiratory tract and no statistical differences were observed (Fig. 6B).
4.4 Discussion

There has been a resurgence of pertussis cases in the last several years, which has been attributed to the waning immunity associated with current aP formulations (6,41,49). Many studies have been devoted to improving the vaccine formulations, such that cell-mediated immune responses, rather than humoral responses, are induced, as these are the responses associated with clearance and long-term protection (19,41,50). Given that B. pertussis is a respiratory pathogen that adheres to the
respiratory epithelium in order to begin its process of infection, it is reasonable to suggest that an intranasally administered vaccine could prove beneficial. In this study, we evaluated the implementation of an IN aP vaccine as a booster formulation. As all currently approved pertussis vaccines are administered IM, if an IN formulation were to be introduced into the current vaccination schedule, it is essential to understand how these two routes would interact. That is, we evaluated a systemic-mucosal (IM/IN) push-pull vaccination strategy. Additionally, we utilized adjuvants that our lab has shown to be effective when given IN to determine if they could improve responses (21).

*B. pertussis* is a strictly mucosal pathogen, as such, the induction of mucosal immune responses is an important aspect in protection (43). Many within the pertussis field have highlighted the importance of cell-mediated immunity in recovery and clearance of the pathogen but few have mentioned the role that humoral responses, particularly mucosal antibodies, play in immunity. In humans naturally infected with *B. pertussis*, anti-*Bp* IgA was detected within nasal secretions approximately 2 weeks after infection and systemic immunization did not give the induction of IgA responses (51). In addition to preventing the pathogen from binding to the ciliated respiratory epithelium, there is evidence that IgA can induce FCαRI-mediated binding, phagocytosis, and killing by human myeloid cells (52,53). Our study demonstrated that just one IN vaccine was able to induce IgA antibody responses, whereas, IM vaccination only induced none (Fig. 3). Interestingly, more IgA was generated in the upper respiratory tract (nasal lavage) when compared to the lower respiratory tract (pulmonary supernatant), which falls into line with the knowledge that *B. pertussis* mostly remains in the upper respiratory tract unless the infection is severe (43). Regardless, certain push-pull strategies were able to generate IgA specific to *B. pertussis*
throughout the respiratory tract. The ability of IRI-1501 to induce high nasal lavage IgA titers can be explained by the inherent ability of β-glucans to induce Th17 polarized responses, as IL-17 has been shown to be important in the production of slgA in mice (43).

Intramuscular vaccination is known to induce systemic protection, which is characterized by the production of both IgM and IgG antibodies within the sera. In regard to pertussis infections, while there is no confirmed correlate of protection, it has been established that anti-PT IgG levels greater than 5 IU/mL are considered to be protective (54,55). Our study established that the push-pull vaccination strategy was able to induce anti-PT IgG antibodies to levels that were similar to strictly IM vaccination (Fig. 2). Further, the use of the IRI-1501 adjuvant dramatically increased the level of anti-PT IgG antibodies above what was seen in all other experimental groups. Previous studies by our lab illustrated the ability of IRI-1501 to induce high levels of systemic antibody titers against both the whole pertussis bacterium as well as pertussis antigens when administered IN (21). These data supports the ability of mucosal vaccines to induce systemic immune responses; therefore, the substitution of an IM booster for an IN administered vaccine would impede the induction of essential humoral responses.

As previously mentioned, cell-mediated immunity has been shown to be essential for the clearance of *B. pertussis* from the respiratory tract (41,50). However, our bacterial burden data demonstrates that IN vaccination is able to reduce bacterial burden to almost negligible levels in both the upper and lower respiratory tracts (Fig. 1). In fact, within the lung and trachea – with the exception of the IM/IN DTaP + IRI-1501 mice – each push-pull group had mice that were at the limit of detection for bacterial burden. That is, they had achieved sterilizing immunity within the lower
respiratory tract. This was not the case for mice that only received IM vaccinations. These results are important, as none of the current aP vaccine formulations induce sterilizing immunity; thus, these results highlight the role of the IN booster.

Finally, we evaluated potential indicators of long-term immunity provided by our experimental vaccination strategies. First, we performed ELISpots on the bone marrow of vaccinated mice to quantify the number of B cells that produced IgG specific to *B. pertussis*. In adults, high levels of antigen-specific memory B cells are correlated to protection against pertussis infections, as these cell populations lead to a rapid production of pathogen-specific IgG and IgA (55). Overall, we found that all of the vaccine groups (except for the IM/IN DTaP mice) had similar spot counts, indicating similar levels of antibody secreting cells (Fig. 5). In fact the IM/IN DTaP mice had a significant reduction in spots when compared to the IM DTaP vaccinated counterparts, which could indicate that the IN vaccine, alone, is not enough to stimulate the formation of antigen-specific B memory cells. Rather, the aP formulation needs to be adjuvanted, such that more robust humoral responses can occur. When we measured the amount of CXCL-13 in the lungs, we observed that the IM/IN DTaP + BECC438b mice had a significant increase in chemokine levels when compared to either MVC mice or IM/IM DTaP mice (Fig. 5). CXCL-13 is a biomarker for the germinal center reaction, which is a process in which B cells undergo somatic hypermutation of their B cell receptor (56). Somatic hypermutation is essential for the production of antibodies that are highly specific for a particular pathogen as well as the production of long-lived plasma cells (57). It is surprising to see that the number of spots does not seem to correlate to the levels of CXCL-13 in the lung/trachea, as these processes are intertwined. Nevertheless, it is promising to see that the IM/IN DTaP + BECC438b vaccination strategy produced large levels of CXCL-13.
This result is most likely due to the immunogenic nature of BECC438b, as it is a TLR4 agonist, and the activation of TLR pathways in follicular dendritic cells promotes the survival of germinal center B cells (58).

The results of this study show promise, but there are some limitations that need to be addressed. To begin, the selected endpoints were limited, as studies initially began with utilizing the IRI-1501 adjuvant, but data prompted the switch to the BECC438b adjuvant. The switch occurred before more data could be collected with IRI-1501, such as antibodies specific to additional *B. pertussis* antigens, T cell analyses, histological analysis, cytokine quantification, and RNAseq analysis. Further, these changes resulted in each vaccine group having an N of 10, while the vaccine containing IRI-1501 had an N = 5, which could have skewed the statistical analyses. Additionally, the only vaccine control group was the IM/IM DTaP vaccinated mice, which served as a comparison point for all experimental vaccines. In order to properly understand how the push-pull strategy improves immunity, studies should have been performed in which vaccines containing the novel adjuvants should have been administered IM only. This would have given us the ability to compare the push-pull strategy for each formulation to its respective IM only counterpart. Because of this, the extent to which we can attribute the efficacy of the vaccines to the IN booster, itself, is limited. Also, many studies that evaluate novel pertussis formulations compare to immunity afforded by wP vaccines, as these provide more robust immune responses when compared to aP vaccines. In order to understand how this push-pull strategy potentially improve responses against *B. pertussis*, these studies should have included a group in which mice were vaccinated with wP. Finally, we only studied parameters that can be attributed to long-term protection, such as antibody secreting cells and CXCL-13. While these data are valuable, the only way to determine the duration
of protection afforded by these vaccination strategies is to perform long-term studies such that we can see if protection is maintained over time.

To conclude, this study demonstrates the importance of IN vaccination and its ability to induce protective immune responses against *B. pertussis*. We observed that systemic immune responses can still occur when an IM booster is replaced with once administered IN. Further, only IN delivered vaccines were capable of eliciting both mucosal immune responses and sterilizing immunity in the respiratory tract. There is still much work to be done before clinical studies that implement an IN booster can occur, but we believe that this preliminary work illustrates that an IN vaccine could enhance the existing immune responses provided by the formulations currently given IM.
Supplemental Figure 1. A depiction of the experimental timeline and vaccination groups. Mice are primed (d0) and boosted 21 days later (d21). At 35 days post-primary vaccination (d35) mice were intranasally challenged with *B. pertussis*. Three days after challenge (d3 p.c.) mice were processed and assays were performed with the collected materials.
4.5 Materials and Methods

Vaccine and adjuvant composition. The Infanrix formulation of DTaP was used and diluted to 1/40th the human dose (12.5µl vaccine diluted to 50µl total volume administered in 0.9% saline) and prepared no longer than 1 h before administration. BECC438b was reconstituted using endotoxin free water. After reconstitution, BECC438b (50 µg per dose) was combined with DTaP, and the mixture was turned end-over-end for 2 hours. IRI-1501 (Immuno Research, Inc.) was prepared with the base vaccine using 100mg per administration. Vaccine antigens (1.25mg of genetically detoxified pertussis toxin [gPT] [catalog number 184; List Biologicals], 1.25mg of filamentous hemagglutinin [FHA] [Enzo Life Sciences], and 0.8mg of pertactin [PRN] [catalog number 187; List Biologicals]) were diluted with endotoxin-free Dulbecco’s phosphate-buffered saline (PBS) (Millipore Sigma) to make the base vaccine (aP).

Vaccine administration. CD-1 (outbred; strain code 022) mice aged four to five weeks were obtained from Charles River Laboratories. Mice were administered 50 µL of vaccine or vehicle control intramuscularly (IM) in the right thigh. For intranasal vaccines, mice were anesthetized with ketamine/xylazine (77 mg/kg of body weight of ketamine and 7.7 mg/kg of xylazine) and 25 µL of the vaccine was administered into each nostril. Mice were boosted with the same vaccine formulations 21 days after priming. All murine infection experiments were performed according to protocols approved by the West Virginia University Animal Care and Use Committee (protocol numbers:1602000797 and 1602000797_R1).

B. pertussis strains and growth conditions. B. pertussis strain UT25Sm1 was used for murine challenge. The B. pertussis strain has been fully genome sequenced (UT25Sm1 NCBI Reference Sequence: NZ_CP015771.1. UT25 was originally isolated in Texas in 1977 (59). UT25Sm1 was
cultured on Bordet Gengou agar (Difco) plus 15% defibrinated sheep’s blood (Hemostat Laboratories) with streptomycin 100 µg/mL. *B. pertussis* was incubated at 36°C for 48 h, then transferred to modified Stainer-Scholte liquid medium. SSM liquid cultures were incubated for 24 h at 36°C, with shaking at 180 rpm until reaching an OD of ~0.6, at which time cultures were diluted for the challenge dose. For challenge with a liquid inoculum *B. pertussis*, the mice were anesthetized with 77 mg/kg of body weight of ketamine and 7.7 mg/kg of xylazine inoculated with 20 µL (10 µL per nostril) of the challenge dose (2 × 10⁷ CFU).

**Vaccine challenge model.** At thirty-five days post prime, mice were challenged with IN 2×10⁷ CFUs of *B. pertussis* (10 µL per nostril or nebulized challenge). At day 3 post-challenge, mice were euthanized, and blood was collected by cardiac puncture. A complete blood cell count with differential was performed using a ProCyte Dx hematology analyzer (IDEXX) and serum was separated by centrifugation through a BD Microtainer blood collector and stored at -80°C until analysis. The trachea and lungs were removed and homogenized together. Lungs and trachea were suspended in 2 mL of sterile PBS in gentleMACS C tubes (Miltenyi; Cat. Number: 130-096-334) using a GentleMACS Octo Dissociator with Heaters (Miltenyi) using the m_lung_02 setting. The lung/trachea homogenates were used for CFU plating, and the remaining sample was centrifuged at 14,000 x g for 4 mins and supernatants were stored at -80°C until cytokine and antibody analyses were performed. The nasal lavage was collected by flushing 1mL of 1x PBS through the nasal cavity and collecting the wash in an Eppendorf tube. Bacterial burden was determined in the lung/trachea and nasal lavage by colony forming units (CFUs) using serial dilutions. Serial dilutions were done in PBS and then plated on BG containing streptomycin (100 µg/mL) to ensure that only UT25 *B. pertussis* was cultured.
Serological analysis of *B. pertussis* specific antibodies. On the day of mouse processing (euthanasia and dissection), a cardiac puncture was performed on each mouse. Approximately 1 mL of blood was removed from each mouse. The samples were placed into serum separator tubes (Cat. Number: 365967; BD) centrifuged for 2 min at 14,000 x g. Serum was collected and stored at −80°C until analyses were performed. Serological responses specific to *B. pertussis* antigens were quantified by ELISA. High-binding microtiter plates were coated with PT (50 ng/well) (PT#180, LIST Biologicals) as described in Boehm *et al* (22). For serological responses to *B. pertussis*, UT25Sm1 was cultured to an OD$_{600}$ of ~0.6 and diluted down to an OD$_{600}$ of 0.245 and microtiter plates were coated with 50 µL of bacteria per well overnight. After coating, the plates were washed with PBS + 0.05% v/v Tween 20 (Cat. Number: P1379-1L, Sigma-Aldrich) (PBS-T) and blocked with 5% non-fat dry (NFD) milk in PBS-T overnight at 4°C. Blocked plates were washed with PBS-T and then serum (1:50) or lung supernatant (1:4) samples were prepared in 5% NFD milk in PBS-T. All samples were serially diluted (1:2). After 2 h incubation at 37°C, plates were washed and incubated with goat anti-mouse-IgG alkaline-phosphatase conjugated antibodies (IgG:1030-04, Southern Biotech) (1:2,000) for 1 h at 37°C. Plates were then washed and incubated with Pierce p-Nitrophenyl Phosphate (PNPP) (Cat. Number: 37620; Thermo Fisher Scientific) following the manufacturer’s instructions. The absorbance of the plates was read at OD$_{405}$ using a Synergy H1 plate reader (BioTek). Positive antibody titers were determined as any values above the baseline (set at two times the average of blanks).

Analysis of mucosal IgA antibodies. On the day of processing, the nasal lavage and lung/trachea supernatants were collected. The nasal lavage was stored at -80°C until analysis and the
lung/trachea homogenates were centrifuged at 15,000 g x 3 minutes. Upon completion of centrifugation, the supernatants of the lung and trachea were collected and transferred to new Eppendorf tubes and stored at -80°C until analysis. For analysis of IgA antibodies, UT25Sm1 was cultured to an OD$_{600}$ of ~0.6 and diluted down to an OD$_{600}$ of 0.245 and microtiter plates were coated with 50 µL of bacteria per well and incubated overnight. After coating, the plates were washed with PBS + 0.05% v/v Tween 20 (Cat. Number: P1379-1L, Sigma-Aldrich) (PBS-T) and blocked with 5% non-fat dry (NFD) milk in PBS-T overnight at 4°C. Blocked plates were washed with PBS-T and then nasal lavage (no initial dilution) or lung supernatant (1:4) samples were prepared in 5% NFD milk in PBS-T. All samples were serially diluted (1:2) down the plate. After 2 h incubation at 37°C, plates were washed and incubated with goat anti-mouse IgA heavy chain secondary antibody [HRP] (Cat. #: NB7504, Novus Biologicals) (1:4,000) for 1 h at 37°C. The plates were then washed and incubated with TMB substrate (Cat. #421101, BioLegend). The absorbance of the plates were read at OD$_{605}$ using the Synergy H1 plate reader mentioned above. Positive antibody titers were determined as any values above the baseline (set at two times the average of blanks).

**Analysis CXCL-13 lung and trachea supernatant.** To quantify inflammatory cytokines at the site of infection, lung/trachea homogenate supernatants were prepared, as suggested by the kit manufacturer, and diluted 1:2. Samples were run on a Magpix (Luminex) instrument. Bead counts below 35 were invalidated.

**ELISpot sample preparation and analysis.** ELISpot assays were performed using a mouse IgA/IgG double-color ELISpot assay (ImmunoSpot) to enumerate the number of B cells that
produced IgA and IgG antibodies specific to *B. pertussis*. UT25Sm1 was cultured and diluted as described above for the ELISA protocol. Polyvinylidene difluoride (PVDF) membrane 96-well plates were coated with UT25Sm1 and incubated overnight at 4°C. Bone marrow cells were isolated from long-term study groups after challenge. Both hind femurs from each mouse were removed and placed into tubes containing Dulbecco’s modified Eagle’s medium (DMEM) (Gibco). The tubes were spun at 1,000 x g for 2 min, and the resulting pellet was resuspended in heat-inactivated (HI), filter-sterilized FBS (Gemini Bio Products). The cells were then filtered through a 70-mm filter and placed in a solution of HI FBS plus 10% (vol/vol) dimethyl sulfoxide (DMSO) and stored at 280°C until the assay was performed. To prepare the cells for the assay, they were first thawed in a 37°C water bath and transferred into a solution of RPMI 1640 (Gibco) plus 10% (vol/vol) HI FBS. The cells were then centrifuged at 350 x g for 6 min, resuspended in CTL test B culture medium (ImmunoSpot), and counted. After coating, the plate was washed with PBS, prior to adding cells. Four serial dilutions were used for each sample (6.25x10^6, 1.25x10^6, 3.13x10^5, and 1.56x10^5 cells/well). Once cells were added, the plate was left to incubate at 37°C overnight. ELISpot plates were imaged and counted with the ImmunoSpot S6 Entry analyzer and CTL software. The counts were analyzed, and a dilution was selected that had spot counts in the range of 10 to 100 per well.

**Statistical analysis.** Statistical analyses were performed using Prism version 9.0 software (GraphPad). Comparisons between three or more groups were performed via a one-way ANOVA.

**Ethics statement.** Animal work in this study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National
Institutes of Health (60). The West Virginia University Institutional Animal Care and Use Committee (IACUC) approved the protocols and this research under the IACUC protocol 1602000797_R1. The West Virginia University Office of Lab Animal Resources is an AAALAC International accredited facility, which is a voluntary accrediting organization. All lab work with *Bordetella pertussis*, including *in vitro* work, infection, *in vivo* studies, and pathogen culture, were completed under an approved IBC (Institutional Biosafety Committee) protocol (#17-01-11).

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Chapter 5

Discussion
5.1 The Big Picture
Chances are, if you are reading this dissertation, you have been vaccinated against *B. pertussis*. Regardless of whether you received the whole cell (wP) or acellular (aP) formulations, the odds that you have ever had pertussis are small. The breadth of *B. pertussis* research has allowed for the development of effective vaccine formulations that have worked to reduce both incidence and deaths. However, with this research, we have gained insight into the shortcomings of aP vaccine formulations and how said shortcomings are contributing to the recent resurgence of pertussis cases. Indeed, as the duration of protection provided by aP vaccines begins to wane, previously vaccinated individuals can become infected with *B. pertussis*. Those infected can exhibit symptoms or act as asymptomatic carriers, unknowingly passing the pathogen onto more susceptible populations, such as those under the age of 1, which is the demographic that is most likely to die as a result of a *B. pertussis* infection. The goal of this body of work was to determine how aP booster formulations could be improved, whether that be via the use of new adjuvants and/or new administration routes, in the hopes that the knowledge gained from these studies could contribute to the next generation of pertussis vaccines.

5.2 Adjuvant studies: Is alum the problem?
There is ample evidence supporting the waning immunity of aP formulations (1–3). Recent studies have demonstrated that the protective immunity induced by a series of five DTaP immunizations quickly wanes, with the odds of getting infected with *B. pertussis* increasing 42% each year post vaccination (Figure 1) (2). Further, after being boosted with Tdap, a large portion of adolescents tested positive in the years following vaccination, as little protective immune responses were still present (1). The alum adjuvant is often said to be the major factor contributing to the inability of aPs to induce longer durations of protection (4). It is well known that alum adjuvants induce Th2
polarized immune responses, giving way to generation of humoral (antibody) responses (5–7). Th2 immune responses certainly provide protection against large extracellular pathogens that cannot be phagocytosed, such as helminths; but in the case of pertussis infections, cell-mediated responses are the most beneficial in terms of clearance and long-term protection (4,8–10). With this information in mind, we aimed to improve upon the aP induced responses by implementing new adjuvants to work alongside alum.

Figure 1. The surveillance of pertussis cases from 2012-2021 in the United States. Adapted from CDC surveillance data. Panel A depicts the percent of pertussis cases per age group (bar graph) as well as the percentage of cases that result in hospitalization per age group (line graph). Panel B illustrates the proportion of cases per age group in an unvaccinated population. Panel C illustrates the proportion of cases per age group in a completely vaccinated population (received the complete DTaP series).
Chapter 2 details a study that utilized the CpG 1018 adjuvant which, in other pre-clinical studies, demonstrated a better response to *B. pertussis* vaccine antigens when combined with alum, when compared to using CpG 1018 by itself. The first objective of the study was to determine the dose of Tdap that would allow improvements afforded by the inclusion of the CpG 1018 adjuvant to be observed (Chapter 2). A previous study performed by our lab titrated DTaP in order to find the sub-optimal such that the improvements of including the ACT antigen could be determined, and we opted to move forward with similar vaccine doses (11). At 1/20th the human dose of Tdap, we observed that the inclusion of CpG 1018 decreased bacterial burden within the respiratory tract and increased serological responses to *B. pertussis* antigens (Chapter 2, Figures 1 and 2). We then moved forward in a time-course study, which utilized the 1/20th dose of Tdap, such that we could evaluate the immune responses that occur over time after *B. pertussis* challenge. Surprisingly, at days 1-, 3-, and 7-post challenge, the Tdap + CpG 1018 vaccine formulation had reduced bacterial burden within the lung and trachea beyond that of the DTP control (Chapter 2, Figure 3). Further, this study evaluated the ability of the combination adjuvant formulation to protect against two different strains of *B. pertussis*: one that expressed PRN and one that did not. We felt this was important, as vaccine-driven evolution – whereby strains evolve to no longer express antigens included in vaccine formulations – is considered another factor in the waning efficacy of aP vaccines. The combination of Tdap + CpG 1018 was able to reduce bacterial burden within the respiratory tract regardless of the challenge strain, and this novel vaccine formulation performed similarly in this regard to DTP (Chapter 2, Figure 4). This data is exciting, as DTP has long been considered to induce responses on par with that of a natural infection but at the cost of deleterious inflammatory responses. With the combination of Tdap + CpG 1018, bacterial clearance exceed that of DTP but the mice did not have a large influx of inflammatory cells into the lungs. These
data suggest that alum does not have to be a hinderance to immunity against *B. pertussis*, but rather, it can work in conjunction with other adjuvants to induce superior protective responses. Further, alum’s Th2 polarization will not necessarily impede the ability of adjuvants to elicit strong cell-mediated immunity.

In chapter 3, we evaluated the ability of another adjuvant, BECC438b, to work in concert with alum to induce protective responses. As BECC438b is a TLR4 agonist, we suspected that it would activate biological processes similarly to the LOS of *B. pertussis*; however, because of the changes in the lipid A structure, the inflammation would not be as prominent. The addition of BECC438b to DTaP decreased bacterial burden within the respiratory tract as well as result in improvements to serological IgG responses. However, the more prominent changes occurred at the genetic level, as the BECC438b adjuvant changed the downstream activation of biological pathways such that genes involved in the activation and regulation of the immune system were activated. The utilization of both alum and BECC438b in a vaccine is akin to AS04, an adjuvant system that is currently incorporated into FDA-approved vaccines (12,13). AS04 is a combination of MPL and aluminum hydroxide, and pre-clinical studies demonstrated that alum neither synergized nor inhibited the functionality of MPL (13). Rather, alum’s depot effect prolonged cytokine responses at the site of injection, allowing for a more prominent activation of APCs (14). Similarly, this is likely the mechanism by which alum and BECC438b work together. With these data in mind, it seems that alum does not necessarily have to be a hinderance to the efficacy of aP vaccine formulations. Instead, alum can work in concert with new adjuvants and allow for the prolongation of the adjuvants local effects, increasing antigen presentation and, thus, the activation of T cells
within the draining lymph nodes. Rather than creating a new formulation that does not utilize alum, the next generation of aP vaccines could build upon its adjuvanticity.

5.3 The importance of intranasal vaccination
While addressing the formulation of aP vaccines certainly paves the way for improved responses against *B. pertussis*, the intramuscular administration of aPs is another limitation that should be addressed. We previously demonstrated that intranasal administration of DTaP – either on its own or with the inclusion of a β-glucan adjuvant – could elicit mucosal immune responses while still inducing systemic protection (15,16). While we were investigating the BECC438b adjuvant, we aimed to determine if it could increase the protection afforded by an intranasal vaccine, as it was capable of improving immune responses over DTaP alone when administered intramuscularly. The work detailed in Chapter 3 supports the importance of intranasal vaccination in reducing the amount of bacteria within the respiratory tract, particularly in the lung and trachea. Further, the addition of BECC438b to the intranasal formulation reduced bacterial burden even further, with some mice achieving sterilizing immunity (CFUs at the limit of detection). The importance of vaccine route was again highlighted when pre-challenge IgG antibody isotypes were enumerated in the sera, as Th2 polarized immune responses were dominant until the BECC438b adjuvant was combined with intranasal vaccination. We hypothesize that presentation of the BECC438b adjuvant to the nasal associated lymphoid tissue within the nasal cavity allowed for an increased production of cytokines associated with TLR4 activation, such as TNF-α, IFN-γ, and IL-12 (17,18) (Figure 2). Further, when RNAseq was used to identify difference in gene expression amongst different vaccines and routes of administration, intranasal vaccination had the highest activation of immunoglobulin associated genes. In particular, we noted that the total number of genes were increased for *Igh* and *Igk*, which demonstrates an increase in both heavy and light immunoglobulin
chains upon intranasal administration of either DTaP alone or DTaP + BECC438b. Again, this suggest that the role of the nasal associated lymphoid tissue, perhaps via increased antigen presentation or via increased cytokine stimulation, is inducing increased germinal center reactions, which would result in the production of more antibody variants.

**Figure 2. The proposed mechanism of the BECC438b adjuvant when administered intranasally.** The DTaP + BECC438b formulation contains both alum as well as the novel lipid A mimetic. When the vaccine is administered into the nasal cavity, microfold (M) cells take up vaccine components and present them to antigen presenting cells (APCs). The depot effect provided by the alum adjuvant allows for a prolonged period of antigen presentation, priming more APCs. This results in increased presentation to T cells and, thus, induction of the germinal center reaction. Further, the binding of BECC438b to TLR4 induces a Th1 polarized immune response and the depot effect of alum combined with the increased number of APCs within the nasal associated lymphoid tissue allowed for an increased release of cytokines associated with the induction of Th1 immunity. The overall effect is an increased activation of APCs and the production of both systemic and mucosal antibodies.
While we have worked to establish the importance of intranasal vaccination, all pertussis vaccines are currently administered into the muscle. With that, we need to determine how an intranasal booster vaccine would interact with the current vaccination schedule. We hypothesized that intramuscular priming followed by an intranasal boost would elicit a systemic-mucosal “push-pull” response, in which the strong systemic immune responses would get pulled into the airway, creating localized protection. The idea of a push-pull vaccination strategy is not novel and has proved to be beneficial in pre-clinical vaccines against influenza, *S. pyrogenes*, and SARS-CoV-2 (19–21). However, this strategy has not been employed into the current pertussis vaccine schedule; therefore, in Chapter 4, we aimed to evaluate the interaction of the two routes and their ability to induce efficacious responses against pertussis vaccine antigens. Additionally, we incorporated new adjuvants into the DTaP formulation to see how they affected the push-pull strategy. In this study, we observed that just one intranasal vaccination was able to improve bacterial clearance in both the upper and lower respiratory tracts and that the induction of systemic immune responses was not affected (Chapter 4, Figures 2 and 3). Further, the intranasal vaccine induced robust mucosal immune responses in both the upper and lower respiratory tracts, particularly when the β-glucan adjuvant (IRI-1501) was used (Chapter 4, Figure 3). The ability to induced localized immune responses is paramount in protecting against *B. pertussis*, as baboon studies have demonstrated that current aP vaccines do not prevent transmission of the pathogen (22). With that, it has been theorized that the remaining nasopharyngeal carriage of *B. pertussis* is contributing to the increased number of cases (23). We know that natural infection with *B. pertussis* induces potent mucosal immune responses and that natural infection gives the longest duration of protection (4). Therefore, intranasal vaccination seems to be a logical, if not essential, step forward in the development of a more efficacious pertussis vaccine.
5.4 The role of humoral immunity in protection against *B. pertussis*

When evaluating the most effective immune responses to combat pertussis infections, most studies focus on the importance of cell-mediated immunity. Indeed, it has been established that, in children, recovery from whooping cough coincides with the activation of Th1 cells that produce IFN-\(\gamma\) (24,25). Both macrophages and dendritic cells activate Th1 cells upon the binding of *B. pertussis* to cell surface receptors, and both IFN-\(\gamma\) and IL-17A induce the killing of the pathogen by macrophages (26,27). Additionally, in a seminal study by Mills *et al*., mice deficient in T cells were unable to clear *B. pertussis* from the respiratory tract after aerosol challenge and that the transfer of spleen cells from wildtype mice resulted in eventual clearance of the pathogen; whereas, when sera from convalescent mice was transferred to the nude mice, there was only a marginal reduction in bacterial loads (10). These studies certainly highlight the role of cell-mediated immunity, but seem to neglect the role that antibodies play in the protective response against *B. pertussis*.

First and foremost, antibodies work to prevent disease. When children with whooping cough were treated with a pertussis-specific immunoglobulin that contained a high dose of anti-toxin, the severity of disease was reduced (28). In particular, there is a strong correlation between high levels of anti-PT serum antibodies and protection against clinical pertussis (29). This is unsurprising because, as mentioned Chapter 1, the fatal cases of pertussis are mediated by the actions of PT and its induction of severe leukocytosis (30,31). Further, in murine studies, the passive transfer of anti-*B. pertussis* serum confers protection in mice, while immunized Ig \(^{-/-}\) mice were unable to clear *B. pertussis* from the respiratory tract (32). In Chapter 3, we performed RNAseq analysis in order to understand the effect that our new aP vaccine formulation had on gene expression. These data demonstrated that the vaccines had the largest effect on gene...
signatures associated an antibody response, such as immunoglobulin heavy and light chains. However, genes associated with T cell activation and regulation, such as Lat, Trat1, Themis, Cd4, and Cd226, did not have large changes in gene expression after vaccination when compared to non-vaccinated, non-challenged mice. Additionally, RNAseq analysis revealed that there were no significant changes in gene expression for genes associated with T-resident memory cells, such as CD103 (Itgae), LFA-1 (Itgal), and VLA-1 (Itga1) (33). Despite this, the addition of the BECC438b adjuvant to DTaP was able to offer a substantial reduction in bacterial burden within the respiratory tract, with some intranasally-vaccinated mice approaching the limit of detection (Chapter 3, Figure 8). These results are similar to another study of ours in which mice were protected from B. pertussis challenge and did not have waning antibody responses against aP vaccine antigens despite the lack of a significant elevation in T-resident memory cells or other T cell responses (16). These data illustrate the importance that humoral immunity plays in protection against B. pertussis, and this is an area of research that warrants further investigation in vaccine efficacy studies.

5.5 Cell-mediated responses are invaluable in combatting B. pertussis infections

Humoral immune responses are important in protecting against B. pertussis infections; however, the main immunological responses of either infection or vaccination are mediated by Th cells (4,34,35). Indeed, both murine and human studies have suggested that the role of Th1 cells are required for clearance of the pathogen from the respiratory tract as well as the induction of a long-lasting duration of protection (10,36). Further, data suggests that, while antibody levels are typically associated with a correlate of protection, in the case of B. pertussis, T cell responses are more indicative of vaccine efficacy (37).
In chapter 2, when titrating Tdap in order to find the sub-optimal dose of the base vaccine, we observed that a Tdap dose of either 1/40th or 1/80th the human dose led to reduced numbers of both anti-PRN, anti-PT, and anti-Bp serum antibodies (Figure 1). However, regardless of the antibody quantity, amongst all Tdap titration doses, the overall bacterial burden levels were similar and still below that of the MVC mice (Figure 2). These data suggest that some aspect of the immune system – other than humoral responses – is playing a role in clearing the pathogen from the NALT, lungs, and trachea. Further, in Chapter 3, RNAseq analysis revealed a marked increase in gene counts for Cd69 in the mice that were vaccinated IN with DTaP + BECC438b (data not shown). This particular cohort of mice, despite having similar serum antibody levels to other groups, had reduced bacterial burden within the lung, trachea, and nasal lavage (Figures 1 and 2). As Cd69 is a marker of T resident memory cells, it stands to reason that the increased bacterial clearance could be due to the localized cell-mediated responses.

Overall, both humoral and cell-mediated immune responses are necessary for protection against B. pertussis: antibodies work to prevent the severity of disease, while T cells work to clear the pathogen and provide long-term protection (4). The chapters in this dissertation worked to highlight the importance of humoral responses, but in doing so, we also revealed the necessity that cell-mediated responses play in protection and how they can compensate for reduced antibody quantities.

5.6 The pathway to a new pertussis vaccine
The aim of this dissertation was to study novel pertussis vaccine formulations and the utilization of different routes in order to determine how the aP vaccine could be improved. These studies concluded that multiple adjuvants, as well as intranasal administration, have the potential improve
the immunogenicity and protection of aP vaccines. But in doing this work, we must ask ourselves: “Is it feasible for these changes to be implemented?” To answer this question, we have to evaluate the scope of either ongoing or recently completed studies of novel pertussis vaccines. The most successful vaccine candidate is that of BPZE1, a live-attenuated strain of *B. pertussis* that is administered intranasally. The attenuated of this strain of *B. pertussis* is due to the deletion of the dermonecrotic toxin encoding gene, its production of an enzymatically inactive PT, and the reduced release of tracheal cytotoxin (34). BPZE1 has made it to phase 2b clinical trials, during which participants will be challenged with *B. pertussis* to determine endpoints associated with protection (ClinicalTrials.gov: NCT05461131). While results of studies have been promising, the use of a live-attenuated vaccine is associated with risks: *B. pertussis* could disseminate in severely immunocompromised individuals or the potential to transmit the attenuated strain to unvaccinated individuals (34,35). Regardless, the success of BPZE1 has led to an appreciation for the introduction of an intranasal vaccine into the current vaccine series.

As previously mentioned, the vaccines tested in both Chapters 2 and 3 still contain the alum adjuvant. Therefore, this negates the need for a complete reformulation of DTaP. CpG 1018 is currently used in a FDA-approved hepatitis B vaccine, HEPLISAV-B, and demonstrates an excellent safety profile (36–39). While BECC438b has yet to be evaluated in a clinical setting, it has proved beneficial in a number of different pre-clinical vaccines (40–42). Further, as most of the deleterious inflammation associated with DTP vaccination stemmed from LOS binding to TLR4, the modified lipid A structure of BECC438b suggests that the inflammation would not be on par with that elicited by DTP (43,44). Before safety and immunogenicity can be determined, there needs to be a rationale to move these vaccine candidates into human clinical trials. All of the
work presented in this dissertation was obtained from female CD-1 mice, which do not adequately model the entirety of pertussis as a disease. To start, mice are unable to exhibit perceivable coughs nor do they have symptoms associated with the catarrhal phase, such as fever or rhinorrhea (45). Additionally, there is a lot of variability in the mouse challenge model. Most studies have utilized an intranasal challenge model, but the dose of \textit{B. pertussis} that is administered can range from $10^3$ to $10^8$ CFUs (46). Studies also challenge mice with different strains, which can then affect the distribution of the bacterium throughout the respiratory tract. With this in mind, the next logical step is to evaluate how these vaccines perform in non-human primate models (46). When \textit{M. rhesus} monkeys (baboons) were challenged with the D420 strain of \textit{B. pertussis}, they exhibited paroxysmal coughing, had prominent leukocytosis, and provided high bacterial counts from nasopharyngeal washes (47). Further, baboons can be used in transmission studies, as they are capable of passing the pathogen onto uninfected cage mates, which allows us to evaluate the ability of vaccines to prevent asymptomatic carriage (48). Indeed, non-human primate models closely mimic both the early and late phases of a pertussis infection as seen in humans and would serve as a great segue into human clinical trials.
5.7 The impact of testing the “push-pull” vaccination strategy

One major takeaway from this dissertation is the importance of intranasal immunization in regard to protecting against a *B. pertussis* infection. From our data, as well as the success of the intranasally administered BPZE1, it seems clear that the ability of a vaccine to induce mucosal immunity and elicit localized protection at the site of infection would be a great attribute of a new booster vaccine. As previously mentioned, all vaccines are administered intramuscularly; thus, in Chapter 4, we aimed to utilize the push-pull strategy to determine how the intranasal booster would interact with or build upon the established systemic immunity. Our lab has previously explored the immune responses elicited by two intranasal vaccinations (a prime and boost) but we had not yet studied the effect of an intramuscular prime followed by an intranasal boost. We first observed
that one intranasal administration of the current Infanrix DTaP formulation was able to reduce bacterial burden in both the upper and lower respiratory tract and induce systemic IgG antibodies against the whole *B. pertussis* bacterium at levels similar to two intramuscular DTaP vaccinations. Additionally, one intranasal booster allowed for the induction of mucosal immune responses, as anti-*B. pertussis* IgA was found in the nasal lavage and within the lung and trachea. These responses could be improved when a new adjuvant was included in the DTaP formulation, such as BECC438b or IRI-1501. For example, both adjuvants increased bacterial clearance within the respiratory tract when compared to the IM/IN administration of DTaP. Further, the IRI-1501 adjuvant significantly increased the induction of systemic IgG antibodies and IgA antibodies within the nasal lavage, while the BECC438b adjuvant significantly increased IgA antibodies within the lung and trachea when compared to the push-pull administration of DTaP. These adjuvants also increased the production of potential indicators of the longevity of the vaccine-induced immune response, such as the number of antibody secreting cells in the bone marrow or the production of CXCL-13. Overall, these data demonstrate the benefit of incorporating an intranasal booster vaccine into the current vaccine schedule. The loss of an intramuscular booster had no impact on the systemic antibody responses and only improved bacterial clearance. Also, the use of an intranasal vaccine is essential for the induction of mucosal immunity. This work serves as a stepping stone for the use of a mucosal pertussis vaccine, regardless of whether that is BPZE1 or one of the formulas tested in this dissertation.

### 5.8 The next generation of pertussis vaccines: What lies ahead?

While it is impossible to predict the future, the evolution of *B. pertussis* strains will continue as will the cyclical nature of pertussis cases. Because current aP vaccines are safe and do save lives, it is very likely that they will remain in place for years to come. However, leaving the current aP
formulations in place will lead to the continued lack of control of a vaccine-preventable disease. The work presented in this dissertation highlights the improvements to vaccine-induced immunity afforded by the adjuvants CpG 1018 and BECC438b. Further, we also demonstrated that the incorporation of just one intranasal vaccine into the current vaccine schedule could induce localized immunity, leading to increased protection. Regardless of what route and formulation moves forward, it will take a great deal of effort to bring a new pertussis vaccine into use: large clinical trials will be needed and vaccine manufacturers will need to be convinced that there is beneficence in making new pertussis vaccines. While there may not currently be a large push to create new pertussis vaccines, we have seen the return of vaccine-preventable diseases before, such as the resurgence of measles and mumps in recent years. When DTP was developed, the idea that the formulation would need to be replaced in 50 years-time was unfathomable. Then, when the aP formulations were put into place, the waning immunity that resulted from their use could not have been predicted. All this to say, the idea of implementing a new pertussis vaccine is not unfounded, and the work detailed in this dissertation provides possible solutions (whether that be in the form of a new adjuvant or a new route) that can improve immunity against *B. pertussis* if/when the need arises.
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