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Development of Improved Acellular Pertussis Vaccines Through Inclusion of the RTX Antigen or Induction of Mucosal Immunity

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**DEVELOPMENT OF IMPROVED ACELLULAR PERTUSSIS
VACCINES THROUGH INCLUSION OF THE RTX ANTIGEN OR
INDUCTION OF MUCOSAL IMMUNITY**

Dylan Tyler Boehm

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

**Doctor of Philosophy
in
Immunology and Microbial Pathogenesis**

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**Keywords: *B. pertussis*, adenylate cyclase toxin, acellular vaccine, pertussis,
vaccine-induced mucosal immunity, DTaP, innate immunity, pertussis vaccine
model**

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ABSTRACT

DEVELOPMENT OF IMPROVED ACELLULAR PERTUSSIS VACCINES THROUGH INCLUSION OF THE RTX ANTIGEN OR INDUCTION OF MUCOSAL IMMUNITY

Dylan T. Boehm

Pertussis is a human respiratory disease, primarily caused by the Gram-negative pathogen *Bordetella pertussis*. The infection is most severe and can be life-threatening in young children and infants where it manifests as a series of paroxysmal coughs. The disease is more commonly known as whooping cough, due to the whoop omitted during a massive inspiratory effort to bring air back into the lungs. Pertussis is a toxin-mediated disease that persists due to an early release of toxins that allow that bacteria to evade the cells of the innate immune response. The inhibition of the host response continues as toxin activity disrupts the signaling generating an adaptive immune response. Fortunately, two generations of vaccines have been developed to combat the spread of the highly contagious disease. The whole-cell pertussis (wP) vaccine was implemented in the 1950s and was successful in controlling cases. However, due to adverse side effects associated with the immunization, wP vaccines were replaced with acellular protein (aP) vaccines absorbed to an alum adjuvant. The second generation aP vaccines induced an immune response capable of neutralizing key virulence factors contained in the vaccine, thereby controlling the symptoms of the disease. Unfortunately, there has been a re-emergence of pertussis incidence in countries using the aP vaccines. Included in this resurgence is an increased number of adolescents and adults with unrecognized pertussis, leading to the potential of adult and adolescent reservoirs of the infection. We reasoned that a pertussis vaccine capable of inducing sterilizing immunity, rather than controlling the symptoms, would decrease the spread of pertussis. In this work, we propose two directions towards this goal. The first was through the neutralization of a key virulence factor not included in any current vaccine formulations, adenylate cyclase toxin (ACT). This was accomplished through the optimization of a mouse immunization and challenge model. In initial experiments using a neutrophil reporter mouse, NeCre luc, we visualized the spatiotemporal localization of neutrophils after *B. pertussis* challenge in wP or aP immunized mice with a vaccine dose known to be protective. In these experiments wP immunized mice elicited an exaggerated immune response, that persisted after clearance of the pathogen, raising concerns to the relevance of the high vaccine dose in characterizing the immune response. In subsequent experiments, we addressed these concerns, by titrating the concentration of a Diphtheria-Tetanus-acellular Pertussis (DTaP) subunit vaccine to a dose that did not induce a protective response in a murine model. In defining a sub-optimal protective dose for mice, the additive effects of potential antigens could be evaluated, that were previously masked by the higher concentrations of vaccine. This model was confirmed through the addition of an ACT toxoid, RTX. The inclusion of RTX into a DTaP vaccine produced an antibody response that neutralized the toxin *in vitro*, and upon challenge with *B. pertussis* reduced the bacterial burden compared to mice only receiving the DTaP vaccine. We continued in the direction of promoting a vaccine-

induced immune response capable of clearing *B. pertussis* from the respiratory tract in future experiments. We hypothesized that early neutralization of the bacterial toxins through the generation of a mucosal immune response in the respiratory tract would increase the clearance of the pathogen. This was tested using a mucosal, intranasally (IN) administered DTaP vaccine. Administration of DTaP through the IN route induced a *B. pertussis*-specific systemic IgG response, and mucosal IgA response, capable of neutralizing the toxins at the site of infection. Furthermore, IN vaccinated mice reduced the bacterial burden to the same levels of intraperitoneal immunized mice. The work completed in this dissertation demonstrates the protective effects of the antigen RTX when incorporated into a current DTaP vaccine. We then altered the route of administration to an intranasal delivered vaccine, demonstrating the effectiveness of inducing a local mucosal response against *B. pertussis* infection. Together these findings establish a foundation for potential vaccine strategies to suppress the toxin-mediated dysregulation of the host response and lead to the more efficient clearance of *B. pertussis* from the respiratory tract.

DEDICATION

This work is dedicated to my parents, David and Beth Boehm, who together have shaped me into the person I am today. Thank you for caring for me in times of trouble, celebrating with me in times of joy, and for being unwavering examples of how to live life on the days between.

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List of Nomenclature

1. WHO: World Health Organization
2. wP,WCV: Whole-cell pertussis vaccine
3. DTP: Diphtheria, Tetanus toxoid and whole-cell pertussis vaccine
4. DT: Diphtheria and Tetanus toxoids
5. aP, ACV, DTaP: Acellular pertussis vaccine
6. FHA: filamentous hemagglutinin
7. PT: Pertussis toxin
8. Fim: Fimbriae
9. PRN: Pertactin
10.vags: virulence associated genes
11.cAMP: cyclic adenosine monophosphate
12.LPF: leukocytosis-inducing factor
13.ACT: Adenylate cyclase toxin
14.AC: Adenylate cyclase domain
15.APC: Antigen presenting cells
16.PAMPS: Pathogen-associated molecular patterns
17.LOS: lipooligosaccharide
18.NET: Neutrophil extracellular traps
19.SIgA: Secretory immunoglobulin A
20.T _{RM} : Tissue resident memory T cells
21.IVIS: <i>in vivo</i> imaging systems
22.RTX: DTaP + RTX antigen
23.p.c.: post-challenge
24.IPA: Ingenuity Pathway Analysis
25.IN: Intranasal
26.IM: Intramuscular
27.IP: Intraperitoneal
28.IP-aP: Intraperitoneal administered DTaP
29.IP-wP: Intraperitoneal whole-cell pertussis vaccine
30.IP-caP: Intraperitoneal DTaP with curdlan
31.IN-aP: Intranasal administered DTaP
32.IN-caP: Intranasal administered DTaP with curdlan
33.IN-curdlan: Intranasal administered curdlan only
34.ELISA: Enzyme-linked immunosorbent assay
35.STING: Stimulator of interferon genes receptor
36.DAMPs: Damage-associated molecular patterns
37.NALT: Nasal-associated lymphoid tissue
38.M cells: Microfold cells
39.DC cells: Dendritic cells

Chapter 1 - Introduction

Disease Overview

Pertussis is an exclusively human pathogen that exists in all populations worldwide. In the pre-vaccine era, the number of cases often exceeded 200,000 annually in the United States, peaking at more than 250,000 in 1934 ¹. The infection occurs in three stages: catarrhal, paroxysmal, and convalescent. In the catarrhal stage, symptoms can include a mild cough, rhinorrhea and lacrimation; similar to other upper respiratory infections ^{2,3}. Over time the cough worsens in frequency and intensity as the disease reaches the paroxysmal phase, normally ranging from 2-8 weeks ³. The paroxysmal phase is defined by repeated coughing fits lasting 5-10 coughs per minute, known as a paroxysm. Following a paroxysm, an individual performs a massive inspiratory effort to refill the lungs with air resulting in the classic whoop ⁴. The coughing fits are associated with production of high amounts of mucus and are commonly followed by post-tussive emesis⁵. Additional symptoms can include elevated white blood cells counts (lymphocytosis or leukocytosis), bronchopneumonia, and hypoxia. In the most severe cases these symptoms can be fatal and from 2000 - 2016 0.1% of cases resulted in fatalities, 88.8% of which occurred in infants ⁶. The transition to the convalescent phase occurs over several weeks or months and is associated with a reduction in severity of paroxysms and other symptoms ⁷.

Pertussis is a highly contagious respiratory disease that is spread through aerosolized *B. pertussis*, a dispersal method capable of infecting surrounding individuals⁸. Household contact studies have shown that transmission between family members is common and capable of causing mild or asymptomatic infections in adults. One study found that 46% of asymptomatic household contacts had antibody titers to *B. pertussis* ⁹. An aerosolized dispersal coupled with an incubation period between 7-10 days leads to a contagious disease capable of infecting multiple individuals ¹⁰. So much so, that pertussis still occurs in cyclical epidemics every 2-5 years; as it did in the pre-vaccine era ⁶. Pertussis surveillance data shows that in United States cases rose from 1,010 in 1976, to a peak of 48,000 cases in 2012 (Figure 1) ¹. The increase of pertussis cases is also occurring in other countries that have adopted acellular vaccines such as the

United Kingdom and Australia ¹¹, suggesting the cyclical pattern of pertussis epidemics is unchanged by modern vaccines.

Figure 1: Incidence of clinical *B. pertussis* cases in the United States from 1922-2017. Data reported from CDC.

History of *B. pertussis* vaccination and the increase in *B. pertussis* incidence

In 1906, Jules Bordet and Octave Gengou were the first scientists to isolate the bacteria *B. pertussis* from an individual. Prior to the isolation of the bacteria, a disease with the same characteristics of Pertussis had been described since the middle ages ¹². The first documented outbreak occurred in Paris during 1578 ¹². Until the 1930s there was still a debate as to whether *B. pertussis* was the causative agent of Pertussis, due to repeated failed attempts to culture the bacteria from a coughing patient ¹³. Before the 1950s, the World Health Organization (WHO) estimates that nearly half of all children that came into contact with *B. pertussis* would develop symptomatic pertussis ¹⁴. The widespread implementation of a killed whole-cell pertussis (wP) vaccine drastically reduced the incidence and mortality of pertussis, to the point that in 1975, it was proposed as a candidate for eradication ¹⁵. However, beginning in the 1980s, the number of cases began to increase in countries with high vaccine coverage ¹⁴. Around this time concerns arose regarding the reactogenicity of wP vaccines. In the late 1970s a landmark study examined the reactogenicity of whole-cell pertussis vaccine combined with diphtheria

and tetanus toxoids (DTP) compared to diphtheria and tetanus toxoids alone (DT) ¹⁶. The authors determined a correlation with redness, pain, and swelling at the injection site; as well as fever, and persistent crying when compared to DT immunization alone. In the early years of DTP vaccination there were reports of serious neurologic events and in some cases death ¹⁷. Understandably, this condition of “vaccine encephalopathy” arose as a major concern against pertussis immunization. As a result of these concerns, neurologic symptoms following pertussis immunization were extensively studied. Initial findings suggested that a correlation existed; however, these experiments lacked proper control groups, and multiple conditions could explain the neurological symptoms. A well-designed study, the National Childhood Encephalopathy Study, concluded there was no correlation between encephalopathy and pertussis immunization, and that cases of vaccine encephalopathy were the first seizures of infantile epilepsy ¹⁸. In the following years DTP formulations were altered to be safer; however, in some cases, immunization was not as effective and resulted in some countries stopping DTP immunizations all together ¹⁹. These inconsistencies in wP formulations and public concern pushed for the development of safer pertussis vaccines composed of acellular proteins with DT toxoids (DTaP) ⁵. It is important to note that, wP pertussis vaccines are still widely used today, especially in low- to middle-income countries; where they are effective at controlling the number of cases.

The first DTaP vaccines were developed in Japan by Sato and colleagues; which consisted mainly of filamentous hemagglutinin (FHA), a membrane-bound adhesin of *B. pertussis*, with lower amounts of chemically detoxified pertussis toxin (PT), over time other adhesins were included, fimbriae (Fim) proteins and pertactin (PRN) ²⁰. As development of DTaP vaccines continued the amount of PT was increased equal to the concentration of FHA ²¹. This generated a more specific immune response capable of neutralizing key virulence factors of *B. pertussis* while reducing fever, and local inflammatory reactions seen with wP immunizations. At the time, the Sato vaccines were the needed solution because they demonstrated low toxicity in mice, but were protective, generating high serum titers to antigens. When DTaP vaccines were moved to humans they were less reactogenic than wP immunizations, but still induced a robust antibody response ²². Evidence from household-contact studies and a decrease in

pertussis surveillance further supported the success of these DTaP vaccines, leading other countries, including the US to pursue DTaP vaccines ²³.

A key difference between the development of wP vaccines and DTaP vaccines was the method used for testing vaccine efficacy. After early inconsistencies in wP preparation, which resulted in ineffective vaccines, a validation method was developed to confirm lot validation ⁵. Pearl Kendrick and others developed the mouse potency test in the mid-1940s that enabled the prediction of wP vaccine efficacy ²⁴. Briefly, this used an intraperitoneal injection of a test vaccine and a standard vaccine into young mice; then mice were challenged with 10^5 of live *B. pertussis* through intracerebral inoculation 14 to 17 days after the immunization. Vaccine-induced protection was determined by survival of the mouse. Vaccine potency was assessed as international units, determined by comparing mouse survival following different lots of wPs compared to survival following a standard vaccine. The problem was that DTaP vaccine-induced protection could not be fully validated using Kendrick's mouse potency test, as DTaP immunizations did not protect against lethality in the intracerebral challenge ²⁵. To address this, a modified Kendrick test was developed to assess vaccine potency of DTaP immunizations ²⁶. This protocol extended the time between immunization and challenge allowing for the generation of an antibody response ^{26,27}. This led to the validity of DTaP vaccines to be determined from antibody titers against the individual antigens composed in a particular vaccine. As opposed, to the survival of mice following intracerebral challenge in wP validation tests. However, evidence now suggests that IgG antibody titers are not a true correlate of protection against pertussis ²⁸. As a result, Guiso *et al.* developed an intranasal mouse challenge model, where *B. pertussis* was capable of colonizing the lung. This protocol led to the validity of DTaP vaccines based on serological titers and clearance of the pathogen from the lower-respiratory system of mice ²⁵.

In the United States vaccine coverage of DTaP has remained high and as of 2016 93.7% of children aged 19 – 35 months have received ≥ 3 doses ²⁹. Nevertheless, a WHO review concluded that the United States, along with Australia, England, Wales and Portugal are experiencing a pertussis resurgence based on increased morbidity and hospitalizations ¹⁴. The increase in incidence of pertussis cases suggests that

current DTaP vaccines do not offer complete protection against *B. pertussis*. Multiple hypotheses have been proposed explaining the resurgence of incidence of pertussis including shortcomings of DTaP vaccines, vaccine-driven evolution, and increased surveillance of pertussis cases.

Waning DTaP Vaccine

Evidence that DTaP vaccine efficacy waned earlier than wP first arose in the early 2000s ³⁰. The mechanisms of protection between DTaP and wP vaccines will be explored at length later in this chapter. Presently, human data exists suggesting differences in the long-term protection from individuals immunized with either wP or DTaP vaccines, suggesting that wP vaccines offer longer protection ³¹. Studies in the US and Australia examining cohorts of children receiving either wP or DTaP priming immunizations, followed by DTaP booster immunizations have allowed for the study of the different responses ³¹. A study comparing protection up to 2 years post-booster immunization found higher IgG titers, memory B-cell numbers, and T-cell cytokine responses in DTaP primed individuals compared to wP primed children shortly after booster immunization ³². At 2 years post-boost DTaP primed children IgG titers remained higher, but only a Th1 cellular response was observed in wP primed children ³². Another study used a similar experimental design; however, they examined protection in wP primed children two years later than they did in DTaP primed children ³³. Their findings suggested that even with an additional 2 years post-boost wP primed children exhibited a higher cytokine response, and more CD4 and CD8 T cell proliferation to *B. pertussis* antigens ³³. A large case-control study in northern California found that DTaP protection waned unexpectedly fast following the fifth dose of DTaP ³⁴. A parallel study also, found that after the fifth dose of DTaP the odds of acquiring pertussis increased on average 42% each year ³⁵. This trend continued as the same group compared pertussis rates among adolescents who received the Tdap booster, which consists of a lower concentration of vaccine antigens with the addition of Fim antigen. These studies found that Tdap effectiveness dropped from 69% the first year after vaccination to an alarming 9% after 4 years ³⁶. Predictably, a study found that individuals who only received DTaP immunizations compared to those who received the wP vaccine schedule were at greater risk of contracting pertussis ³⁷. Together these

data clearly demonstrate the waning of protection provided by DTaP vaccines following a completed vaccine schedule.

Asymptomatic Transmission

Recently, data has emerged suggesting asymptomatic transmission in humans based on five areas of research ³⁸. First, it is known that subclinical levels of pertussis exist in countries such as the United States and the United Kingdom^{1,14}. Secondly, household contact is a well-documented mode of transmission of pertussis ^{5,9,23}. However, based on compiled data from household contact studies a symptomatic individual was only identified as the source of infection in 42% of cases ¹¹. Third, the process termed “cocooning” an infant (vaccinating those that will have early contact with the infant), has not been found to increase protection among infants ^{39,40}. Fourth, is the unexpected high number of cases in infants, given the high vaccination rate in countries such as the United States ⁴¹. Lastly, is the persistence of the cyclical epidemics of pertussis similar to the pre-vaccine era ¹⁴.

Up to this point, human data suggesting asymptomatic infection is only correlative; however, in the non-human primate model it has been shown that asymptomatic transmission following DTaP immunization can occur ⁴². Warfel *et al.*, demonstrate that while convalescent or wP vaccine-induced protection inhibited colonization by *B. pertussis* and blocked transmission to an unprotected animal, DTaP immunized baboons were colonized and transmitted the infection ⁴². It is then conceivable to think that the correlations stated above by Althouse and Scarpino ³⁸ may be occurring in humans, and asymptomatic infection could be accounting for the resurgence of pertussis in countries with strictly DTaP immunization schedules.

Vaccine-driven evolution of *B. pertussis*

B. pertussis is often described as an organism with limited genetic variability, although recent data has revealed substantial variation in circulating strains across different countries ^{43,44}. Weigand *et al.* find that this variation occurs mostly in genes that encode for immunogenic proteins, such as *ptxA*, *fimH*, genes encoding for PT and FHA, respectively ⁴⁵. Furthermore, these strains quickly spread through the population, leading researchers to conclude that these mutations may be a result of vaccine-driven

selection ^{44,46}. Sealey *et al.*, note the increased mutation rate in antigen-encoding genes found in various DTaP vaccines, and further mention that the frequency of the mutations has increased since the widespread incorporation of DTaP vaccines ⁴⁷. Perhaps the most recognized mutation is the rise of PRN deficient strains, which have spread at alarming rates across the United States and Europe ^{48,49}. In the United States a 2015 study, found that of 753 clinical isolates, 85% were PRN-deficient, a staggering statistic when PRN is considered a major immunogen in current vaccine formulations ⁵⁰.

Increased Surveillance and Diagnosis

As mentioned earlier, cyclical outbreaks of pertussis continue to occur. In the pre-vaccine era, these outbreaks were associated with high fatalities, and this was common in low and high-income countries ^{11,14}. Today, these high fatality outbreaks continue to occur mainly in low-income countries without established infant immunization programs. In these countries, positive diagnosis occur from observation of classical pertussis clinical symptoms ⁵¹. However, the resurgence of pertussis cases has been observed in high-income countries with well-established vaccine programs. In these countries, cases of pertussis are now diagnosed using quantitative real-time (RT)-PCR using insertion sequences allowing for the identification of *Bordetella* species, such as IS481, IS1001 and IS1002 ⁵². PCR diagnosis was first implemented for pertussis testing in the late 1980s and early 1990s and has since become the standard in developed countries ^{52,53}. It must be considered that the increased sensitivity of pertussis diagnosis is playing a role in increased pertussis case incidence. When the WHO assessed the re-emergence of pertussis they did not support a global increase in pertussis cases due to the increasing global population and increased diagnosis sensitivity ¹⁴. However, taking these factors into consideration they did determine a pertussis resurgence in the United States and other developed countries ¹⁴.

Together these proposed hypotheses are supported by evidence that suggests that the switch to DTaP immunizations is playing a role in the resurgence of pertussis. A proposed strategy to circumvent for this resurgence of cases is a vaccine capable of inducing a Th1/Th17 mediated immune response more similar to wP vaccines. The rationale behind this strategy is that priming with a more pro-inflammatory vaccine will

lead to the generation of an immune response more similar to the natural infection of *B. pertussis*. The hope being, that this response extends the duration of protection and leads to clearance of the pathogen from the respiratory tract.

Pathogenesis of *B. pertussis*

Much of the work performed in understanding the pathogenesis has been performed using animal models of *B. pertussis* infection. *B. pertussis* colonizes the respiratory tract and much of the work has focused on the establishment of colonization following inhalation of the bacteria. Aerosolized *B. pertussis* is transmitted through respiratory droplets⁵⁴. The bacteria attach to cilia, expressed on respiratory airway epithelial cells, through bacterial adhesins, such as Fim and FHA⁵⁵.

Once in the host, a currently unknown natural signal activates the sensor kinase (BvgS), of the *bvgAS* locus, a two-component regulatory system responsible for many of the virulence factors of *B. pertussis*. The activation of the histidine kinase BvgS and the phosphorylation of the response regulator BvgA results in the transcriptional regulation of BvgAS regulated genes, known as the Bvg⁺ phase⁵⁵. Virulence factors activated in the Bvg⁺ phase are known as vags (virulence-activated genes) and include *ptx* (PT), *cya* (adenylate cyclase toxin), *bteA*, (type III secretion system), *fha* (FHA), *fim2* and *fim3* (Fim); and have been classically considered essential for infection^{56–59}. After the establishment of infection *B. pertussis* produces bacterial toxins that either enter the bloodstream or effect surrounding cells.

Pertussis toxin

Pertussis toxin (PT) is an AB₅ toxin, that is exclusively expressed by *B. pertussis*. In earlier stages of the infection, PT plays a role in adherence to ciliated respiratory cells⁶⁰. In later stages, PT inhibits chemotaxis of phagocytic cells to the site of inflammation, where it is believed to play a critical role in the clinical symptoms of pertussis⁶¹. PT binds to glycoproteins on the cell surface of a wide variety of host cells, and the entire holotoxin is phagocytosed. In the endoplasmic reticulum, the S1 subunit is released into the cytoplasm, where it catalyzes ADP-ribosylation of G_i proteins, holding them in their inactive state^{62–64}. This results in 1) cytoplasmic increases of cyclic adenosine monophosphate (cAMP), and 2) a dysregulation of G_i protein-coupled signaling

pathways; PT is specifically known to inhibit macrophage and neutrophil recruitment to the site of infection ^{65,66}. In early studies by Morse and associates, PT was termed, leukocytosis-inducing factor (LPF), where they determined LPF was responsible for the increase in leukocytosis and lymphocytosis following intravenous administration of the toxin ⁶⁷. It was not until the work of Margaret Pittman demonstrated that LPF was an essential toxin for the pathogenesis of *B. pertussis* was the toxin referred to as PT ^{5,68}. It has been shown in numerous studies that neutralization of PT alone results in vaccine-induced protection. Therefore, detoxified toxin is included in all acellular vaccines ^{60,69–72}.

Figure 2: Pathogenesis of *B. pertussis*.

1) Aerosolized *B. pertussis* contained in expelled droplets is inhaled by individual. 2) Infection is initiated by adherence of bacteria to cilia, present on airway epithelial cells by bacteria adhesins; FHA, PRN, and Fim. 3) Activation of BvgAS phosphorelay, leads to expression of vags critical for infection, such as PT and ACT. 4) Secretion of PT and ACT dysregulate the innate immune response. PT suppresses systemic signaling of G_i protein-coupled signaling pathways utilized by chemokine receptors, resulting in leukocytosis. ACT acts locally at the site of infection, through catalytic activity of adenylate cyclase domain and hemolysin activity of RTX domain on APCs. The immunomodulatory activity of PT, ACT along with other produced by *B. pertussis* allow for evasion of the innate immune response and persistence of infection.

Adenylate cyclase toxin

Adenylate cyclase toxin (ACT, CyaA) is a large 177kDa monomeric protein, that consists of five regions: 1) N-terminal adenylate cyclase domain (AC), 2) a central hydrophobic domain, 3) a modification region responsible for acylation of the protein, 4), a repeat in toxin (RTX) hemolysin binding domain, and 5) a C-terminal secretion signal^{61,73–76}. ACT is secreted through a type I secretion system, through a channel formed by the CyaBDE proteins⁷⁷. Following secretion, ACT is modified by acylation at sites K860 and K983.

This process is essential for toxin activity and it has been shown that acylation enhances binding to complement receptor 3 (CR3, α M β integrin, CD11b/CD18)⁷⁸. A recent hypothesis proposes that FHA may play a role in the delivery of ACT by binding to newly secreted ACT; thereby, retaining the toxin on the surface of bacteria⁷⁹. This hypothesis is supported by findings that suggest delivery of ACT requires a close association of live bacteria with target host cells⁸⁰. Additionally, activation of the toxin also requires calcium, which binds in the RTX motifs of the binding domain, forming beta rolls⁸¹. Upon, this activation the RTX region can then bind CR3, leading to the penetration of host cell membrane by the hydrophobic region of ACT⁸². This allows for the translocation of the AC domain into the cytoplasm of the host cell⁸³. Calmodulin present in the cytoplasm of the host cell increases the catalytic activity of ACT, which drastically increases cellular cAMP to supraphysiological levels⁸⁴. The increases in cAMP caused by AC activity of ACT has been shown to inhibit activation of antigen-presenting cells (APCs), as well as induce apoptosis^{84–86}. Thus, ACT, along with PT has significant immunomodulatory properties, leading to the classification of these two toxins as the two most important virulence factors of *B. pertussis* pathogenesis⁶¹. Despite the immunomodulatory properties of ACT, it has yet to be included in newer generation pertussis vaccines. Limited studies have evaluated ACT as a potential antigen. Guiso *et al.* demonstrated purified ACT was protective in a murine intranasal challenge against *B. pertussis*⁸⁷. Furthermore, the same group demonstrated anti-ACT antibodies were capable of neutralizing ACT and inhibiting ACT activity on neutrophils⁸⁸. These studies yielded encouraging results; however, after the widespread

incorporation of DTaP into vaccine schedules progress on Pertussis vaccine research had slowed. Yet, ACT remains a strong antigen candidate for future pertussis vaccine development.

Adhesin factors of *B. pertussis*

FHA is a large, 232-kDa adhesin produced by all *Bordetella* spp. Studies suggest that the protein has multiple binding sites, which facilitate binding of *B. pertussis* to monocytes, macrophages, ciliated respiratory cells, and nonciliated epithelial cells^{89–91}. FHA has been shown to be a strong immunogen and serum antibodies to FHA have been found in patients following natural infection and after immunization⁹². Due to the protein's immunogenicity and role in attachment, it is considered a major virulence factor and included in most DTaP vaccines.

Another group of antigens included in current 5-component DTaP formulations is the Fim proteins. Fim proteins are filamentous, polymeric cell surface proteins, that bind host cells. They are also expressed during the Bvg⁺ growth phase, and like other vags have been shown to play a critical role in *B. pertussis* pathogenesis and adhesion to host cells⁹³. *In vivo* studies using Fim-negative strains have demonstrated reduced growth in the trachea and nasopharynx of mice⁹⁴, and reduced bacterial adherence *in vitro*⁹⁵. Two serotypes of fimbriae are most commonly observed in clinical isolates, Fim2 and Fim3, and are expressed in a serotype-specific manner⁹⁶. It has been demonstrated that Fim proteins are involved in bacterial agglutination, and included in current DTaP formulations as agglutinogens⁹⁷.

The continued research of *B. pertussis* pathogenesis has driven antigen selection for current acellular vaccines. As the field pushes for the development of the next generation of pertussis vaccines there is evidence that other virulence factors of *B. pertussis* have the potential for inclusion into new vaccines.

Immunity to *B. pertussis*

Pertussis cases are most severe in infants and can even be fatal. However, the majority of individuals are able to clear the infection. Studies using animal models have suggested that cells of the innate immune response are able to control the early infection in naïve animals⁹⁸. The innate immune response generated following

challenge with *B. pertussis* has been shown to consist of antimicrobial peptides, components of the complement system, and effector cells such as macrophages, neutrophils, natural killer cells, and dendritic cells⁹⁹. However, complete clearance requires the development of an adaptive, *B. pertussis*-specific response. Immune subversion of the innate immune response and persistence of *B. pertussis* infection are capable of delaying the generation of an adaptive immune response, suggesting that the interface between the innate and adaptive immune responses is crucial to controlling infection.

In the natural immune response, adherent bacteria colonize the respiratory epithelium. *B. pertussis* virulence factors act as pathogen-associated molecular patterns (PAMPS) such as: lipooligosaccharide (LOS), ACT, FHA, PT. These PAMPS bind pattern recognition receptors present on mucosal epithelial cells, macrophages and dendritic cells (DCs) leading to a cascade of pro-inflammatory responses^{100,101}. Cytokines such as: interleukin-6 (IL-6), interleukin-1 β (IL-1 β), interleukin-12 (IL-12), tumor necrosis factor alpha (TNF- α) and interleukin-17 (IL-17) have been shown to be produced early in *B. pertussis* infection and lead to recruitment of macrophages, neutrophils, DCs, and natural killer T cells to the respiratory tract^{69,98,102–104}.

Macrophages are long-lived phagocytic cells capable of residing in the tissue. Thus, they are one of the first cell types that play a role in recognition of *B. pertussis*. In animal studies it has been shown that upon challenge with *B. pertussis* a large percentage of the bacteria are cleared early in infection, suggesting that macrophages have a role in clearance of the pathogen^{65,105}. Studies in the mouse model have highlighted the role of macrophages in early *B. pertussis* infection, by showing that depletion of airway macrophages enhanced the infection⁶⁵. It was previously mentioned that macrophages are susceptible to toxins, PT and ACT. There is evidence that some *B. pertussis* survives phagocytosis and can persist in macrophages. This suggests that dysregulation caused by bacterial toxins could lead to an intracellular niche and persistent infection^{106–108}. Thus, there is potential that neutralization of both toxins would be a beneficial property of future pertussis vaccines.

Neutrophils have been shown to have an interesting role in the clearance of *B. pertussis*. In the mouse model following natural infection by the bacteria, neutrophils were shown to not play an essential role in bacterial clearance ¹⁰⁹. However, when bacteria are opsonized, neutrophils do play a significant role in bacterial clearance ¹⁰⁹. Furthermore, the formation of neutrophil extracellular traps (NETs) has been observed following infection with *B. pertussis* ¹¹⁰. Like macrophages, neutrophils as well are affected by *B. pertussis* toxins through inhibition of chemotaxis, phagocytosis, NET formation from ADP-ribosylation and cAMP accumulation ¹¹¹. The role of neutrophils in pertussis has not been fully explored and provides an interesting avenue for future research.

Adaptive immune response

The generation of an adaptive immune response is necessary for complete protection against *B. pertussis*. DCs from the infected respiratory tract migrate to lymph nodes to present antigen to naïve T cells. The lungs of naïve adult mice do not contain high numbers of T cells; however, following infection of *B. pertussis* T cells are recruited to the lung which correlates with bacterial clearance from the tissue ¹⁰¹. A critical role of CD4⁺ T cells in pertussis immunity was shown using athymic *nu/nu* mice, as challenge resulted in persistent infection or death ¹¹². Adoptive transfer of convalescent splenic T cells into these athymic mice cleared the infection. Conversely, similar experiments using transfer of *B. pertussis*-specific CD8⁺ T cells to sublethally irradiated BALB/c mice did not result in clearance of infection, suggesting cellular responses mediated by CD4⁺ T cells play an important role in immunity to *B. pertussis* ¹¹². In particular, CD4⁺ T cells from infected mice produce IFN- γ and IL-17, and not Th2 associated cytokines ¹¹³. Furthermore, IFN- γ ^{-/-} mice have a significantly reduced ability to clear the pathogen, and lethal disseminating *B. pertussis* infections have also been observed ^{113,114}. Studies using IL-17A ^{-/-} mice, also reached similar findings, determining greater bacterial burden, and decreased neutrophil concentrations in the lungs of infected knockout mice compared to WT mice ¹¹⁵. Data from baboons infected with *B. pertussis* correlate with data from mouse model as both models generate Th1 and Th17 immune responses following infection and colonization of *B. pertussis* ¹¹⁶. Taken together, data from mouse

and baboon models demonstrate that the natural infection of *B. pertussis* is cleared through the induction of a mixed Th1 and Th17 immune response¹¹⁶.

Mucosal immune response

B. pertussis is strictly a pathogen of the respiratory system. In humans all but the most severe cases occur in the upper respiratory tract⁵. In the mouse model, *B. pertussis* infection also remains in the respiratory tract and only in immunocompromised models or neonatal mice has dissemination into other organs been documented^{114,117}. Yet today, all commercially available pertussis vaccines are given parenterally and have not been observed to induce local immune responses in the respiratory system. Therefore, it seems that this under-explored avenue of immunity could play an important role against *B. pertussis*. Findings from the non-human primate pertussis model have strengthened this possibility. As mentioned early, Warfel *et al.*, demonstrate DTaP immunization in baboons did not inhibit transmission of *B. pertussis* to another animal¹¹⁸. While wP immunization inhibited transmission, *B. pertussis* colonization persisted for more than 19 days. Only in baboons that had been previously challenged with *B. pertussis* and convalescent at the time of challenge, was colonization inhibited. In another study by the same group, a strong IL-17 response was measured in nasopharyngeal wash fluid following *B. pertussis* infection¹¹⁶. Upon, re-challenge of convalescent baboons Th-17 associated cytokines were increased early in infection. The strength of the baboon work comes from relevance to *B. pertussis* pathogenesis in humans. However, non-human primate models are unobtainable for most researchers. Mice share similar T cell responses to humans and through the mouse model protection from the mucosal immune response can be currently addressed.

Immunoglobulin A in the respiratory tract

The induction of a mucosal immune response is associated with production of IgA antibody. In the mucosal surfaces IgA is the predominate isotype, where it exists in the dimeric form as secretory IgA (SIgA). Mucosal IgAs are known to block adhesion of bacteria and neutralize toxins, and serum IgA can facilitate receptor-mediated phagocytosis in other bacterial respiratory infections¹¹⁹. In a study using IgA^{-/-} mice, it was shown that upon challenge with *B. pertussis* infected mice did not exhibit a

statistically different bacterial burden compared to WT ¹²⁰. More so, upon secondary challenge there was still no difference, suggesting that IgA did not play a critical role in protection in a natural infection or a primed response. Interestingly, IgA antibodies did appear to play a significant role in protection against, the closely related, *Bordetella bronchiseptica* clearance in the upper-respiratory tract; however, not in the lower-respiratory tract ¹²⁰. The authors do state that this finding may be due to *B. pertussis* infecting human hosts, while *B. bronchiseptica* effects mainly other mammals. However, recent data has determined contradicting findings ¹²¹. SIgA antibodies are transported across the mucosa through the polymeric Ig receptor. Using a mouse model deficient in this receptor, the specific role of SIgA in the respiratory tract was determined in the aforementioned study. Solans *et. al.* found that following intranasal administration of a live-attenuated *B. pertussis* vaccine, BPZE1, that in SIgA-deficient mice protection was strongly diminished compared to WT mice ¹²¹. Additionally, the authors demonstrate that IL-17 plays a critical role in the production of nasal SIgA following BPZE1 immunization, as IL17^{-/-} mice produced only background levels of the antibody. Furthermore, in these mice the vaccine did not inhibit colonization. In human data, infection has been shown to be correlated with *B. pertussis*-specific IgA in the nasal secretions of infected patients ¹²². It was found that these antibodies were detected at 2-3 weeks after infection, but the response is reduced to low levels around 6 months. A possible protective role of human IgA was shown *in vitro* where anti-*B. pertussis* IgA antibodies from serum and nasal washes of convalescent individuals were shown to inhibit adherence of *B. pertussis* to human respiratory cells ¹²³.

Local cellular mucosal immune response

In the pathogenesis of *B. pertussis*, the first cells to encounter the pathogen are those of the lung-resident innate immune cells. The process of antigen-presentation via macrophages and DCs to naïve T cells in the draining lymph nodes is well understood, as described earlier. The role of Th1 and Th17 immune responses to *B. pertussis* are also well described though, most of this data comes from peripheral T cells ^{114,115,124}. It has been shown that T cells from the lung and those from the spleen respond differently to bacterial antigens, and suggests a compartmentalization between T cells of periphery

and those of the lung ¹²⁵. More recently, it has been shown that lung CD4⁺ specific to *B. pertussis* do still secrete IFN- γ and/or IL-17 ^{112,126}.

The group of Kingston Mills has identified a portion of the CD4⁺ T cells secreting IFN- γ and IL-17 are from a population known as tissue-resident memory T cells (T_{RM}) ¹²⁷. These cells exhibit classical memory T cell effector markers, CD44⁺ and CD62L⁻, along with expression of surface markers associated with tissue retention CD69⁺ and/or CD103⁺ ^{127,128}. An innovative method was used to confirm that these T_{RM} cells are indeed not circulating and retained in tissue. An anti-CD45 labeled-antibody was administered intravenously prior to euthanasia, thus labeling cells that were circulating as CD45⁺, while cells retained in the tissue would not be labeled. Then using another anti-CD45 labeled-antibody with a different fluorophore, cells were labeled in single-cell suspension along with other markers, T_{RM} cells that were negative for the first anti-CD45 antibody, and positive for the second were classified as cells that were resident in the tissue ¹²⁷. In the study described above, these T_{RM} cells provide a protective role following the secondary infection of a mouse previously infected with *B. pertussis*. Additionally, these studies were repeated and found that these cells persisted in the lung tissue for several months following the initial infection, and then expanded after re-challenge. The finding that these mucosal resident T_{RM} are capable of secreting IL-17 and IFN- γ , suggest that this population may play a critical role in recruitment of phagocytes during the secondary immune response. More evidence suggesting a critical role was shown when migration of CD4⁺ T cells was chemically inhibited during secondary challenge with *B. pertussis*, and the infection was still cleared due to the presence of T_{RM}s in the lung, this was also shown using adoptive transfer with similar findings ¹²⁷. The findings described in this section provide strong evidence that the generation of a local mucosal immune response plays a role in the clearance of *B. pertussis* following previous challenge. However, the generation of a mucosal immune response has not been observed following parenteral immunization with DTaP vaccines ¹²⁹.

Pertussis vaccine-mediated immunity

It is clear that widespread use of wP vaccines was effective. By the 1970s the average yearly rate of pertussis cases in the United States dropped to <1 per 100,000 population, compared to 157 cases per 100,000 population in the 1950s¹³⁰. These vaccines, still used today, are composed of killed whole *B. pertussis* organisms. As a result the vaccines consist of numerous potential antigens and adjuvants, creating a highly immunogenic vaccine. However, this creates a degree of reactogenicity, mostly attributed to the presence of LOS¹³¹. It has been discussed that the adverse effects of wPs caused the switch in vaccine schedules to DTaP vaccines, and that following the change pertussis cases are re-emerging. Following the resurgence of pertussis, work has focused on understanding the differences in the vaccine-induced immune response following DTaP or wP immunization. While, it is known that DTaP-induced protection wanes significantly each year, initially strong IgG serum titers are observed¹³². A recent study demonstrated DTaP-induced IgG levels remain higher than individuals immunized by wP until 6 years of age; however, wP protection persists longer¹³³. Data characterizing the T cell responses in children following DTaP or wP priming suggests greater differences between the immunizations. Children receiving DTaP demonstrated Th2 responses, while children receiving wP preferentially induce Th1 cells^{134,135}. Interestingly, current data suggests that the T cell responses induced by initial vaccine priming are imprinted. Data from human patients suggests that Tdap booster immunizations do not alter the T cell responses or IgG antibody subtypes induced by wP, as these patients still produce the response elicited by the wP vaccine^{133,136}.

Table 1: Immune response to *B. pertussis* following previous infection or vaccination. Summary of current literature in mice, baboons, and humans. Figure adapted from Wilk *et al.*¹¹, Kapil and Merkel¹⁰², and Brummelman *et al.*¹⁰¹.

	Infection	Pertussis Whole-cell vaccine (wP)	Pertussis acellular vaccine (DTaP)
Immune Response:	Th1/Th17	Th1/Th17	Th2
Antibody Response:	IgG2a	IgG2a IgG1	IgG1 IgE
Mucosal Response:	T _{RM} IgA	T _{RM} IgA	None
Mechanism of Protection:	Robust proinflammatory response	Robust proinflammatory response	Toxin neutralizing antibodies
Protection against colonization:	High	Moderate	Low
Persistence of Protection:	Good	Moderate	Poor

The vaccine-induced responses following challenge with *B. pertussis* have been well characterized in mouse models. The response to *B. pertussis* in a wP immunized mouse mirrors the response to natural infection. A strong pro-inflammatory response is elicited in response to infection, resulting in enhanced bactericidal activity of macrophages and neutrophils^{102,124,137}. In mice, the IgG profile following wP immunization is made up primarily of IgG2a/c, as well as IgA, and IgG1 antibodies, suggesting a Th1 response, whereas the IgG antibody isotype IgG2a/c (depending on

mouse strain) ^{105,115}. This pro-inflammatory response has been shown to be dependent upon IFN- γ , as is natural infection, wP-primed IFN- $\gamma^{-/-}$ mice could not clear a challenge with *B. pertussis* ^{115,138}. Similarly, IL-17 has been shown to be induced following wP-induced protection, and clearance of *B. pertussis* was delayed in IL-17 $^{-/-}$ compared to WT mice, although mice did clear the infection eventually ¹¹⁵. Recent work by Wilk *et al.* has demonstrated a novel finding suggesting that clearance of *B. pertussis* from the nasal cavity is dependent upon IL-17, as the T_{RM} population was not detected in IL-17 $^{-/-}$ mice, nor could they clear the infection in the nasal cavity ¹²⁹. Interestingly, the T_{RM} population and more so bacterial clearance in the nasal cavity only occurred following natural infection with *B. pertussis* or with intraperitoneal wP immunized mice. Clearance from the nasal cavity and the T_{RM} population were not found following challenge of DTaP or Tdap primed mice.

DTaP on the other hand consists of high doses of 3-5 *B. pertussis* antigens mainly: PT, PRN, FHA, Fim2 and Fim3. Thus, DTaP immunizations induce a more specific response that is capable of neutralizing the symptoms of the disease; however, it does not inhibit colonization or transmission ¹¹⁸. In mice, DTaP induces a predominantly IgG1 driven humoral immune response. It is known that in mice DTaP induces a Th2 response, but also a Th17 mediated response. This was further shown through the finding that DTaP immunized IL-4 $^{-/-}$ mice were fully protected from infection, and the protection was mediated through IL-17. The Th17 response is known to be induced by aluminum used as an adjuvant. The induction of a Th17 response has not been shown to occur in humans or non-human primates and is specific to mice ^{101,115,118}. Although a Th17 immune response is observed in DTaP immunized mice, it is not sufficient to induce the T_{RM} population that was induced with wP immunization, which may play a role in the duration of memory between the vaccine-induced responses. It is clear that DTaP-vaccine induced immunity has various shortcomings including narrow specificity, different Th-cell subtype response, and different memory T cell populations.

The goal of this body of work was to better understand and improve upon the current DTaP vaccine-induced immune response. The development of DTaP vaccines occurred at a time when an answer to the reactogenic wP vaccines was desperately needed.

Thus, the development process occurred quickly, the duration and type of immune response elicited were not designed to be tested in the safety and efficacy trials. We aimed to characterize the DTaP vaccine-induced response through a mouse model capable of addressing vaccine efficacy beyond production of *B. pertussis*-specific IgG antibodies. In chapter 2, we characterize the neutrophil response to *B. pertussis* following immunization and challenge. Using, a neutrophil reporter mouse line, NeCre luc we observed the drastic neutrophil response following challenge of a wP-primed mouse, compared to DTaP protection and even natural infection. We used RNAseq analysis to observe transcriptional changes between the vaccinated groups to further characterize pro-inflammatory response. The work in chapter 3, discusses the titration of DTaP immunization dose to a relevant immunization dose for murine studies. In this chapter, we aimed to determine a sub-optimal vaccine dose. Defining this dose would enable us to determine effects of potential antigens. Furthermore, we implemented an antigen of a key virulence factor of *B. pertussis*, ACT. The antigen (RTX) induced a strong serum IgG response that was capable of neutralizing the toxin *in vitro*. Inclusion of the antigen resulted in increased bacterial clearance and a reduction in the pro-inflammatory response following *B. pertussis* infection. Data from non-human primates and mice studies suggest that the lack of clearance of *B. pertussis* from the upper-respiratory in DTaP protected mice was a shortcoming of the vaccine. In chapter 4, we address the mucosal immune response by characterizing DTaP vaccine-induced immunity when the vaccine was administered intranasally. In this work we determined that intranasal immunization with DTaP can generate a local mucosal immune response. When the vaccine was administered with a Th17 promoting adjuvant we detected a local IL-17 response at the site of infection and production of IgA in the respiratory tract. In chapter 5, the implications of these works in the generation of future pertussis vaccines are discussed. The current state of pertussis vaccine research is at a crossroads due to the uncertainty of which vaccine avenue to pursue, this work establishes a safe, intranasal vaccine with parameters that could be used to establish protection in mice and non-human primates.

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Chapter 2 - Characterizing the innate and adaptive responses of immunized mice to *Bordetella pertussis* infection using in vivo imaging and transcriptomic analysis

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Abstract

Bordetella pertussis (*B. pertussis*) is the causative agent of pertussis (whooping cough). Since the 1990s, pertussis has re-emerged in the United States despite an estimated 95% vaccine coverage. Our goal was to characterize neutrophil responses and gene expression profiles of murine lungs in the context of vaccination and *B. pertussis* challenge. We utilized a bioluminescent neutrophil mouse model (NECre luc) to track neutrophil recruitment. NECre luc mice were immunized with whole cell vaccine (WCV), acellular vaccine (ACV), or a truncated adenylate cyclase toxoid (RTX) vaccine. Neutrophil recruitment was measured in live mice across time and corroborated by flow cytometry and other data. WCV immunized mice showed signs of neutrophilia in response to *B. pertussis* challenge. Mice immunized with either ACV or WCV cleared the challenge infection; however immunization with RTX alone was not protective. RNA sequencing revealed distinctive gene expression profiles for each immunization group. We observed an increase in expression of genes associated with responses to infection, and changes in expression of distinct genes in each vaccine group, providing a complex view of the immune response to *B. pertussis* infection in mice. This study suggests that combination of immunological analysis with transcriptomic profiling can facilitate discovery of pre-clinical correlates of protection for vaccine development.

Introduction

Pertussis is a human disease primarily caused by a respiratory infection of the Gram-negative pathogen *Bordetella pertussis* (*B. pertussis*). The hallmark of pertussis is a distinctive whooping cough. What is surprising about pertussis is that the cause of the cough has never been elucidated, which highlights the fact that there are many under-researched aspects of this disease. Aerosolized *B. pertussis* bacterium are inhaled and adhere to airway respiratory epithelial cells through bacterial adhesins such as filamentous hemagglutinin (FHA), fimbriae and pertactin¹. After colonization *B. pertussis* express multiple toxins including pertussis toxin (PT) and adenylate cyclase toxin (ACT). *B. pertussis* releases PT, which dysregulates the immune response through ADP-ribosylation of the G-protein α -subunit of cytokine receptors present on a range of leukocytes²⁻⁵. The secretion of PT has long range effects, ACT is thought to act locally on host cells by converting ATP into supraphysiological levels of cAMP, further dysregulating the host immune response⁶.

In the 1940s, an effective whole cell vaccine (WCV) was developed and as a result, basic research efforts on *B. pertussis* decreased. The WCVs were highly reactogenic and caused prolonged and unusual crying after administration, hyporesponsiveness, and febrile convulsions⁷⁻⁹. These issues led to the development of acellular vaccines (ACV), known today as DTaP/Tdap (hereafter referred to as ACV). The ACVs utilize an alum adjuvant and induce a Th2 response to several key virulence factors such as PT, FHA, fimbriae, and pertactin depending on vaccine formulations. Whereas the ACV induces a Th2 response in human and mice, WCV immunization and *B. pertussis* infection promote a Th1/Th17-type response¹⁰⁻¹⁴. Since the replacement of the WCV with the ACVs there has been a remarkable increase in the number of cases in the US, with the number of reported cases in 2012 equaling that of 1954. Multiple factors potentially played a role in the return of pertussis in the US. It has been documented that the Th2 response induced by ACV immunization may be inferior to the Th1/Th17 response directed by the WCV¹⁵. Additionally, ACV protection wanes significantly as early as one year after vaccination, and by 4 years after vaccination vaccine effectiveness is only 9%¹⁶. Furthermore, in a non-human primate model it has been shown that ACV immunized baboons challenged with *B. pertussis* are capable of

carrying the infection and transmitting aerosolized *B. pertussis* to naïve baboons¹⁷. Additionally, the findings of an epidemiological study suggest that asymptomatic transmission of pertussis is indeed occurring in the human population, and has a role in the increase of pertussis incidents¹⁸. These recent findings demonstrate the shortcomings of the ACV. Utilizing new technologies that were not available during the developments of the WCV or ACVs we can further investigate differences between WCV and ACV protection. Adenylate cyclase toxin (ACT) is an essential virulence factor of *B. pertussis* and a known protective antigen^{19–21}. ACT contains two main domains: adenylate cyclase and the Repeats-in-Toxin (RTX). In the absence of the AC domain, the protein is non-toxic and referred to simply as RTX²². In a previous study, sera from mice immunized with RTX contained antibodies capable of neutralizing ACT. ACT antigens are not included in any commercial formulations of ACVs and many have proposed that its inclusion could improve protection⁶.

Neutrophils play a role in both the innate and adaptive arms of the immune system. Following *B. pertussis* infection of a naïve mouse, neutrophils reach peak recruitment between 5 and 7 days after challenge^{23,24}. This occurs after the bacterial burden has begun to decrease. Subsequent studies have determined that neutrophil depletion prior to *B. pertussis* infection in naïve mice does not increase bacterial burden. However, when immunized mice are challenged with *B. pertussis* following depletion of neutrophils the *B. pertussis* bacterial burden increase, demonstrating a protective role of neutrophils in immunized mice²⁵. As previously mentioned, *B. pertussis* toxins PT and ACT both affect neutrophils and their recruitment^{6,26–28}. These toxins contribute to a significant rise in the number of circulating white blood cells and in severe cases leukocytosis is linked to fatality¹. Leukocytosis was first described in pertussis patients as early as the 1890s^{29,30}. In the 1960's, Stephen Morse observed leukocytosis following WCV immunization³¹, which he hypothesized was caused by PT, then known as leukocytosis-inducing factor³². Therefore, it can be conceived that the toxin associated effects on neutrophils would be neutralized following ideal *B. pertussis* immunization, and this raises the question of where and when neutrophils are recruited in an ACV or WCV protected mouse?

In this study, we utilized *in vivo* imaging systems (IVIS) to characterize the neutrophil responses to *B. pertussis* challenge in mice vaccinated with ACV or WCV. We implemented NECre luc mice, a luminescent neutrophil reporter mouse strain, previously used in a *Bacillus anthracis* infection model to track neutrophil recruitment following *B. pertussis* challenge³³. To do so, we: 1) validated the NECre luc mouse as a model for tracking neutrophil recruitment in response to *B. pertussis* infection, 2) tracked the spatiotemporal localization of neutrophils in response to *B. pertussis* challenge in ACV and WCV vaccinated mice, and 3) and characterized vaccine-associated gene expression profiles of the infected lungs from each group using RNA sequencing. The NeCRE luc mouse³³ was used to measure the presence and relative quantities of neutrophils in live mice during *B. pertussis* challenge. We then corroborated our findings with flow cytometry analysis to quantify neutrophils in the blood and airway of naïve and immunized mice. WCV immunization resulted in robust neutrophil recruitment and clearance of *B. pertussis*, but also resulted in morbidity of the NeCre luc mice. RNAseq analysis was performed on the lungs of naïve or immunized mice at both early and late time-points after challenge with *B. pertussis*. We observed specific gene signatures for each vaccine, and we also identified gene expression profiles that had not been associated with *B. pertussis* or immunization. Neutrophil specific gene expression corroborated our cellular analysis of the respiratory tract demonstrating increased expression of neutrophil specific genes in WCV immunized mice. Furthermore, we determined the T-helper cell gene expression profiles of lung transcriptomes and corroborated the activation of Th1 specific gene expression to increased levels of Th1-associated cytokine production in the lungs of WCV immunized mice. Utilizing the depth of data generated through RNAseq, we could classify specific B cell clonotypes present in the lung of each immunization group. This technique allowed for analysis of the diversity and frequency of the immunoglobulin repertoire of immunized groups. We hypothesize that these approaches can continue to be applied to other pathogen to host interactions to characterize the underpinnings to disease progression and immunological responses.

Results

ACV and WCV immunization of NECre luc mice results in clearance of *B. pertussis*, however WCV immunized mice experience increased morbidity and mortality.

To characterize the effects of vaccination on neutrophil recruitment, NECre luc mice were immunized with the vaccines as indicated in Supplementary Table S1, and then boosted with the same vaccine 21 days later by intraperitoneal injection. We then compared the neutrophil recruitment in naïve, ACV, WCV, and RTX-only vaccinated NECre luc mice during *B. pertussis* challenge. At day 35 post initial immunization, NECre luc mice were challenged with virulent *B. pertussis* strain UT25 (Fig. 1) by intranasal instillation. A challenge dose of 2×10^7 CFUs *B. pertussis* does not typically cause morbidity in immunocompetent mice such as CD-1 or C57B6/J (data not shown). We originally planned to euthanize 4 mice per group on each experimental day (1, 2, 4, and 9). However, 2 of 13 naïve challenged NECre luc mice were euthanized at day 2 due to morbidity (Fig. 2). At day 6, we observed additional unexpectedly morbidity in the WCV group, which required euthanasia of all remaining WCV immunized NECre luc mice (Fig. 2). Conversely, no morbidity was observed in the ACV and RTX immunized mice 9 days post *B. pertussis* challenge (Fig. 2). We were surprised to observe morbidity in the WCV immunized mice. Upon determining viable bacterial burden from lung, trachea, and nasal lavage, we observed clearance of *B. pertussis* to our limits of detection in both ACV and WCV immunized mice compared to naïve infection (Fig. 3abc). Taken together, these data suggested that morbidity of the WCV immunized mice was not fully attributed to bacterial burden. Additionally, we determined that vaccination with RTX resulted in 100 percent survival out to 9 days post-challenge (pc), while bacterial burden were similar to naïve infected mice, suggesting that RTX immunization did not improve bacterial clearance (Fig. 3abc).

Analysis of serological responses of immunized mice.

As expected, the ACV immunized mice were protected against challenge as they rapidly cleared the *B. pertussis* challenge dose (Fig. 3abc). WCV immunized mice cleared *B. pertussis*, but this response was delayed compared to the ACV mice. To determine the

efficacy of immunization on antibody production, we performed serology analysis. PT is a common antigen of both the ACV and WCV and we observed a significantly higher concentration of anti-PT in the ACV mice compared to the WCV (Fig. 3d). Immunization of NECre mice with RTX with alum adjuvant resulted in high anti-RTX antibody titers (Fig. 3e) despite the fact that we did not see increased clearance due to RTX immunization. In another study, we immunized CD1 mice with RTX and alum adjuvant and we also saw no clearance of *B. pertussis* compared to non-vaccinated mice (data not shown). Our data suggest that, while immunization with RTX induces antibody production, it is not sufficient to protect mice from *B. pertussis* infection as a single, antigen vaccine.

Spatiotemporal IVIS imaging of neutrophils in naïve and vaccinated NECre luc mice.

Neutrophil accumulation in NECre luc mice were visualized on days 1, 2, 4, 6 and 9 post-challenge. Mice were IP injected with the high sensitivity luciferin CycLuc1³³ before imaging. A Lumina II IVIS (Xenogen) was used to capture luciferase-driven neutrophil luminescence at indicated time-points (Fig. 4 and Supplementary Fig. 1). Luminescent signal of the whole body (Fig. 4d) or nasal cavity (Fig. 4e) of vaccinated mice was then compared to signal from naïve, non-infected mice and calculated as fold change. At day 1 post-challenge, we detected significantly higher luminescence in naïve challenged mice compared to naïve non-challenged mice, suggesting that there is a higher neutrophil accumulation in challenged mice. Furthermore, whole body luminescence in WCV immunized and challenged mice was significantly higher than the whole body luminescence of naïve, ACV and RTX *B. pertussis* challenged mice at day 1 (Fig. 4d). In general, luminescence signals of all groups decreased across days 2 and 4. However, in the WCV group at day 6, we detected an increase in signal in the nasal cavity (71-fold) and the whole body (47-fold) compared to naïve not challenged mice (Fig. 4de). Due to variability between animals, this increase was not significant compared to other groups. At this point WCV immunized mice were morbid and required euthanasia. Taken together this data suggests that WCV immunization prior to challenge resulted in increased neutrophil accumulation compared to other vaccination

groups. We next aimed to corroborate our IVIS luminescence data with flow cytometry analysis of respiratory tissues.

Validation of neutrophil infiltration in respiratory tissue by flow cytometry

Luminescent signal of luciferase-expressing cells correlates with the localization of neutrophils in NECre luc mice³³. To confirm this, we measured the relative number of neutrophils by performing flow cytometry analysis on respiratory tissue following IVIS imaging. Single cell suspensions from lung tissue or nasal lavage were labelled with antibodies recognizing cell surface markers. We defined neutrophils as CD11b+GR-1+ live cells (Supplementary Fig. 2). Similar to the IVIS data, all *B. pertussis* challenged groups had significantly higher neutrophil percentages in nasal lavage compared to naïve non-challenged mice (Fig. 4f). At day 1 post *B. pertussis* challenge, WCV immunized mice had a significantly higher percentage of neutrophils than ACV immunized mice in nasal lavage. The percentages of neutrophils decreased from day 1 to day 2 in all vaccinated groups, as well as the naïve group. At day 4, WCV immunized mice exhibited higher levels of neutrophils than those observed at day 2 (Fig. 4g). Correlating with the nasal lavage, WCV mice exhibited significantly higher percentages of neutrophils in the lungs than ACV mice. Lung neutrophils at day 4 were not statistically significant between any of the groups. However, by day 6 there was a 53% increase in lung neutrophils compared to day 4 in the WCV group, which directly correlates with the increased luminescence signals measured by IVIS imaging (Fig. 4g).

WCV immunized NECre luc mice induce a more robust Th1 immune response compared to ACV immunized mice.

It has been previously documented that upon *B. pertussis* challenge, WCV immunized mice elicit a Th1/Th17 immune response, similar to a naïve infection while ACV immunized mice generate primarily a Th2 response^{10–12,34}. We hypothesized that similar responses are triggered by WCV and ACV in the NECre luc model. To test this hypothesis, we determined the cytokine profiles in lungs of immunized and challenged mice using electrochemiluminescent sandwich immunoassays. The Th1 response was determined by increased levels of IFN- γ , IL-12p70, IL-1 β and TNF- α (Fig. 5ab), while

the Th17 response was qualified by increased IL-17A and IL-6 (Fig. 5cde). Th2 responses were associated to increased levels in IL-4 and IL-5.

As expected, we observed an increased Th1 response in the WCV vaccinated NECre luc mice compared to the ACV group. We also observed an increase in the Th1 response in naïve challenged mice compared to non-challenged mice or the ACV group. Interestingly, the highest levels of IL-17 were observed in WCV NECre luc mice (Fig. 5c). The levels of Th2-associated cytokines, IL-4 and IL5 were similar between the WCV and ACV groups. (Supplementary Fig. 3). The lung cytokine profile from NECre luc mice immunized with RTX resembled a profile of naïve *B. pertussis* challenged mice with the exception of IL-6, which was lower in the RTX group. Taken together these data from the NECre luc mouse model corroborates previous findings by displaying a strong Th1/Th17 response in response to *B. pertussis* in WCV vaccinated mice.

Characterizing the gene expression profiles of the lungs of immunized and naïve mice after *B. pertussis* challenge

Current and past *B. pertussis* immune response studies have focused on a limited set of known immunological responses to *B. pertussis* challenge such as: bacterial burden, antibody response, recruitment of phagocytes, cytokine profiles, and others^{35,36}. Whooping cough is a toxin-mediated disease, due mainly to the activity of pertussis and adenylate cyclase toxins. These toxins are released and affect a wide array of host cells^{4,25}. It is therefore conceivable that *B. pertussis* infection would have a broader effect on the respiratory tissue beyond the induction of predictable immune response factors. To address this, we employed RNA sequencing (RNAseq) to observe the effect of *B. pertussis* challenge on the lungs of immunized and naïve NECre luc mice at a transcriptomic level. This allowed us to characterize the transcriptome response to infection and vaccine-induced responses. We hypothesized that regardless of the vaccine administered, we would identify a set of genes differentially regulated in all challenged mice. Additionally, we sought to discern the unique gene expression profiles in response to challenge in each immunized group.

To perform RNAseq analysis, lungs from NECre luc mice were harvested at an early time-point (day 1) following *B. pertussis* challenge and at a late time-point (day 6 or 9).

RNA from the lungs of the mice was isolated. Libraries were prepared and sequenced on the Illumina HiSeq 1500 platform. To characterize transcriptional responses to *B. pertussis* challenge in naïve or vaccine mice (ACV, WCV, RTX), we compared the challenged samples to control mice that were not immunized nor challenged. Based on this, we expected to determine both the immunological (innate and adaptive) and the non-immunological responses to *B. pertussis* challenge. We determined the differentially expressed genes (DEGs) for both early (day 1) and late (day 6 or 9 depending on group) time-points. The total numbers of differentially regulated genes are shown in Fig. 6ac. Overall, there were more differentially genes at the later time-point than early after challenge (Fig. 6ac). At day 1 post-challenge, the WCV group had the most genes activated (Fig. 6a, Supplementary Fig. 5). Of the genes that were upregulated at day 1 in the WCV, 29.7% were not found upregulated in the other groups (464 of 1,560). At the late time-points, there were more common upregulated genes and only 14.1% of the genes were unique to the WCV. We found that 12.6% of unique upregulated genes (196) were common to all groups either challenged with *B. pertussis* following immunization or naïve challenge, suggesting that these genes were common to infection (Fig. 6b). At the later time-point, 19.8% of unique upregulated genes (867 of 6,595) were shared by the WCV, ACV, RTX groups, while this number was much lower at the early time point (8.5% of DEGs, 133 of 1,560) (Fig. 6bd). Cumulatively, this data suggests that: 1) WCV vaccination induces a more diverse early response to *B. pertussis* challenge due to the higher number of unique genes compared to other groups, and 2) at the later time-point all vaccinated groups have a more similar lung transcriptome.

Ingenuity Pathway Analysis (IPA) was performed to determine enrichment analyses of gene expression signatures in the context of each immunization. Using IPA Knowledge-based enrichment analysis, statistically significant genes were grouped into disease and function categories. A system of genes was considered enriched based on Fisher's exact test on the ratio of represented genes. At the early time-point we, observed that the largest changes were associated with genes involved in immune cell trafficking of the WCV group (Fig 7a). Genes with the highest fold changes within this system were associated with activation and migration of leukocytes (Fig. 7b). Additionally, we

observed significant enrichment of genes associated with the humoral and cell-mediated immune responses (Fig. 7b). Pertussis toxin affects multiple aspects of the cardiovascular system such as blood pressure, leukocyte migration, etc^{7,37,38}. In both naïve and immunized mice, we observed gene expression signatures related to cardiovascular diseases (Fig. 7ab). ACV immunization results in high amounts of anti-PT antibodies (Fig. 4d), and we observed fewer genes significantly altered in the ACV immunized mice, suggesting positive effects of anti-PT antibodies on neutralization of toxin activity. At the late time-point, immune cell trafficking gene expression seemed to decrease in naïve, ACV, and RTX groups; however, the expression of these genes was still high in the WCV group. As expected, immune-related gene signatures dominated the responses to *B. pertussis* infection. However, thousands of genes significantly dysregulated due to infection at both time-points were not related to immune response or were not sufficiently characterized to be assigned a function. Analysis of the lung transcriptomes corroborated the findings of flow cytometry and cytokine profile analyses, further supporting that there is an increase in leukocyte activity in the respiratory tissue of WCV immunized mice compared to ACV or RTX immunized mice following *B. pertussis* challenge.

Analysis of neutrophil-specific gene signatures

Since we observed that neutrophils accumulate in the lung of WCV immunized NECre luc mice, we chose to delve further into the changes in expression of genes associated with neutrophil activity. To compare the neutrophil response between the groups, we selected genes with GO terms associated with neutrophil migration and activation and plotted the relative fold changes for this subset of genes for each immunization group (Fig. 8). The expression levels of these genes from either the naïve challenged, or immunized and challenged groups was compared to naïve, not infected control mice (Fig. 8).

Cxcl2 is a chemokine produced by monocytes and neutrophils at sites of infection³⁹. Previously it has been shown that convalescent mice previously challenged with *B. pertussis* express lower amounts of *cxcl2* when re-challenged with *B. pertussis* than naïve mice⁴⁰. This suggests that the adaptive responses in these mice negate the need

for the innate production of *Cxcl2*. Supporting this hypothesis, we observed the highest expression levels of *Cxcl2* in naïve and RTX immunized mice, which had similar bacterial burdens (Fig. 7bd and Fig. 8be). The induction of *Cxcl2* expression was lower in ACV vaccinated mice than naïve mice (Fig. 8ac). The expression of *Cxcl2* also increased in the WCV mice, but remained high at the late time-point (Fig. 8b). Our serological analysis indicated that WCV immunization only induced minimal anti-PT production, whereas ACV induced high amounts of anti-PT (Fig. 3d). PT has been shown to induce production of *Cxcl2* in TY10 cerebral endothelial cells which further suggests a causal relationship between non-neutralized PT and *Cxcl2* production⁴¹.

All of the groups showed increases in a number of other C-X-C and C-C family chemokines (*cxcl3*, *cxcl1*, *ccl3*, *ccl4*, *ccl6*, and *ccl17*). *Cxcl1* gene expression was increased at day 1 in all groups compared to the non-infected control (Fig. 8). We also observed an increase in *IL-23* expression in the WCV group (Fig. 7b), which might be associated with the IL-17 cytokine response measured in the WCV lung tissue. Tumor necrosis factor (*tnf*) gene expression was increased in WCV, RTX, and ACV mice compared to non-infected mice. However, *Tnf* expression levels decreased in ACV mice and were lower than those observed in uninfected mice at day 9 (Supplementary Fig. 4). Ross *et al.* demonstrated that IL-17A promotes production of CXCL1 during *B. pertussis* challenge¹⁰. In our cytokine analysis, only the WCV mice had sufficient IL-17 to be detected in the lung homogenates (Fig. 5c). Taken together these data suggest that although we did not observe high *IL-17A* gene expression in the transcriptome or in the IL-17 cytokine analysis, there could still be sufficient IL-17A in the Naïve, ACV, and RTX challenged mice that could account for the increased *Cxcl1* gene expression.

We observed that *S100a9* and *S100a8* were highly upregulated in WCV and RTX groups (Fig. 7 and Fig. 8b). *S100a9* is a major calcium-binding protein of neutrophils, which forms a heterodimer with *S100a8*, and promotes polymerization of microtubules. Interestingly, *S100a8/a9* is produced mainly by neutrophils and high plasma levels of *S100a8/a9* correlate with higher blood neutrophil blood levels in healthy human adults⁴². *S100a8/a9* promotes transendothelial phagocyte migration by promoting tubulin polymerization⁴³. Additionally, monocytes isolated from human patients with cardiac

injury have been found to be particularly responsive to S100a8/a9 and secrete increased amounts of TNF- α and IL-6⁴⁴. S100a8/a9 can induce neutrophil chemotaxis and adherence, but PT can block this function of S100a8/a9⁴⁵. These data suggest that PT induction of leukocytosis may be related to the role of S100a8/a9 in leukocyte recruitment. S100a8/a9 expression was lowest in the ACV mice which had the highest amount of serum anti-PT.

Overall the WCV mice had higher expression of more neutrophil genes than any of the other groups (Fig. 7). Expression of these neutrophil signatures did not return to uninfected levels. These observations corroborates IVIS (Fig. 4de) and flow cytometry analyses (Fig. 4fg). Neutrophil gene expression in ACV mice was dynamic and returned to levels lower than uninfected mice by day 9. ACV mice did not have detectable *B. pertussis* in their lungs at day 9, indicating that the initial challenge does had been cleared (Fig. 3). ACV and WCV signatures were the most dissimilar, while the RTX and naïve mice transcriptome profiles were the most similar (Fig. 7). This correlates with the fact that RTX mice still have detectable *B. pertussis* in their nares, trachea and lungs at day 9 at levels similar to those observed in non-vaccinated mice. These data confirm our neutrophil observations but also enhance our analysis of the neutrophil dynamic in the context of vaccination status and *B. pertussis* challenge.

B cell gene expression signatures in relation to immunization status.

It has been established that B cells are required for the clearance of *B. pertussis* in mice and that ACV immunization protects through neutralizing antibodies^{46,47}. To further investigate antibody production between WCV and ACV immunized mice, we aimed to determine the diversity of B cell clone expansion between vaccinated and non-vaccinated challenged mice. Using MiXCR software, the same illumina sequenced reads mentioned above were analyzed specifically for immunoglobulin profiling⁴⁸. Individual B cell clones were identified by B cell receptor sequence and clonal expansion was accessed by immunoglobulin diversity and frequency between the vaccinated and challenged, naïve challenged, and non-challenged control groups. At the early time-point day 1 post-challenge, immunoglobulin diversity and frequency of B cells depicted a close relation between the ACV and RTX vaccination groups, as also

did WCV and naïve challenged mice (Supplementary Fig. 6a). This response shifted by the late time-point (days 6 or 9), when we observed that WCV and ACV groups were more similar. Using this approach, we were able to determine the individual variable and joining segments abundance, and the frequency of which a particular was found. Not surprisingly, we observed the highest degree of diversity at the late time-point. WCV and ACV were the most similar due to a higher number and abundance of clonotypes (Supplementary Fig. 6b). We focused our analysis on the diversity of the third complementarity-determining region (CDR3) of the heavy Ig chain, because this region has been shown to be necessary for antigen specificity⁴⁹. The highest abundant CDR3 region sequence, NQHFW, was present only in the WCV immunized mice, while the other 10 highest CDR3 regions were shared across vaccine groups at the early and late time-points (Supplementary Table 6 and Supplementary Table 7). Together this data suggests that clones generated from acellular based vaccines (ACT and RTX) are more closely related because they are generated to a limited number of antigens. However, by the late time-point, proliferation of specific B cell clones generated from WCV has created the same diversity seen in the ACV clones. The B cell clonotype analysis suggests that we can aim to further understand these populations in the future, but we will need to increase our sequencing depth by directly isolating the cell populations from the immunized mice instead of analyzing the full lung tissue. It may also be interesting to probe the T cell receptor clonotypes using the same method.

Discussion

In this study, we compared the innate and adaptive immune responses of immunized NECre luc mice vaccinated with ACV or WCV vaccines, to mice immunized with a truncated ACT toxin absorbed to alum (RTX), or non-vaccinated naïve mice following challenge with *B. pertussis*. ACV and WCV immunized mice cleared *B. pertussis* challenge but distinctive immune responses were observed. We also determined that the RTX antigen alone was not protective as a single antigen vaccine with alum adjuvant in NeCre luc mice. We utilized IVIS imaging to track the recruitment of neutrophils to the respiratory tract of challenged mice from days 1 to 9 post-challenge. The data obtained illustrated a dramatic increase in the amount of neutrophils recruited

to the lungs and nasal cavity of WCV immunized mice compared to ACV immunized mice when followed by *B. pertussis* challenge. These data were also supported by flow cytometry and cytokine analysis. Furthermore, by performing RNA sequencing on the lung transcriptome during infection in vaccinated or naïve mice, we described unique gene expression profiles depending on the vaccination status of the mice. We also used RNA sequencing to begin to describe the immunoglobulin diversity induced by each vaccine.

Upon initial *B. pertussis* challenge, we observed activation of neutrophil associated genes in all groups, however it was only in the WCV immunized mice that we saw increased neutrophil genes expression still elevated at the late time-point. Neutrophilia and elevated neutrophil related genes expression at the late time-point, when bacterial numbers in the airways are greatly reduced, indicates either over activation of neutrophil “chemokine storm,” or possibly a lack of neutralization of bacterial toxins. Multiple studies suggest that PT is responsible for neutrophilia and leukocytosis following *B. pertussis* infection^{13,32,50,51}. Immunization with the ACV induced 1,146-fold more anti-PT antibody production than WCV immunization at day 2 (Fig. 3d). It is possible that the low levels of anti-PT production in WCV mice resulted in insufficient neutralization of PT, resulting in exacerbated neutrophilia. Surprisingly, the highest neutrophil accumulation was determined at day 6 post-challenge when bacterial levels were similar to those of ACV protected mice (Fig. 3). These findings suggest that while WCV immunization is protective at clearing infection in NECre luc mice, it also induced severe neutrophilia. Interestingly, the neutrophil response in WCV mice decreased at days 2 and 4 but highly increased at day 6 (Fig. 4). At day 2, we observed a significant increase in IL-17 in the lungs of WCV mice (Fig. 5c) that could result from the increased IL-17 expression in these mice. Stephen Morse observed dose-dependent leukocytosis in mice following vaccination with killed *B. pertussis*³¹. In this study, NECre luc mice were immunized with 1/5th the human dose of WCV. We now realize that this dose is likely well above the proportional weight of a mouse compared to a human. It is possible that this high vaccine dose induced the hyper leukocytosis, similar to the dose-dependent increase that Morse observed. It is known that hyperleukocytosis is associated with death in infant cases, and that PT is responsible for inducing

leukocytosis^{1,7,52}. Furthermore, mice and baboons infected with *B. pertussis* and then treated with anti-PT antibodies had lower levels of leukocytosis compared to non-infected controls⁵⁰. An epidemiological study found that in unvaccinated individuals with pertussis, 72% of patients experienced leukocytosis⁵³. In this study, we observed that WCV immunized NECre luc mice experienced neutrophilia and morbidity, which highlights the potential issues of using WCVs. It would be interesting to determine the relationship of neutrophilia and WCV immunization in epidemiological past studies but we have not been successful in identifying a study that specifically looked at neutrophilia because studies most note general leukocytosis.

In NECre luc mice, the ACV was clearly more protective and less detrimental to the mice than the WCV. Truncated and full-length ACT purified from *B. pertussis* culture has been shown to be a protective antigen²¹. Wang *et al.* described the RTX region of ACT as highly immunogenic and easily purified as a recombinant protein²². We have observed that immunization of CD1 mice with RTX and alum adjuvant results in high anti-RTX titers (data not shown). Here, we immunized NECre luc mice with RTX and alum, but no protection was observed (Fig. 3abc). The previous studies were performed with strain 18323 and the antigen was directly isolated from *B. pertussis*^{21,54}. It is now known that strain 18323, is an outlier compared to most other global *B. pertussis* strains and Guiso *et al.*²¹ noted that strain 18323 produces less PT than the Tohama I type strain. From our work, we know that UT25 produces more PT and ACT than Tohama I (data not shown). It is possible that due to the increased PT levels produced by UT25, vaccination with RTX was not sufficient to block colonization and proliferation *in vivo*. Although RTX immunization did not result in clearance of *B. pertussis* from NECre luc mice, we did observe a reduction in the pro-inflammatory cytokine IL-6 in the RTX group compared to not vaccinated and WCV vaccinated and challenged mice (Fig. 5e). PT and ACT have both been shown to induce the production of IL-6 in human cell lines^{55,56}. The anti-ACT antibodies generated by RTX vaccination may play a role in reducing the levels of IL-6 due to reducing the activity of ACT. We hypothesize that it is necessary to neutralize both PT and ACT to provide optimal protection and in future studies, we will test RTX as an antigen in a multivalent ACV containing PT antigen.

Using standard immunological analyses, we observed typical Th2 and Th1/17 responses in ACV and WCV immunized mice respectively. *B. pertussis* utilizes PT and ACT to facilitate survival in the host and most studies about the PT/ACT specific effects have been performed on cell cultures *in vitro*. Microarray analysis has been used to profile the lung transcriptome of mice challenged with *B. pertussis*^{40,57}. Here, we sought to use RNAseq to investigate the overall gene expression profiles of the murine lung in response to *B. pertussis* challenge. Analysis of the lung transcriptome 1 day after *B. pertussis* challenge exhibited a distinct profile in mice vaccinated with WCV compared to ACV, RTX, or naïve infected mice. This response was consistent with our WCV cytokine profile demonstrating a strong pro-inflammatory response. Similarly, transcriptome data from others during early *B. pertussis* infection noted an increase in chemokines such as Cxcl2, Cxcl10, Cxcl3, Ccl3, Cxcl1, and Ccl4^{57,58}. In Raeven *et al*, convalescent mice previously infected with *B. pertussis* exhibited higher expression of these chemokines compared to naïve mice, similar to our whole cell transcriptome where these chemokines are consistently higher than the naïve mice (Fig. 7 and Fig. 8). We observed the highest expression of these chemokines in the naïve and RTX mice groups, where these groups had a similar gene profile of genes belonging to the immune cell trafficking and cardiovascular disease annotations (Fig. 7). These findings, along with higher bacterial burden (Fig. 3) suggest that RTX-alum immunization does not provide sufficient protection.

Our overall transcriptomic profiling revealed thousands of significant gene expression changes (Fig. 6). After analyzing the neutrophil (Fig. 8), and innate gene changes (Supplementary Fig. 4), we then sought to specifically characterize B cell clone diversity. The RNAseq reads were re-processed with the MiXCR algorithm and we analyzed the abundance and diversity of VDJ clonotypes (Supplementary Fig 5). Besides the T cell response differences of the ACV and WCV (Th2 v. Th1/Th17) another significant difference between these two vaccines is the number of antigens. ACVs have 3-5 antigens (PT, FHA, PRN, FIM2/3) but the WCV hypothetically has ~3,000 antigens. In light of this, it would be logical to hypothesize that the WCV would induce a vast antibody repertoire as measured by many VDJ clonotypes. However, we observed a greater diversity and abundance in the ACV group compared to the WCV at

both early and late time-points (Supplementary Fig. S6). It is important to point out that this analysis was performed on total lung RNA. If we were to isolate B cells and sequence deeper we would expect to more thoroughly characterize the repertoire. These data are interesting but we do not know which clone types result in functional antibodies that protect against *B. pertussis*. Further analysis is required to bridge the gap between clonotypes and functional/protective antibodies.

Using NeCRE luc mice, we performed tracking neutrophil recruitment during a *B. pertussis* respiratory infection. These data demonstrate how analysis of cellular responses through *in vivo* imaging can be used to provide a quantitative parameter throughout a study. This model can be applied to other bacterial infection models or cancer tumor progression models where following the same mouse throughout a study would be beneficial. In this study, we also added next generation sequencing technology to expand upon the immunological findings. RNAseq analysis can be further employed to understand key cell populations and how they are impacted by both immunization and *B. pertussis* challenge. Our current goal is to continue to refine the murine challenge models with new technological approaches in order to facilitate formulation of new pertussis vaccines. The findings of this study suggest that by integrating RNAseq analysis with classic immunological techniques, it is possible to illuminate novel intricacies of vaccine induced immunity to *B. pertussis*.

Methods

Bacteria and culture conditions

B. pertussis strain UT25 (UT25Sm1) were cultured on Bordet-Gengou (BG) agar (1906) supplemented with 15% defibrinated sheep blood (Hemostat Laboratories) for 48 h at 36°C⁵⁹. *B. pertussis* was then transferred from BG plates to three flasks of 12 ml of modified Stainer-Scholte liquid medium (SSM)⁶⁰. SSM cultures were not supplemented with cyclodextrin (Heptakis(2,6-di-O-methyl)- β -cyclodextrin). SSM cultures were grown for ~22 h at 36°C with shaking at 180 rpm until the OD₆₀₀ reached 0.5 on a 1 cm path width spectrophotometer (Beckman Coulter DU 530). The cultures were then diluted to 1 x 10⁹/ ml with SSM.

Mouse Strains

All mouse strains used were bred in a specific pathogen-free experimental conditions within the Office of Laboratory Animal Resources vivarium at West Virginia University. Mice were aged 8 – 12 weeks, male and female sex mice were equally assigned to all vaccination groups. 129-Elane^{tm1(cre)Roes}/H mice (Medical Research Council, London, UK) were crossed with FVB.129S6(B6)-Gt(ROSA)26Sortm1(luc)kael/J mice (Jackson labs; 0051225) resulting in NECre luc progeny. NECre luc mice were injected with CycLuc 1 luciferin (EMD Millipore, Darmstadt, Germany) and confirmed to be luminescent using Xenogen Lumina II⁶¹. Upon intraperitoneal injection of a luciferase substrate (luciferin), neutrophils emit luminescence that is detectable using a high sensitivity live animal imaging system (Xenogen IVIS Lumina II).

Vaccines used in study and administration

All vaccines were formulated into 200 µl doses with the antigen content described below. 100 µl of INFANRIX (GSK) human vaccine (DTaP) which is 1/5 human dose of the vaccine, was mixed in 100 µl of PBS. The NIBSC WHO standard *Bordetella pertussis* whole-cell vaccine (NIBSC code 94/532) was received lyophilized and reconstituted in 1ml of PBS. At this concentration one human dose is 100 µl therefore, 20 µl was mixed with 180 µl of PBS, which is 1/5 of the human dose (66 µg of total protein). A truncated ACT protein (RTX) was purified as previously described²² 5.6 µg of RTX was combined with 100 µl alum adjuvant (Alhydrogel®, InvivoGen) corresponding to 1 mg of aluminum hydroxide. All immunizations occurred by intraperitoneal injection. Unvaccinated mice received 200 µl of sterile PBS.

Vaccination and Challenge with *Bordetella pertussis*

NECre luc mice were bred to ages ranging from (8-12 weeks). Mice were vaccinated at day 0, and then boosted 21 days later. Thirty-five days post initial vaccination, *B. pertussis* UT25 was grown as described above, and provided as a challenge dose at 2×10^7 CFU in 20 µl. Mice were anesthetized by intraperitoneal injection of 200 µl of ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) in 0.9% saline. Two 10 µl doses of bacteria were administered through nasal inhalation into each nostril of the mouse. Mice

from each of the groups were challenged WCV (8), ACV (8), RTX (6), and naïve control (PBS injection) (5).

Collection of murine samples and determination of bacterial burden

On days 1,2,4,6, and 9 pc, mice were euthanized by intraperitoneal injection of pentobarbital and dissected in a biosafety cabinet under BSL-2 conditions. Blood was collected by cardiac puncture, serum was separated by centrifugation through a BD Microtainer SST blood collector (BD), or blood for complete blood cell counts were collected in BD Microtainer Tubes with K₂EDTA (BD). Trachea and lungs were removed, placed in 1 ml PBS, and then homogenized. Trachea tissue was homogenized by a Brinkman Homogenizer (Polytron), while lung tissue was dissociated using a Dounce homogenizer (Kimble Chase). To determine viable *B. pertussis* in the nares, 1 ml of PBS was flushed through the nares and collected. In order to determine bacterial burden 100 µl of homogenate or nasal lavage was serially diluted in sterile PBS. Four 10 µl aliquots of each serial dilution were plated on BG containing streptomycin (100 µg/ml) to ensure only UT25 *B. pertussis* grew on the plates. After 72 h at 36°C colony forming units (CFUs) were counted and the bacterial burden per tissue was calculated. Due to the serial dilutions plated our limit of detection was 10³ CFUs per ml or organ. CFUs from experimental groups at each time-point were compared to the naïve (PBS injected) and challenged group by a two-tailed unpaired t test using the software package Prism 7 (GraphPad, La Jolla, CA). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All murine infection experiments were performed per protocols approved by the West Virginia University Institutional Animal Care and Use Committee (protocol number Damron 14-1211).

IVIS imaging

NECre luc mice received 83 mg/kg of CycLuc1 luciferin by IP injection (100 µl). Five minutes after injection mice were anesthetized with 3% isoflurane, mixed with oxygen from the XGI-8 gas anesthesia system supplied with a Xenogen IVIS Lumina II. Luminescent signals were acquired during a five-minute exposure. Acquisition was performed using Living Image 2.5 software (Xenogen). Images were acquired with a

binning of 4. Following imaging, mice were either euthanized for dissection and extraction of tissue samples or returned to the vivarium. Luminescence was determined by quantification of photons emitted per second generated in each region of interest (ROI): whole mouse image or nasal cavity. The background of each image was subtracted from the ROI, background photons were defined by the average of two ROIs away from the mouse, for the same image. Data was expressed as relative fold change between target ROIs from experimental groups to an average of ROIs from control mice that were not vaccinated or challenged. Group comparisons were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparison test using Prism 7.

Preparation of tissue and flow cytometry analysis

Blood, lung, and cells isolated from nasal lavage were analyzed by flow cytometry. A 100 µl sample was removed from lung homogenate of all samples and filtered through a 70 µm cell strainer, then centrifuged at 1000 x *g* for 5 mins to pellet cells. Supernatant was removed, and RBC lysis buffer (BD Pharm lysis) added incubated at 37°C for 2 min, then pelleted using the same centrifugation conditions. Cells were resuspended in 500 µl of PBS + 1% FBS, 100µl was aliquoted for antibody staining. After cardiac puncture blood was placed in EDTA containing tube (BD Bioscience), RBC were lysed using Pharmlyse (BD Biosciences) with a 15 min room temperature incubation and then prepared in a similar manner to other tissues. All cell suspension samples were incubated on ice in PBS and 1% FBS for blocking. Antibodies against specific cell surface markers: PE-conjugated GR-1 (BD, 553128) Alexa Fluor 700-conjugated CD11b (Biolegend, 101222) were added to cell suspensions and incubated in the dark for 1 h at 4°C. Lung, blood, and nasal wash suspensions were pelleted, and resuspended in PBS prior to analysis. Samples were read using LSR Fortessa (BD), and analyzed using FlowJo v10 (FlowJo, LLC). PMNs were classified as CD11b⁺Gr-1⁺ single, live cells.

Cytokine Quantification

Lung homogenates were pelleted by centrifugation and then supernatant was collected and stored at -80°C until analysis. Concentration of cytokines in the lungs of vaccinated

and challenged mice were determined by quantitative sandwich immunoassays, Meso Scale Discovery (Rockville, MD) V-PLEX Proinflammatory Panel (K15048G-1) and Mouse IL-17 Ultra-Sensitive kits (K152ATC-1), following manufacturer's instructions. Data was analyzed by one-way ANOVA, with a Tukey's multiple-comparison test for each time-point.

Serology

Vaccinated and challenged mouse serological responses to RTX, and PT were determined by qualitative ELISA. High-binding 96-well ELISA plates were coated overnight at 4°C with 50 µl of purified RTX or Pt in PBS at a concentration of 1 µg/mL. Purified *B. pertussis* antigens, were obtained from Dr. Jennifer Maynard. Serum samples from NVNC, PBS, ACV, and WCV serum titers were analyzed for PT, as the RTX group had no PT in vaccine. Samples from NVNC, PBS, WCV, and RTX were analyzed for serum antibody titers to RTX, as ACV would not be expected to have RTX titers because no RTX was included in vaccine. Plates were then washed with PBS + 1% Tween 20 (PBS-T), then blocked with 5% milk in PBS-T for 1 hour at room temperature. Sera were diluted to a concentration in the linear dose range for each antigen. Plates were incubated for 2 h at 37°C with serial diluted serum samples from vaccinated groups. Following 3 PBS-T washes, 1:4000 goat anti-mouse IgG-AP (Southern Biotech), secondary antibody was added and incubated 1 h at 37°C. Plates were washed, then developed for 30 min with 100 µl p-nitrophenyl phosphate. Colorimetric signal was measured using Spectramax i3 (Molecular Devices) at 450nm. An average of blanks was subtracted from all absorbances and used as a baseline detection limit. The minimum detection limit above the baseline was analyzed by one-way ANOVA, with a Tukey's multiple-comparison test for each time-point using Prism 7.

Isolation of Lung RNA, illumina library preparation, and sequencing

Lung RNA was prepared similar to previously reported⁶² with the following modifications. The freshly isolated NECre luc mice lungs were homogenized and RNA was prepared immediately using RNeasy purification kit (Qiagen). Each lung was placed in 1 ml of sterile PBS and then 2 ml of TE lysozyme (1mg/ml) was added and allowed to incubate for 10 min on ice. 2 ml of RLT buffer was added and incubated for

another 10 min on ice. The homogenate was then pushed through a syringe needle. The homogenates were pelleted by centrifugation at 20,800g (max speed microfuge) for 10 min. The supernatant was extracted and then 2.8ml of 100% EtOH was added to each tube. This supernatant of each mouse sample was then disturbed to four RNeasy tubes for RNA isolation. The RNA was eluted and pooled into one sample per mouse. The resulting RNA was quantified on a Qubit 3.0 (ThermoFisher) with the high intensity assay kit. Next, the RNA integrity was assessed using Agilent BioAnalyzer RNA Pico chip. All samples were then submitted Ribo-zero rRNA depletion (illumina) and reassessed for RNA integrity. rRNA depleted mRNA samples were then fragmented and prepared into libraries using illumina ScriptSeq Complete Gold (Epidemiology). Libraries were checked for quality control with KAPA qPCR QC assay (KAPA Biosystems). The libraries (33 total) were then sequenced on an illumina HiSeq at the Marshall University Genomics Core facility on 2 lanes of 2x50B. *pertussis*. Sequencing data were deposited to the Sequence Read Archive (SRA) and are available under the reference number SRA587785, BioProject number PPJNA394758.

RNAseq bioinformatics analyses

The reads were analyzed using the software CLC Genomics workbench 9.5. *Mus musculus* genome was downloaded from NCBI (version GRCm38.78). Reads were mapped against the genome using the following settings for mapping: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8. RPKM values were generated using default parameters for CLC Genomics. On average ~20 million reads were obtained for each sample and with stringent parameters ~73% mapped to the murine genome with our mapping parameters. Fold changes in gene expression and statistical analyses were performed using an Extraction of Differential Gene Expression (EDGE) test p value. Expression data for each gene was considered significant if the p value was less than 0.05. Venn diagrams were generated using Venny 2.1⁶³. Fold-change of gene expression data was plotted relative to non-challenged control groups at each time-point. Supplementary Table 8 contains the exported gene expression analysis worksheets and statistical analyses. Gene list were created using GO terms acquired through AmiGO 2 database including neutrophil

(CL:000075), neutrophil activation (GO:0042119), neutrophil migration (GO:1990266), innate immune response (GO: 0045087), T-helper 1 type immune response (GO:0042088), type 2 immune response (GO:0042092), and T-helper 17 type immune response (GO:0072538)⁶⁴.

Pathway Enrichment Analysis

Ingenuity Pathway Analysis (Qiagen) was utilized to map lung transcriptomes to biological and disease functions. Lung transcriptomes were loaded to IPA, and comparative analyses were performed at early and late time-points. Gene expression fold-changes were mapped to higher-order disease and function defined by the IPA knowledge base, and a gene enrichment analysis was performed on early and late time-points. A Fisher Exact T-test was used to determine statistically significant ($p < 0.05$) p-values of the functions that made up the higher-order disease category and represented as a range of p-values. The relative fold-change of these identified genes was determined, and the 10 highest fold-changes were represented as heat maps.

Immunoglobulin and T-cell receptor profiling

B cell clones were identified using MiXCR software (MiLaboratory), capable of generating quantitated clonotypes of immunoglobulins⁴⁸. The same paired-end Illumina sequenced reads mentioned above were merged using concatenation, then imported into MiXCR software. Merged reads were aligned to each other to generate clonotypes of based on VDJ segment regions of unique immunoglobins specific to each sample. Clone data for each sample was grouped based on vaccine received, and time-point. Clonotypes from each sample were then separated into T and B cells, based on T-cell receptor or B-cell receptor specific sequences. Prepared data files were then imported into VDJtools (MiLaboratory), for data representation according to established protocol⁶⁵. Briefly, data was represented to show V-J diversity, and quantify unique clonotypes using dendrograms and chord diagrams.

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Author contributions

All authors participated in the composition and review of the manuscript. D.T.B. designed experiments, performed IVIS imaging, raised and dissected mice, analyzed immunological data, mapped NGS read data and calculated expression analysis. M.E.V. developed flow cytometry panels, and performed analyses. T.W. coordinated murine trials, and prepared RNA for NGS analysis. E.S.N. and E.S.K. analyzed RNAseq and developed visualizations. J.M.H. performed serological analysis. C.E., M.B., S.B., K.D., J.B., and M.E prepared and analyzed samples to measure correlates of protection on each experimental day. J.M. provided the RTX antigen. J.M., E.L.H, M.B. and F.H.D.

designed the overall strategy of the studies. M.B. and F.H.D. formulated vaccines, directed experiment days, performed dissections, and analyzed RNAseq data.

Competing financial interests

The authors declare no competing financial interests.

Supplemental Information

Supplemental information is available under Boehm et al Supplemental Information.

Figures

Figure 1: Experimental workflow for vaccination, *B. pertussis* challenge, sample acquisition, and analysis of this study.

Figure 1

Figure 1: Experimental workflow for vaccination, *B. pertussis* challenge, sample acquisition, and analysis of this study. Schematic diagram of experimental design showing vaccination schedule, immunization groups, *B. pertussis* challenge, euthanasia, sampling, and analysis method on days 1,2,4, 6, and 9 days pc in NECre luc mouse model. Mice were initially vaccinated and then given a booster dose of the same vaccine at day 21. A challenge dose of 2 x 10⁷ viable bacteria was administered by intranasal inhalation at day 35. IVIS imaging of neutrophils was monitored throughout study. The overall numbers of mice per group are indicated. At time-points shown bacterial burden, complete blood cell counts, lung cytokine profiles, lung and nasal wash neutrophil cells, serum antibody titers, and lung transcriptome were determined. To characterize the effects of vaccination, the vaccinated groups were compared to a group of not vaccinated and not challenged controls.

Figure 2: Kaplan- Meier survival curves of NECre luc mice according to immunization group.

Figure 2: Kaplan- Meier survival curves of NECre luc mice according to immunization group. The survival percentage of remaining NECre luc mice (prior to scheduled euthanasia) immunized with WCV, ACV, RTX, and *B. pertussis* challenged, or not vaccinated and challenged with *B. pertussis*. Log-rank (Mantel-Cox) test: *** $p < 0.0005$. In Fig. 1 the time-points where mice were euthanized for analysis are indicated. In this survival curve, we are only showing the mice became morbid throughout the study timeframe.

Figure 3: Bacterial burden in respiratory tissue and serological responses to *B. pertussis* challenge of immunized and naïve NCR luc mice.

Figure 3: Bacterial burden in respiratory tissue and serological responses to *B. pertussis* challenge of immunized and naïve NCR luc mice. Mice were vaccinated with PBS control vehicle, WCV, ACV, or RTX then *B. pertussis* challenged. At days 1,2,4,6 and 9 pc the bacterial burdens were determined by culturing of (A) nasal lavage, homogenates of (B) trachea and (C) lung on BG agar. The dashed line at 1000 CFUs represents the lower limit of detection, due to plating. Data in each group were compared to PBS control using an unpaired two-tailed t-test. Significant differences are not indicated on the graphs for clarity (Supplementary Table 2 indicates all statistics performed).

Figure 4: IVIS imaging and flow cytometric analysis of naïve and immunized NECre luc mice post challenge with *B. pertussis*.

Figure 4: IVIS imaging and flow cytometric analysis of naïve and immunized NECre luc mice post challenge with *B. pertussis*. IVIS imaging of luminescent neutrophils in anesthetized mice following luciferin injection. (A) Representative images of naïve, not *B. pertussis* challenged NECre luc mice. (B) Representative images of

PBS control, WCV, ACV, and RTX immunized and *B. pertussis* challenged NECre luc mice at day 1 pc and (C) day 6 pc (D and E). Luminescence was measured on Xenogen IVIS Lumina II. Relative luminescence levels quantified by fold change of emitted photons/second of NECre luc mice following *B. pertussis* challenge (N=3-5) compared to average luminescence of naïve, not challenged NECre luc mice (N=5). Neutrophil luminescence of (D) whole animal signal and (E) nasal cavity was determined at days 1,2,4,6, and 9 pc. (f) Quantification of the percentage of live, single cells classified as neutrophils (GR-1+CD11b+) detected in nasal lavage (f) and lung homogenate (g). Significant differences are not indicated on the graphs for clarity, but are included in Supplementary Table 3.

Figure 5: Analysis of cytokine profiles from lungs of naïve and immunized NCR luc mice post challenge with *B. pertussis*.

Figure 5: Analysis of cytokine profiles from lungs of naïve and immunized NCR luc mice post challenge with *B. pertussis*. Th1 associated cytokines from the supernatant of lung homogenates were analyzed at days 1,2,4 and 9 pc. Cytokines (a) IFN- γ , (b) IL-12p70, (c) IL-17, (d) IL-6, (e) IL-1 β , (f) TNF- α were quantified using electrochemiluminescence immunoassays. Significant differences are not indicated on the graphs for clarity but, are included in Supplementary Table 4. ND: Sample not determine, #: data above upper limits of detection.

Figure 6: Lung transcriptome profile of vaccinated and challenged NECre luc mice.

Figure 6: Lung transcriptome profile of vaccinated and challenged NECre luc mice. RNA sequencing was performed on total RNA from homogenized lung on days 1 (Early) or 6/9 (Late) following challenge with *B. pertussis*. (a) The total number of statically differentiated genes found in lung transcriptome at early (a) or late (c) time-points. Statistically differentiated genes were categorized as those that were activated or repressed. Venn diagram of statistically differentiated genes either unique or common to vaccinated or naïve groups at early (b) or late (d) time-points.

Figure 7: Enrichment analysis and relative fold-changes of genes upregulated following *B. pertussis* challenge.

Figure 7: Enrichment analysis and relative fold-changes of genes upregulated following *B. pertussis* challenge. Early (day 1)(a), and late (day 9)(b) IPA comparative analysis of lung transcriptomes from vaccinated and non-

vaccinated/challenged groups was performed, gene enrichment p -values are represented in $-\log$ scale. P -value was calculated by IPA software based on the number of genes found in a certain data set to the total number of genes associated to a particular function in the IPA knowledge base. Threshold indicates the significance ($p < 0.05$, Fisher's exact t-test) Black-PBS, Blue – WCV, Yellow-ACV, Red – RTX. Relative fold changes of ten highest genes associated with a particular function annotation present in either of the experimental groups.

Fig. 8: Differentiated genes associated with neutrophil recruitment at early and late time-points following *B. pertussis* challenge.

Fig. 8: Differentiated genes associated with neutrophil recruitment at early and late time-points following *B. pertussis* challenge. Gene expression profiles are shown for (a) Naïve, (b) WCV, (c) ACV, and (d) RTX immunized and challenged mice. Blue and gold bars indicate the fold change compared to control non-challenge non-vaccinated mice at early and late time-points, respectively. Genes shown are the 25 genes with the highest variance across all groups between early and late time-point. Genes were ranked based on variance in fold change across groups for the early and late time-points, separately.

Supplemental Information

Characterizing the innate and adaptive responses of immunized mice to *Bordetella pertussis* infection using *in vivo* imaging and transcriptomic analyses

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Supplementary Fig. 1: IVIS imaging of naïve and immunized NECre luc mice post challenge with *Bp* as image was acquired from Xenogen IVIS Lumina II.

Supplementary Fig. 1: IVIS imaging of naïve and immunized NECre luc mice post challenge with *Bp* as image was acquired from Xenogen IVIS Lumina II. Images at day (a) 1, (b) 2, (c) 4, and (d) 6 – WCV or 9 – Control (Non-vaccinated/ no challenge), PBS, and ACV. Relative luminescence was quantified based on region of interest luminescence in these non-adjusted images.

Supplementary Table 1. Compositions of vaccines of this study

Vaccine component*	Vaccine groups		
	aP (1/5th human dose)	wP**	RTX + alum
Pertussis Toxoid***	5	0.4	0
Filamentous Hemagglutinin	5	3.5	0
Pertactin	1.6	0.3	0
RTX	0	0.2	10
Aluminum hydroxide	125	0	1 (mg)
Other antigens/adjuvants	0	62	0

*All masses of antigens or adjuvant are indicated in µg

** This estimate is based on the number of peptides identified for each antigen in the wP. The percentage is then used to estimate the potential mass based on the fact that the wP dose used in this study contained 66 µg of total protein.

*** For total pertussis toxin, the sum of PtxA,B,C,D peptides was combined.

Supplementary Table 2. Statistical analysis from Figure 3abc.

	Day 1		Day 2		Day 4	
Trachrea	ACV	0.0524	WCV	0.0341		
	WCV	0.059				
	RTX	0.0729				
Nasal Lavage	ACV	0.0984	ACV	0.0764	ACV	0.0567
			WCV	0.0209	WCV	0.0567

Unpaired two-tailed T tests to PBS challenged group

Supplementary Table 3: Relevant statistical differences in relative fold changes of neutrophil luminescence of Bp challenged mice.

Luminescence was analyzed by one-way ANOVA with a Tukey's multiple comparison test at each time point.

	Day 1	P value	Day 2	P value	Day 3	P value	Day 4	P value	Day 6	P value	Day 9	P value
Whole Mouse	non-infected vs. PBS	0.0002	non-infected vs. PBS	0.0004	non-infected vs. PBS	0.002	non-infected vs. ACV	0.013	non-infected vs. WCV	0.0001	non-infected vs. PBS	0.0006
	non-infected vs. WCV	<0.0001	non-infected vs. WCV	0.0002	non-infected vs. WCV	<0.0001			non-infected vs. ACV	0.0035	non-infected vs. RTX	0.0076
	non-infected vs. ACV	0.0004	non-infected vs. ACV	0.0003	non-infected vs. ACV	0.0003			non-infected vs. RTX	0.0042	PBS vs. ACV	<0.0001
	non-infected vs. RTX	0.0007	PBS vs. RTX	0.0009	non-infected vs. RTX	0.0001					ACV vs. RTX	0.0003
	PBS vs. WCV	0.0007	WCV vs. RTX	0.0004	PBS vs. WCV	0.01						
	WCV vs. ACV	0.0004	ACV vs. RTX	0.0006	WCV vs. ACV	0.0102						
	WCV vs. RTX	0.0004										
	Day 1	P value	Day 2	P value	Day 3	P value	Day 4	P value	Day 6	P value	Day 9	P value
Nasal Cavity	non-infected vs. PBS	0.0002	non-infected vs. PBS	<0.0001	non-infected vs. PBS	0.0017	non-infected vs. ACV	0.0047	non-infected vs. PBS	0.0247	non-infected vs. PBS	0.0132
	non-infected vs. WCV	<0.0001	non-infected vs. WCV	<0.0001	non-infected vs. WCV	<0.0001			non-infected vs. WCV	0.0004		
	non-infected vs. ACV	0.0006	non-infected vs. ACV	0.0005	non-infected vs. ACV	0.0004			non-infected vs. ACV	0.0009		
	non-infected vs. RTX	0.0005	non-infected vs. RTX	0.0005	non-infected vs. RTX	0.0002			non-infected vs. RTX	0.0038		
	WCV vs. ACV	0.0262			WCV vs. ACV	0.0314						

Supplementary Fig. 2: Representative images and gating strategy for neutrophils from lung and nasal lavage tissues.

Lung homogenate

Nasal Lavage

Supplementary Fig. 2: Representative images and gating strategy for neutrophils from lung and nasal lavage tissues. Neutrophils were gated as live, single cells (data not shown), quadrants demonstrate positively labeled cells determined by single marker flow cytometry controls. Region of interest shows neutrophil population (Gr-1⁺CD11b⁺). (a) Lung and (b) nasal lavage representatives.

Supplementary Fig. 3: Analysis of Treg and Th2 associated cytokines from lungs of naïve and immunized NERCre luc mice post challenge with *B. pertussis*

Supplementary Fig. 3: Analysis of Treg and Th2 associated cytokines from lungs of naïve and immunized NERCre luc mice post challenge with *B. pertussis*. Cytokines were analyzed at days 1, 2, 4 and 9 pc, then quantified using electrochemiluminescence immunoassays. (a) Cytokines IL-10 and IL-5 associated with Treg immune response. (b) Cytokines IL-2 and IL-4 associated with a Th2 immune response. ND: Sample not determined.

Supplementary Table 4: Relevant statistical differences between cytokine production in lungs of immunized and *Bp* challenged NECre luc mice.

Samples were analyzed by one-way ANOVA with a Tukey's multiple comparison test at each time point.

Day 1			Day 2	
		P value		P value
IL-6	NV/NC vs. WCV	0.0251		
	WCV vs. RTX	0.0361		
IFN- γ	NV/NC vs. WCV	0.0175	NV/NC vs. WCV	0.0022
	PBS vs. WCV	0.0261	PBS vs. WCV	0.004
	WCV vs. RTX	0.0408	WCV vs. ACV	0.0162
	WCV vs. ACV	0.0257	WCV vs. RTX	0.0039
TNF- α	NV/NC vs. WCV	0.0232		
	WCV vs. RTX	0.033		
IL-1 β			NV/NC vs. WCV	0.0044
			PBS vs. WCV	0.0072
			WCV vs. ACV	0.0288
			WCV vs. RTX	0.011
IL-2	NV/NC vs. WCV	0.0224		
	WCV vs. RTX	0.0321		

Supplemental Fig. 4: Volcano plot analysis of differentiated genes at early and late time-points of immunized or naïve mice following challenge.

Supplemental Figure 4: Volcano plot analysis of differentiated genes at early and late time-points of immunized or naïve mice following challenge. Upregulated genes are represented in red, repressed genes are in blue. Relative fold change was calculated compared to not infected control mice.

Supplementary Fig. 5: Gene expression profiles of T helper cell immune responses at early time point.

Supplementary Fig. 5: Gene expression profiles of T helper cell immune responses at early time point. Differentiated genes associated with (a) Th1, (b) Th2, and (c) Th17 immune responses. Fold changes are relative to NVNC mice. Genes represented are significantly different in at least one group, genes were sorted based on variance of fold change among the experimental groups.

Supplementary Fig. 6: Repertoire of B cell clones identified from experimental groups at late time point.

Supplemental Fig. 6: Repertoire of B cell clones identified from experimental groups at late time point. (a) Relativeness of immunoglobulin profiles between vaccinated and challenged, challenged, or non-challenged groups at late time point. (b) Immunoglobulin diversity and frequency of non-challenged control, experimental groups at late time points. Chord diagrams are used to visualize variable – joining segment diversity. Outer arcs demonstrate overall count of a particular variable (upper portion of chord diagram) or joining segment (lower portion of chord diagram) in each group. Inner ribbons represent the linked segments of a clonotype, while the thickness of the ribbon signifies frequency of the clonotype.

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Chapter 3 - Evaluation of adenylate cyclase toxoid antigen in acellular pertussis vaccines using a *Bordetella pertussis* challenge model in mice.

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Abstract

Bordetella pertussis (*B. pertussis*) is the primary causative agent of pertussis (whooping cough), which is a respiratory infection that leads to a violent cough and can be fatal in infants. There is a need to develop more effective vaccines because of the resurgence of cases of pertussis in the US since the switch from the whole cell pertussis vaccines (wP) to the acellular pertussis vaccines (aP; DTaP/Tdap). Adenylate Cyclase Toxin (ACT) is a major virulence factor of *B. pertussis* that is: 1) required for establishment of infection, 2) an effective immunogen, 3) a protective antigen. The C-terminal Repeats-In-Toxin domain (RTX) of ACT is sufficient to induce production of toxin neutralizing antibodies. In this study, we characterized the effectiveness of vaccines containing the RTX antigen against experimental murine infection with *B. pertussis*. RTX was not protective as a single-antigen vaccine against *B. pertussis* challenge, and adding RTX to 1/5th human dose of aP vaccine did not enhance protection. Since the doses of aP used in murine studies are not proportionate to mouse/human body masses, we titrated the aP vaccine from 1/20th to 1/160th of the human dose. Mice receiving 1/80th human aP dose had bacterial burden comparable to naïve controls. Adding RTX antigen to the 1/80th aP base resulted in enhanced bacterial clearance. Inclusion of RTX induced: production of antibodies recognizing RTX, enhanced production of anti-PT, decreased secretion of pro-inflammatory cytokines such as IL-6, and decreased recruitment of total macrophages in the lung. This study shows that adding RTX antigen to an appropriate dose of aP vaccine can enhance protection against *B. pertussis* challenge in mice.

Introduction

Whooping cough is a disease caused by an upper respiratory tract infection that is primarily caused by the Gram-negative pathogen, *Bordetella pertussis* (*B. pertussis*) and several other *Bordetellae*. These infections, commonly referred to as pertussis, can be fatal in infants, but can also cause significant suffering in children, adolescents, and adults. Fortunately, two generations of vaccines have been developed and have greatly reduced the incidence of pertussis in the US and worldwide. Killed whole cell vaccines (wP) were first reported in 1933 ¹. With the development of the Kendrick test in 1947, wPs were standardized leading to an increase in vaccine efficacy such 80% of those immunized developed long-term immunity to pertussis ². Use of the wP resulted in drastic reduction of the incidence of whooping cough in the second half of the twentieth century. However, in the 1990s, due to reactogenicity and public concern, wPs were replaced with acellular vaccines (aP; DTaP; Tdap) containing 3-5 pertussis protein antigens and alum adjuvant ³. Since the introduction of aPs, increases in pertussis cases throughout the US and Europe have been observed and a number of reasons have been postulated to explain their lack of complete efficacy of the aPs ⁴.

One of the major issues with the aPs is that their efficacy wanes each year after administration ^{5,6}. Recent data from the non-human primate model of pertussis demonstrates that while the aPs prevent the paroxysmal whooping cough, they do not protect from colonization and transmission ⁷. In light of the increased incidence and our appreciation of the lack of efficacy of the aP, there is a need to develop another generation of vaccines with improved efficacy and duration of protection. Various approaches have been considered for the development of the new generation of pertussis vaccines, including: 1) addition of new antigens to the current aPs, 2) repolarization of the host Th-2 response triggered by alum-adsorbed aPs to a Th-1/Th-17 response by changing the adjuvant and adding new antigens, or 3) re-introduction of wPs (live attenuated or killed) either as primary immunization or as a re-polarizing booster.

B. pertussis expresses pertussis toxin (PT) and adenylate cyclase toxin (ACT) to facilitate infection and impair the host immune response in a variety of ways. Detoxified PT antigens are included in all commercial aPs. When the aP was developed, ACT was recognized as an essential virulence factor, but as of the late 1980s, ACT had not been extensively evaluated as protective antigen and was not available in purified form. It was therefore not included in aP formulations. The *cyaA* gene encodes ACT, a soluble toxin of 1,706 amino acids ⁸, comprised of an adenylate cyclase (AC) domain and a Repeats in Toxin (RTX) domain ⁹. The RTX domain is responsible for the hemolytic activity of ACT ¹⁰. The cyclase domain can be delivered to eukaryotic cells where it catalyzes ATP into supraphysiological concentrations of cAMP, which impairs many cell functions and leads to cell death ⁹. *B. pertussis* requires ACT to establish lethal infection in neonatal mice ¹¹, where the toxin targets macrophages, neutrophils, dendritic cells, and other integrin $\alpha_M\beta_2$ expressing cells ⁹. ACT has both adenylate cyclase and hemolytic activities and its hemolytic activity is required for *B. pertussis* penetration into the lung parenchyma ¹². Underscoring its role in disease, AC enzyme activity has been detected in airway samples from a non-human primate model of pertussis infection ¹³, and humans ¹³, and convalescent humans have antibodies against ACT ^{14,15}. Furthermore, adenylate cyclase activity can be detected in wPs ¹⁶ and in our own mass spectral analysis we have confirmed the presence of ACT in wP vaccines (unpublished).

In light of the role of ACT in virulence and the fact that *B. pertussis* infection induces production of anti-ACT antibodies, ACT has been evaluated as a protective antigen in pre-clinical murine immunization/challenge models. Before aPs replaced wPs in the US and Europe, Guiso *et al.* demonstrated that a polypeptide consisting of the AC domain purified from *B. pertussis* was a protective antigen in mice ¹⁷. The same group demonstrated that full length toxin purified from *B. pertussis* was protective (16). Subsequent studies showed that recombinant ACT was also a protective antigen ^{18,19} and an adjuvant ²⁰. While immunization with ACT results in toxin neutralizing antibodies, the presence of these antibodies do not correlate directly with protection. However, antibodies that recognize the C-terminal domain (RTX) are protective ¹⁹. These studies

were further supported by evidence that the hemolytic domain only antigen (RTX) was protective in mice against sub-lethal and lethal challenge with *B. pertussis* ¹⁹. In all of the aforementioned studies, ACT antigens were evaluated as single antigen formulations without any of the aP antigens. Cheung *et al.* observed that inclusion of enzymatically inactive recombinant ACT enhanced protection of the aP ²¹, and that unlike previous single antigen studies, neither active nor enzymatically inactive ACT protected alone ²¹. The study also showed that inclusion of ACT in the aP skewed the T-helper cell responses from a humoral Th2 response to a more cell-mediated Th1 response ²¹.

Multiple studies have demonstrated the protective capacity of ACT ⁸. In order to determine the appropriate form of ACT for use in a human vaccine, it is necessary to identify a protective toxoid form that would be readily purified for mass production. Genetic mutation of the ACT coding sequence results in an enzymatically inactive form/molecule that is protective in mice ²¹. However, the use of a full length ACT would be problematic to for use in human vaccines due to: 1) the susceptibility of the toxin to proteolytic degradation ²² and 2) overexpression of ACT leads to formation of inclusion bodies in the *E. coli* cytoplasm ²³⁻²⁵. A recent study confirmed that ACT is prone to aggregation and proteolysis ⁹. Wang *et al.* characterized a recombinant RTX antigen that was 1) readily purified, 2) immunodominant, and 3) elicited neutralizing antibody production ²⁶. While these results are very promising, it is essential to determine the efficacy of recombinant RTX antigen in pre-clinical models of pertussis, both alone and in combination with aP. It is likely that a new aP vaccine would still include PT, FHA, and FIM antigens. Our objective was to investigate the RTX antigen with the standard aP antigens as a multivalent formulation. In this present study, inclusion of the RTX antigen into an aP formulation was evaluated with a murine immunization and challenge model. We observed that immunization with RTX alone was not protective and furthermore when either RTX or full length ACT was added to the 1/5th human dose, no significant enhancement of protection occurred. The use of high amounts of the human aP in mice (such as 1/4th, 1/5th, or 1/10th human dose) relates back to doses intended for commercial vaccine lot validation. It is known that as little as 1/50th human dose of

aP is protective in mice ²⁷. We hypothesized that identification of the minimal protective and maximal non-protective aP doses would allow us to investigate enhancement of protection by inclusion of new antigens, thereby refining this evaluation platform. By performing a vaccine titration study using two-fold dilutions, we were able to identify 1/80th human dose of aP as a minimal protective dose. When the RTX antigen was added into the 1/80th human dose, improved bacterial clearance was observed. Inclusion of RTX antigen also enhanced antibody production to PT demonstrating the adjuvant capacity of RTX. The enhanced protection correlated with 1) decreased pro-inflammatory cytokine production, 2) decreased blood and lung neutrophils, and 3) decreased macrophages in the lung. This study begins to redefine murine immunization and *B. pertussis* challenge model for evaluation of pertussis vaccines and sheds light on the positive effects of including RTX as an antigen in the acellular vaccines.

Materials and Methods

ACT and RTX antigen expression and purification

ACT and RTX antigens were expressed and purified as previously described ⁹. Following purification, antigens were stripped of endotoxin with Dextoxi-GelTM Endotoxin Removing Gel (Thermo Scientific, 20399) and Zeba Spin columns (Thermo Scientific, 89896). Endotoxin purity was quantified with Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, 88282).

Composition of vaccines used in study and administration

The INFANRIX (GSK) human vaccine (diphtheria, tetanus, and pertussis) was the aP used in this study. For the purpose of vaccinating mice, 1/5th of the human dose was used (100 µl). To test ACT as an additional antigen, full length ACT or RTX-ACT were purified as previously described ⁹. Equimolar amounts of full length ACT (10 µg) or RTX (5.6 µg) were used for vaccination. Vaccine compositions are shown in Table S1. Alum and MPLA were obtained from InvivoGen. The National Institute for Biological Standards and Control WHO standard whole cell pertussis vaccine (NIBSC code 94/532) was used as the wP. One ampule of NIBSC wP contains 40 IU (10 human

doses). For murine vaccination, the whole ampule was diluted in 1 ml of sterile PBS and 20 μ l (1/5th of the human dose, 66 μ g of total protein) were used. The NIBSC wP standard used in this study has been used in other studies as a wP vaccine ²⁸. The NIBSC wP does not contain alum adjuvant, whereas commercial wP vaccine contain alum to induce antibody production to the Diphtheria and Tetanus toxoids. The adjuvant alum was only added to single antigen vaccines (Table S1). INFANRIX is formulated with alum, thus we did not include additional alum for aP titration and aP supplementation with RTX. Unvaccinated mice received 200 μ l of sterile phosphate buffered saline (PBS) as vehicle control. Prior to administration, aP, aP-ACT, aP-RTX, and wP doses were all diluted into a final volume of 200 μ l with sterile PBS and administered by intraperitoneal injection.

***B. pertussis* strains and growth conditions**

B. pertussis strain UT25 (UT25Sm1) ²⁹ was cultured on Bordet Gengou (BG) agar (Remel) ³⁰ supplemented with 15% de-fibrinated sheep blood (Hemostat Laboratories) for 48 h at 36 °C. Bacteria were transferred from BG plates to a flask with 20 ml of modified Stainer-Scholte liquid medium (SSM) ³¹. SSM cultures were not supplemented with cyclodextrin (heptakis). *B. pertussis* cultures in SSM were grown for ~22 h at 36°C with shaking at 180 rpm at which time the OD₆₀₀ was ~0.6.

Vaccination and challenge with *B. pertussis*

Four week old CD-1 (outbred; strain code 022) mice were obtained from Charles River. Mice were vaccinated at 5 weeks of age with the vaccines described (Table S1), and then boosted with the same vaccines 21 days later. Thirty-eight days post initial vaccination, *B. pertussis* UT25 was grown as described above, and cultures were diluted to provide a challenge dose of 2×10^7 colony forming units (CFU) in 20 μ l. Mice were anesthetized by intraperitoneal injection of 200 μ l of ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) in 0.9 % saline. Mice received 20 μ l total challenge dose by pipetting 10 μ l directly into each nostril of the mouse. Four to eight mice per group were challenged, and at 1, 3, and 8 days post infection, mice were euthanized. Blood was

collected by cardiac puncture and serum was separated by centrifugation through a BD Microtainer blood collector and stored at -80°C until analysis. Trachea and lungs were removed and separately homogenized with Dounce homogenizers for determination of bacterial burden in each tissue. To determine the number of *B. pertussis* in the nares, 1 ml of PBS was flushed through the nares and collected. Serial dilutions in PBS were plated on BG containing streptomycin (100 µg/ml) to ensure that only UT25 *B. pertussis* were cultured. All murine infection experiments were performed according to protocols approved by the West Virginia University Animal Care and Use Committee (protocol number 14-1211.10).

Cytokine analysis

Lung homogenates were pelleted by centrifugation and the supernatants were collected and stored at -80°C until analysis. The concentration of cytokines in the lungs and serum of vaccinated and challenged mice was determined by Raybiotech Mouse Inflammation Array C1 (AAM-INF-1-8) following the manufacturer's instructions. A total of 100 µl of lung homogenate supernatants or serum were pooled from 4 mice for each analysis. Quantitative analysis of the concentration of cytokines in the lungs mice was determined Meso Scale Discovery V-PLEX Proinflammatory Panel (K15048G-1) or Mouse IL-17 Ultra-Sensitive kits (K152ATC-1) per the manufacturer's instructions.

Adenylate cyclase toxin neutralization assay

ACT is, through a combination of mechanisms, cytotoxic for J774A.1 (ATCC® TIB-67™) macrophage-like cells and protection against this effect is the basis for the toxin neutralization assay ³². J774A.1 cells were cultured in Dulbecco's modified Eagle's medium with 25 mM glucose (Gibco). J774A.1 cells (30,000 in 90 µl) were seeded in each well of a 96-well plate and allowed to attach overnight. ACT was incubated with serum samples for 10 min with mixing at 4°C, and added to the cells at a final ACT concentration of 80 ng/ml. Cells were then incubated at 37°C for 2 h. The number of viable cells was determined using the CCK8 assay (Dojindo Laboratories), which measures reduction of WST-8, a water-soluble tetrazolium salt, by dehydrogenases in

viable cells. Percent viable cells was determined for ACT alone and for serum + ACT compared to control cells by the following equation $[(\text{treatment} - \text{media without cells}) / (\text{control} - \text{media without cells})] \times 100$.

Preparation of flow cytometry samples and analysis

Blood, lung, and cells isolated from nasal lavage or lung were analyzed by flow cytometry. A 100 μl sample was removed from lung homogenate of all samples and filtered through a 70 μm cell strainer, then centrifuged at 1,000 $\times g$ for 5 min to pellet cells. Supernatant was removed, Pharmlyse red blood cell (RBC) lysis buffer (BD Biosciences) was added, and samples were incubated at 37°C for 2 min before being pelleted by centrifugation at 1,000 $\times g$ for 5 min. Cells were finally re-suspended in PBS + 1% Fetal Bovine Serum (FBS). To prepare blood samples for flow cytometry, blood collected by cardiac puncture was placed in ethylenediaminetetraacetic acid (EDTA) containing tube (BD Biosciences) and RBC were lysed using Pharmlyse with a 15 min room temperature incubation. Cells were then pelleted by centrifugation at 1,000 $\times g$ for 5 min and re-suspended in PBS + 1% FBS. Cells isolated by nasal lavage are in a single cell suspension by nature, thus only required centrifugation at 1,000 $\times g$ for 5 min to concentrate samples. All cell suspension samples were incubated on ice in PBS and 1% FBS for blocking for 15 min. Antibodies against specific cell surface markers were added to cell suspensions and incubated in the dark for 1 h at 4°C: PE-conjugated GR-1 (BD, 553128), Alexa Fluor 700-conjugated CD-11b (Biolegend, 101222), PerCP/Cy5.5-conjugated F4/80 (Biolegend, 123128). Lung, blood, and nasal lavage suspensions were pelleted and resuspended in PBS prior to analysis. Samples were read using an LSR Fortessa (BD) flow cytometer, and analyzed using the software FlowJo v10 (FlowJo, LLC). Neutrophils and macrophages were classified as CD-11b⁺Gr-1⁺ single, live cells or CD-11b⁺F4/80⁺ single, live cells, respectively.

Serological analysis of *B. pertussis* specific antibodies

Vaccinated and challenged mouse serological responses to RTX ²⁶, PT (LIST Biologicals), filamentous hemagglutinin (FHA; Enzo Life Sciences), and

lipooligosaccharide (LOS; LIST Biologicals) were determined by enzyme-linked immunosorbent assays (ELISA). High-binding 96-well ELISA plates were coated overnight at 4°C with 50 µl of purified RTX, PT, FHA in PBS at a concentration of 1 µg/mL or LOS at 5 µg/ml. IgG2a monoclonal m1B7³³ (anti-PT) and IgG2a monoclonal M1H5²⁶ (anti-RTX) were obtained from Dr. Jennifer Maynard. To measure FHA or LOS responses, polyclonal murine anti-FHA (Cat #JN1H-11, NIBSC) or murine IgG3 monoclonal antibody D26E (Thermo Scientific) were used, respectively. Plates were washed with PBS + 1% Tween 20 (PBS-T), then blocked with 5% milk in PBS-T for 2 hr at 37°C. Sera were diluted to a concentration in the linear detection range for each antigen. Plates were incubated for 2 hr at 37°C with serially diluted serum samples from vaccinated groups. Following 3 PBS-T washes, goat anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (AP) (Southern Biotech) was diluted at 1:2,000 in blocking buffer and added to each well, and incubated 1 h at 37°C. Antibody standards were prepared from anti-PT, anti-FHA, anti-LOS, anti-RTX by 2-fold dilutions from 100 µg/mL. Plates were washed, then developed for 30 min with 100 µl *p*-nitrophenyl phosphate. Colorimetric signal was determined by measuring sample absorbance at 450nm using Spectramax i3 (Molecular Devices). Serum antibody concentration was calculated by plotting absorbance values of samples to absorbance of known concentrations of control antibodies. Values were plotted using a logarithmic scale, and fit to a four-parameter equation. Relative EC50 was determined by fitting the absorbance values of half of the maximal response to the four-parameter equation. Dilution of EC50 was determined by plotting the calculated antibody sample concentrations to sample dilution factors and fit to a four-parameter logistic curve³⁴. Dilutions were accounted for by multiplying antibody concentration by dilution factor.

Statistical Analysis

Experiments in this study were performed with a minimum of four biological replicates. Data were statistically analyzed with GraphPad Prism 7. Comparisons between single groups were performed using unpaired two-tailed *t*-tests and comparisons between multiple groups were performed using one-way ANOVA with a Tukey test for multiple comparisons.

Heat map modeling and visualization of the effects of immunization on correlates of protection

A master file containing all of the data collected during this project was compiled and linear regressions were performed for each variable measured in response to vaccination and challenge (Matlab, Mathworks) to identify variables that correlate with overall protection against *B. pertussis* challenge. A subset of those variables were then extracted and used to plot visual representations of the overall correlates of protection. For each variable, modulations were calculated as the difference between the averaged responses of the non-challenged / non-vaccinated mice, and the averaged response of the mice in each experimental group. To establish a colorimetric scale, green was set as equal to the non-challenged / non-vaccinated mice, red was used for the maximal relative increase for that variable, and blue was used for the minimal relative value for that variable.

Results

Immunization with RTX alone does not enhance clearance of *B. pertussis* in CD-1 mice.

ACT plays a key role in the pathogenesis of *B. pertussis* and many studies have shown that both native and recombinant ACTs can be adjuvants and/or antigens for protection against pertussis in murine challenge studies^{17-20,35-39}. In this study, we aimed to systematically evaluate the RTX antigen (toxoid version of ACT) for inclusion into the acellular pertussis vaccine. Wang *et al.* previously described that recombinant RTX antigen was immunogenic and readily purified, making it an attractive candidate for further evaluation. In our previous study using NECre luc mice (transgenic strain specifically used for tracking neutrophils in live mice⁴⁰), we did not observe protection when mice were immunized with RTX + alum adjuvant but we did observe the presence of antibodies that recognize RTX antigens via ELISA (manuscript in review). Here, we immunized outbred CD-1 mice with the RTX antigen (Table S1) formulated with alum, which is a Th2 adjuvant, or monophosphoryl lipid A, (MPLA) which is a TLR4 agonist

that induces Th1 responses. CD-1 mice were immunized intraperitoneally with PBS vehicle control (naïve) or 1/5th human dose of aP as a positive control for protection. Twenty one days later the mice were boosted with the same vaccines. Two weeks after boost, each of these groups of mice were challenged with 2×10^7 CFU of *B. pertussis* strain UT25 by intranasal administration. When this challenge dose of *B. pertussis* is intranasally administered to anesthetized mice, 26% of the initial dose is recoverable from the lung at 30 min post challenge and only 5% (10^6 CFU) is recoverable at 4 h post challenge (data not shown). These data suggest that many of the *B. pertussis* that were instilled by intranasal administration are killed by innate immune responses during the early stages of infection. However, this dose is sufficient to establish respiratory infection, as 4×10^6 CFU can be recovered in the airways 24 hr post challenge in naïve (PBS control immunized) CD-1 mice (Fig. 1A).

At one and three days post *B. pertussis* challenge, we enumerated the viable bacteria in the lungs, nares and trachea of naïve, aP, RTX+alum, and RTX+MPLA immunized CD-1 mice (Fig. 1A and Fig. S1). The aP immunized mice had 99.75% less *B. pertussis* at day 1 post challenge than naïve mice (Fig. 1A), and by day 3, the bacterial load was at or below our detection limit of 100 CFUs. However, mice immunized with RTX+alum or RTX+MPLA, were not significantly protected as they contained the same amount of viable *B. pertussis* as naïve mice (Fig. 1A and Fig. S1AB). Interestingly, ELISAs were performed to measure anti-ACT antibodies and showed that mice vaccinated with RTX in combination with alum or MPLA induced detectable levels of antibodies in sera and lung homogenates (data not shown).

Vaccination with wP, aP, aP+RTX, and or aP+ACT protect against *B. pertussis* challenge. We hypothesized that inclusion of RTX into a standard acellular formulation would enhance protection against *B. pertussis* challenge in mice. To test this hypothesis, we compared aP and wP to aP supplemented with ACT or RTX in a murine vaccination challenge study. CD-1 mice were immunized as indicated in Table S1 and boosted three weeks post initial vaccination. At day 38 post initial vaccination, the mice were challenged with the *B. pertussis* strain UT25. At 1, 3, and 8 days post challenge, mice were euthanized for determination of bacterial burden and collection of tissues and

samples for analysis. Naïve mice harbored similar amounts of *B. pertussis* in the lungs (Fig.1B), trachea and nares (Fig. S1CD) as those observed previously (Fig. 1A). wP and aP-vaccinated mice had significantly lower bacterial loads in the airways than non-vaccinated mice, and aP mice had the lowest bacterial burden at day 1 and 3. By day 8, all wP or aP immunized mice had cleared the infection to our detection limit. (Fig. 1B). Overall these data show that in CD-1 mice with UT25 as challenge strain, the 1/5th human dose of aP is protective and that at that dose, adding RTX or ACT does not enhance bacterial clearance.

Serological analysis of naïve, wP, aP, aP+ACT, and aP+RTX immunized mice.

ELISAs were performed and we observed that alum and MPLA formulated RTX or ACT vaccines, induced antibodies that recognize ACT (data not shown). Additionally, 1/5th aP supplemented with either RTX or ACT also induced detectable antibody production. Surprisingly, we did not observe any antibodies that would recognize ACT in the wP immunized mice (data not shown). The lack of anti-ACT in wP immunized mice is likely due to the fact that when we analyze the wP with shotgun proteomics, we only observe 4 peptides of ACT per 1,000 total peptides in the whole cell vaccine, which shows ACT makes up a minor amount of the total protein content (data not shown).

AC toxin neutralizing capacity of serum antibodies.

Due to the lack of noticeable effects on the rate of clearance of *B. pertussis*, it was unclear that those antibodies influenced clearance of *B. pertussis* in the mouse. ACT has potent toxic effects against CD-11b/CD-18-expressing phagocytes, such as macrophages or neutrophils⁴¹⁻⁴³. To determine the functional capacity of the serum antibodies induced via immunization, we utilized the AC toxin neutralization assay with J774A.1 cells³². Only sera obtained from mice vaccinated with aP+ACT or aP+RTX were able to neutralize the toxicity of ACT in J774A.1 macrophages (Fig. 1C). We did not observe detectable anti-ACT antibodies in the serum from wP mice, and this serum did not neutralize ACT *in vitro* (Fig. 1C). The AC toxin neutralization data suggest that immunization with RTX can induce production of antibodies that can neutralize the AC toxin *in vitro*.

Identification of the optimal aP murine dose to determine synergy between aP and RTX. Our data and previous published studies ⁹ show that RTX is immunogenic and could contribute to protection against *B. pertussis* when used as an antigen. However, we hypothesized that the dose of aP used (1/5th human) in these studies is too high to observe synergy between the aP and RTX. Therefore, we sought to define a physiologically appropriate aP dose, for the mouse, that could be used to evaluate the effects of inclusion of new antigens into the aP. Guiso et al. demonstrated that 1 full human dose of DTaP was most protective (SmithKline Beecham Biologicals) ⁴⁴ and that 1/4th and 1/10th of the human dose were protective yet required more time to result in sterilizing immunity compared to a full human dose. Alvarez *et al.* ⁴⁵ used 1/5th human dose and supplemented with an iron regulated protein, AfuA (*BP1605*) and observed modest improvement in bacteria clearance compared to 1/5th human DTaP (INFANRIX®; GSK). In other recent studies, 1/50th human human DTaP (INFANRIX®; GSK) was partially protective in BALB/c mice challenged with B3 lineage strain B1917 ^{27,46}. These two studies encouraged us to determine if lowering the aP dose could allow us to detect measurable improvements in bacterial clearance when additional antigens improve the vaccine.

CD-1 mice were immunized mice with aP doses of 1/20, 1/40, 1/80, and 1/160th human dose (Table S2) to titrate the vaccine and identify both the effective or non-effective doses. These mice were challenged with *B. pertussis*, and viable bacteria in the lungs, trachea, and nasal lavage were determined at 1 and 3 days post challenge (Fig. 2). At day 1 post challenge, the 1/20th aP group had the least bacterial burden and at day 3, both the 1/20th and 1/40th were near our detection limit (Fig. 2AB) in the lungs. A similar trend was observed in the trachea and nasal washes (Fig. 2CDEF). However, the 1/80th aP group had 3.5 log more viable *B. pertussis* in the lungs than the 1/40th aP group, suggesting 1/80th was below the aP dose for maximum protection in CD-1 mice (Fig. 2B). The non-effective dose for trachea seemed to be higher than 1/160th (Fig. 2CD), whereas the non-effective dose for the nasal lavage was 1/160th (Fig. 2EF). Based on these data, we selected the 1/40th (minimal effective dose) and the 1/80th (maximal non-effective dose) to supplement with AC toxoid antigen (RTX).

We hypothesized that neutralization of ACT *in vivo* would enhance bacterial clearance and that inclusion of RTX would improve the protection provided by the 1/80th dose as demonstrated by decreasing viable bacteria in the lung. Testing the inclusion of RTX in the 1/40th dose would allow for evaluation of other subtler variables such as cytokines production, phagocyte recruitment, and serology. CD-1 mice were therefore immunized with either 1/40th or 1/80th dose aP supplemented with RTX antigen (Table S2). Inclusion of RTX into the 1/40th aP did not affect the bacterial burden at day 3 in the lungs (Fig. 2B) compared to 1/40th aP alone, as we predicted. When RTX was added to 1/80th aP, we saw a significant decrease in viable bacteria in the lungs at day 3 (Fig. 2B). In light of this observation, it was necessary to measure other metrics in addition to viable bacteria to further support this finding.

In the trachea and nasal lavage, we observed the similar inverse relationship between bacterial burden and decreasing vaccine concentration as in the lungs. In the trachea, there was minimal improvement of bacterial clearance with inclusion of RTX to the 1/40th or 1/80th base aP. In the nares, adding RTX to 1/40th aP decreased the bacterial burden at day 1 but this difference was not significant ($p = 0.29$), and no differences were observed at day 3. Overall, these data highlight the need to consider the location of *B. pertussis* in the host in respect to vaccine efficacy in a challenge trial and also the utility of identifying sub-effective murine vaccine doses to determine if there are effects of adding new antigens into a formulation.

Serological analysis of antibodies recognizing PT and RTX antigens.

In our aP immunization titration strategy, we expected that as the aP dose decreased, the concentration of anti-PT in the serum of immunized and challenged mice would also decrease. Given that mice immunized with 1/40th aP had minimal bacterial burden in all respiratory tract locations (Fig. 2), we hypothesized that 1/40th aP would induce production of high concentrations of anti-PT antibodies in serum. We also hypothesized that inclusion of the RTX antigen into the 1/40th dose would result in production of significant amount of anti-RTX antibodies. As expected, immunization with RTX in both 1/40th and 1/80th aP doses induced production of antibodies that recognize the RTX

antigen (Fig. 3AB). Surprisingly, there was very little difference between the amounts of detectable anti-RTX in 1/40th and 1/80th despite the fact that there was 50% less alum adjuvant in 1/80th compared to 1/40th. We also observed that as we decreased PT antigen and alum adjuvant by diluting the vaccine, less anti-PT was produced in response to *B. pertussis* challenge (Fig.3CD). At day 1 we saw a dose response curve for anti-PT respective of the aP dose (Fig. 3C). Anti-PT decreased as would be expected since PT antigen and alum adjuvant are decreasing respective to the vaccine dose.

The addition of RTX into the 1/40th and 1/80th doses significantly increased antibody production to PT compared to same dilution of aP alone ($p = 0.045$ and $p = 0.0039$, respectively) at day 1 post challenge. Yet these levels of anti-PT drastically decreased at day 3 post challenge (Fig. 3D). This was a puzzling observation. There are several possibilities to explain this phenomenon. These ELISAs are from serum samples and it is possible that the high amounts of anti-PT have left the serum and are in the lung or upper respiratory tract closer to where the pathogens are infecting. The second possibility is that neutralization of ACT *in vivo* could decrease antibody production, which is less required due to bacterial clearance. More kinetics studies should be performed on these dual antigen considerations, especially with antigens like PT and RTX which both are classified as immunogens due to their adjuvant properties.

Analysis of cytokine secretion as a function of aP immunization and inclusion of RTX antigen.

Due to the fact that including RTX into the 1/80th aP dose helps lower the bacterial burden in the lungs, we then characterized lung cytokine profiles to better understand the type of immune signaling factors released during infection and their association with bacterial clearance. To do so, we measured cytokines in lung homogenate of vaccinated and naïve mice at 1 and 3 days post-challenge. IL-6 is one of the most important cytokines involved in the immune response to *B. pertussis*. Respiratory epithelial cells and macrophages have increased IL-6 mRNA and protein in response to *B. pertussis in vitro* ^{47,48} and C57BL/6 mice have high IL-6 secretion in response to *B.*

pertussis infection ⁴⁸. Specifically, ACT has been shown to induce IL-6 expression ⁴⁹. Furthermore, IL-6 knockout mice have increased *B. pertussis* burden in the respiratory tract compared to wild type mice due to decreased recruitment of leukocytes ⁴⁸. Based on these studies and our preliminary cytokine data (Fig. S2), we anticipated that as we titrated the aP dose down, we would observe increased IL-6 correlating with the increased bacterial burden (Fig. 4AB). If antibodies against RTX can neutralize ACT *in vivo*, we expected to observe decreased IL-6 levels in RTX vaccinated mice. Our data confirmed this idea at day 1, but at day 3 post challenge, RTX did not affect IL-6 secretion in the 1/80th vaccine dilution group (Fig. 4B).

IL-6 is related to induction of Th17 responses, and in the case of *B. pertussis* infection, PT facilitates induction of IL-17 production typically around 7 days post *B. pertussis* challenge ⁵⁰. At day 1, there were no statistically significant changes were observed in IL-17 across the groups. However, at day 3, as the aP dose decreased, IL-17 was slightly increased until it was significantly different at 1/160th human dose (Fig. 4D). It was surprising to observe that naïve infected mice did not have significantly more IL-17 than mice vaccinated with the 1/160th dose. We interpret these data to mean that: 1) 1 and 3 days are early for IL-17 responses to *B. pertussis* as we saw biological variability within the groups and 2) these diluted vaccines are likely capable of eliciting PT-neutralizing antibodies. We did not observe any effect on IL-17 concentrations due to including RTX into either the 1/40th or 1/80th (Fig. 4CD).

B. pertussis challenge of mice induces interferon gamma (IFN- γ) expression, which is necessary for clearance of *B. pertussis* from mice ⁵¹. Furthermore, IFN- γ receptor deficient mice challenged with *B. pertussis* display atypical disease leading to dissemination of *B. pertussis* and death of the mice within several weeks ⁵². Since aP immunization results in Th2 polarization, we expected that as the aP vaccine was titrated to less effective doses, we would see an increase in IFN- γ induced by the natural Th1 response to *B. pertussis* infection ⁵³. At day 1, the 1/40th aP and the 1/80th aP displayed similar IFN- γ levels in the lung. However, when RTX was added to the 1/80th we observed a striking reduction of IFN- γ at day 1 only. It is also interesting to note that naïve infected mice had lower IFN- γ than 1/40th, 1/80th, or 1/160th aP groups at

day 1 (Fig. 4E), yet high IFN- γ was observed in the naïve infected mice at day 3 (Fig. 4F).

IL-1 β mediates clearance of *B. pertussis* and resolution of inflammation in mice ⁵⁴. IL-1 β deficient mice have high amounts of IL-17 that are observed at 7 days post challenge and high IFN- γ at 14 days post challenge ⁵⁴. We wondered if neutralization of ACT *in vivo* would alter IL-1 β production in the lungs of the mice. All challenged mice had very similar IL-1 β levels at day 1 (Fig. 4G) and the levels of IL-1 β were lowest in the 1/20th aP group at day 3. Addition of RTX did not significantly decrease IL-1 β which suggests IL-1 β may be induced independently of ACT activity or bacterial burden.

We measured other cytokines such as IL-5, TNF- α , KC Gro, IL-12p70, IL-10, IL-2, and IL-4, in the lung homogenates of the vaccine groups (Fig. S3 and S4). Vaccination with aP+RTX resulted in lower IL-5 at day 1 in both 1/40th and 1/80th groups, yet these groups returned to base aP IL-5 levels at day 5 (Fig. S4). IL-2 followed a similar trend as IL-5 (Fig. S4). We observed that Th1 related responses such as IFN- γ were decreased by the addition of RTX to the aP.

Neutrophil infiltration kinetics are not affected by inclusion of RTX into the aP.

Pertussis is characterized by an increase in the number of circulating white blood cells. This phenomenon known as leukocytosis can lead to lung hypertension and death in the most severe cases. ACT targets the CR3 receptor expressed on many cell types including neutrophils ⁵⁵. Based on this, we hypothesize that neutralization of ACT *in vivo* would alter neutrophil kinetics and recruitment profiles in the blood and airway. Andreassen and Carbonetti reported that in naïve mice, neutrophils did not play a role in clearance of *B. pertussis* from the airway ^{50,56}. On the other hand, neutrophils played an essential role in immune mice in the same study. Another study showed: 1) that PT inhibited recruitment of neutrophils to the lung and neutrophils played a role in antibody directed clearance of *B. pertussis* by the Fc γ receptor ⁵⁷. As the dose of the aP decreased, we observed an increase in lung and blood neutrophils by the 1/160th neutrophil percentages were similar to those of naïve *B. pertussis* challenged mice. It is

likely that as the aP dose decreased, there was less PT neutralization due to a decrease in anti-PT titers. Lung neutrophil populations of mice immunized with inclusion of RTX into the 1/40th and 1/80th doses were significantly reduced ($p = 0.0142$ and $p = 0.008$, respectively) compared to mice immunized with aP only at the same doses (Fig. 5C); however, by day 3, this reduction was no longer significant because neutrophil levels had returned to levels observed in non-challenged mice (Fig. 5D). We observed lower circulating neutrophil populations in the blood at both days when RTX was included compared to aP only doses (Fig. 5F) (1/40th: $p = 0.018$, 1/80th: $p = 0.071$). Collectively, these data suggest that inclusion of RTX into the aP vaccine impacts the neutrophil populations in the respiratory tissues and circulating neutrophils in the blood.

Decreased macrophages in the respiratory tract due to inclusion of RTX into the aP.

Macrophages are a primary target of ACT and are readily killed by the toxin ^{41,43}. We hypothesized that as ACT is neutralized *in vivo*, that there would be an increase in macrophages due to their increased survival. However, our data did not support that hypothesis, and instead we saw that both RTX supplemented aP groups had lower macrophages in the nasal lavage at day 1 compared to aP only. Inclusion of RTX decreased the overall numbers of macrophages in both the 1/40th and 1/80th base aP groups (Fig. 6AB). At day 3, we observed that mice vaccinated with 1/40th, 1/80th and 1/160th aP showed increasing amounts of macrophages compared to naïve and naïve challenged mice. As observed for other parameters measured, this effect might be due to an induction of a naïve Th1 response due to insufficient aP driven Th2 as a result of our decrease in aP dose.

Visualizing and modeling the relationships between: antibodies against *B. pertussis* toxins, pro-inflammatory signals, phagocytes, and clearance of *B. pertussis* from the murine respiratory tract.

The Kendrick test was used to establish units of efficacy for whole cell vaccines in mice to estimate the dose to be used in humans ². The murine intranasal infection model was

then developed to validate aP vaccines ^{44,58}. Since the introduction Tdap, numerous pre-clinical studies have been performed to evaluate new antigens or adjuvants to improve aPs. However, we argue that there is a need for standardization of the murine evaluation platform to facilitate the development of these formulations, and transition to other models such as the baboon. In this study, we titrated DTaP and found a dose ($1/80^{\text{th}}$ human) that was partially protective with the *B. pertussis* strain (UT25) and the challenge dose of 2×10^7 CFU that were used. It is clear that decreases in bacterial burden are indicative of protection; however, we aimed to visually represent all the data collected to support our conclusions. For this comparison, we represented the increases and decreases for each variable relative to the no vaccine / no challenge group using a 64 color scheme (Fig. 7). If the relative units increases, the color changes to red, or if it decreases then the value is represented in blue. We then plotted the average values per each immunization group ($1/20$ - $1/160^{\text{th}}$) in relation to the RTX supplemented groups. As one visually compares $1/80^{\text{th}}$ to $1/80^{\text{th}}$ +RTX at day 1 or 3, it is apparent that adding RTX relatively decreases all values towards the green or no relative change, which we interpret as overall protection. It is also important to note that values at day 1 are relatively higher than day 3 and this relates to the fact that the total amount of bacteria are decreasing. The amount of anti-PT or anti-RTX is shown in black to grey to white color scale (Fig. 7). There is a clear correlation with amount of anti-PT and overall protection. In addition, it is interesting to see that inclusion of RTX enhanced the anti-PT levels in the aP+RTX groups. Overall, variables associated with inflammation such as cytokines or phagocytes tend to return to green or naïve non-infected levels in mice vaccinated with aP+RTX. We only observed five relatively large decreases (indicated in blue). The $1/20^{\text{th}}$ aP group had relatively lower KC/GRO which could be associated with the anti-inflammatory Th2 response due to the high amount of alum in the immunized mice. We also saw a relative decrease in number of macrophages present in the nasal wash at day 3 in the naïve group. This decrease could be attributed to high amounts of PT and ACT secreted during infection which could be impairing normal macrophage recruitment in the airway. We also saw large relative decreases in macrophages in the RTX supplemented $1/40^{\text{th}}$ and $1/80^{\text{th}}$ groups at day 3 and reasons for these changes were described above. Combining these data allow us to consider the overall

inflammatory response in relation to the vaccination status, to further demonstrate the effects of including RTX into aP formulations, and to begin to establish a multi-variable approach to determining efficacy of experimental vaccines in the mouse.

Discussion

There is a resurgence of whooping cough that coincides with the switch to the use of aP vaccines. In this study, we aimed to accomplish two goals. First, we sought to define an aP vaccine dose, or doses, that allow the evaluation of synergy between the current aP and potential new antigens. The second goal was to evaluate RTX as an antigen for inclusion in acellular pertussis vaccines in the murine challenge model. As a result of these studies, we now better appreciate the need to continue to refine the methodologies of improving aP vaccines.

We initiated this study by evaluating the efficacy of ACT and RTX as single vaccine antigens. Immunization with ACT or RTX alone did not induce significant protection in CD-1 mice with the UT25 *B. pertussis* challenge strain (Fig. 1). The AC domain of ACT was first described as a protective antigen by Guiso *et al*¹⁷. In that study, the antigen preparation that was used consisted of two polypeptides of 43 and 45 kDa. We now know that these polypeptides corresponded to the AC domain of ACT. In those studies, BALB/c mice were vaccinated twice with AC polypeptide absorbed on aluminum hydroxide adjuvant and it was observed that 80% of the AC-vaccinated mice survived *B. pertussis* challenge with strain 18323¹⁷. The aforementioned study was then further supported by a subsequent study by the same group that showed the protective capacity of the AC domain and full length recombinant ACT protein³⁵. Several more studies showed that: 1) CyaC acylation of ACT was required for the protective activity¹⁸ and 2) that the C-terminal domain was also required for protection with recombinant ACT. Full length recombinant ACT was protective when 15 µg of antigen were mixed and absorbed with 250 µg of alum¹⁹. We used less antigen and adjuvant than Betsou *et al*. (either 10 µg of ACT or 5.6 µg of RTX with 125 µg of alum) and we did not observe protection in CD-1 mice (Fig. 1A), and the use of the MPLA adjuvant did not improve bacterial clearance compared to alum. An apparent difference between our study and

that of Betsou *et al.* is that strain 18323 was used, which has been suggested to be an outlier compared to most other global *B. pertussis* strains. Guiso *et al.* noted that strain 18323 produces less PT than the Tohama I type strain ³⁵. From our own studies, we know that UT25 produces more PT and ACT than Tohama I (data not shown). This information highlights the importance of the challenge strain. Another caveat of our study is that Betsou *et al.* absorbed the ACT antigen to alum, which we did not with RTX. It is possible that absorbing RTX to the aP could further enhance the synergistic protection observed in this study when RTX was combined with the alum-containing aP. Another key aspect to consider is the potential endotoxin contamination in recombinant ACT preparations. Villarino *et al.* used recombinant ACT but did not observe the same bacterial clearance as Betsou *et al.* ¹⁹. ACT used by Cheung *et al.* was highly purified of endotoxin ²¹, as was the RTX that we used in this study ⁹. It is possible that endotoxin levels in ACT preparations may influence the overall protection afforded by such antigens as they may induce a more Th1-biased response.

Several of the aforementioned studies indicated that single antigen vaccines containing ACT were protective but the study by Cheung *et al.* and our RTX-only study (Fig. 1) were not in concordance. Other recent studies testing single antigen vaccines composed of proteins expressed during biofilm or in response to iron starvation showed that very few of these single antigen formulations were protective on their own ^{27,46}. Given that the aP antigens of either DTaP or Tdap (PT, FHA, PRN, and +/- FIM) do provide some protection against disease, we hypothesize that new antigens to be included in the aP should be tested not only alone, but also in combination with aP antigens. Only one study has investigated ACT as an antigen in combination with the aP in mice ²¹. Cheung *et al.* reported that adding 12.5 or 25 µg to 1/8th aP, significantly decreased bacterial burden in mice at 7 days post challenge with strain 18323 ²¹. PT and FHA are formulated in the DTaP (INFANRIX®) at 25 µg each in 1 human dose. Therefore, the use of 25 µg ACT is roughly equal to 1 human dose of antigen (assuming no adjustment for molar mass of each antigen). We chose to test the utility of RTX antigen with lower antigen doses in an effort to best model what would be scaled towards a commercial vaccine dose. To do so, we identified a partially protective dose

of aP, so synergistic effects of addition of the antigen to be tested. We titrated the aP vaccine from 1/20th to 1/160th human dose. As decreasing aP doses were tested, we saw an increase in innate immune responses to levels observed in naïve challenged mice (Fig. 7). From the bacterial burden in the lung, we determined that 1/40th dose of aP is protective, while 1/80th is only partially protective. However, it is important to note that 1/40th aP immunization was non-protective in the nares at day 1, but at that dose, *B. pertussis* was cleared by day 3 (Fig. 2EF). These data show that the protective capacity of an aP dose in the mouse is dependent on the location in the airway and the time elapsed since *B. pertussis* challenge. Using total clearance in all locations, 1/40th aP immunization results in sterilizing immunity against our challenge dose as observed at three days post challenge. Based on these data, we selected the 1/40th (minimal effective dose) and the 1/80th (maximal non-effective dose) to supplement with AC toxoid antigen (RTX).

We identified the 1/80th aP dose that would allow us to tease out RTX antigen effects that would otherwise be masked by the use of a high mouse to human aP dose (e.g. 1/5th). With the 1/80th aP dose, it is clear that innate responses are occurring due to the lack of protection provided by the immunization. We hypothesize that decrease in innate responses would correlated with induction of antibody production that either: neutralizes the effects of the pathogen (e.g. toxins: PT or ACT) or results in killing of the pathogen (e.g. opsonization, phagocytosis, or complement killing). We were able to detect antibodies that recognized RTX (Fig. 3AB) and we saw that adding RTX enhanced production of anti-PT (Fig. 3A). When we supplemented the 1/80th aP dose with RTX, we were able to observe positive effects on the response to *B. pertussis* challenge such as decreased: bacterial burden (Fig 2B), IL-6 secretion (Fig. 4A), IFN- γ (Fig. 4E), IL-5 (Fig. S4), IL-2 (Fig. S4). We speculate that the neutralization of ACT *in vivo* leads to a decrease in the innate immune responses at day 1 post challenge, but at day 3, the levels of these pro-inflammatory cytokine were not affected by the inclusion of RTX (Fig. 4BF and S4). We observed that *B. pertussis* challenge increased neutrophil levels in the nasal lavage and that these levels were not reduced to non-challenged levels during our study (Fig. 5AB). Neutrophils in both the nares and lungs were overall decreased by day

3 post challenge in the RTX supplemented groups. Adding RTX antigen to the 1/40th and 1/80th vaccines decreased the total neutrophils in the lungs at day 1 and same trend was observed for blood neutrophils (Fig. 5EF). Interestingly, we observed that adding RTX to aP (1/40th nor 1/80th) did not affect nasal neutrophils amounts (Fig. 5AB). We speculate that ACT may have specific effects in the lung and not in the nares of mice, but more experiments are necessary test this hypothesis.

ACT can induce apoptosis in macrophages ⁵⁹ and it is also known that IL-12 production by macrophages plays a role in vaccine-mediated clearance of *B. pertussis* ⁶⁰. We measured the number of macrophages in the lungs and nasal lavages of aP or RTX supplemented aPs and saw less macrophages when RTX was added to the aP (Fig. 6ABCD). This data was somewhat counterintuitive but Skopova *et al.* have shown that cAMP signaling and hemolysin activity synergize and provoke PMN and macrophage influx into the lungs ¹². Our macrophage observations suggest anti-RTX may be neutralizing ACT *in vivo*. There are also two more possibilities: 1) there may be less macrophage recruitment because there are less bacteria or 2) if macrophages are less impaired (due to ACT neutralization), they may be effectively migrating to the lymph nodes to present antigens. At this point, we do not have sufficient data to fully understand the relationships between the amount of macrophages in the airway and if that is an indication of protection and/or an effect of ACT neutralization.

B. pertussis strains have evolved in response to aP immunization ⁶¹. Pertactin is no longer expressed by circulating *B. pertussis* strains ⁶² and recently, a *B. pertussis* strain was isolated and sequenced that lacked PT ⁶³. *B. parapertussis* is not capable of expressing PT ⁶⁴ but causes as many as ~15% of whooping cough cases. *B. holmesii* is an underappreciated pathogen that cause whooping cough-like illness and does not express PT ⁶⁵. In addition, there are unpublished reports of strains sequenced by the CDC that lack expression of filamentous hemagglutinin. Given the potential rise of triple mutant strains that do not make pertactin, FHA, or PT, it is possible that the efficacy of aP protection will further decline if strains arise that lack the main antigens that most aP vaccines contain. Based on these possibilities, we propose that adding additional antigens that are both immunogenic and important for bacterial virulence is necessary

to slow the progression of *B. pertussis* evolution ⁶⁶, and the resistance to aP-mediated immunity.

We aimed to investigate ACT as a vaccine antigen and we developed a new evaluation approach involving analysis of multiple correlates of protection (Fig. 7). Our data suggest that RTX antigen may enhance protection of aP vaccines, based on what we observed in CD-1 mice. The different protection levels observed in each of the regions of the airway (nares, trachea, and lungs) suggest that it may also be advantageous to consider the development of mucosal *B. pertussis* vaccines. This would allow for the pathogen to be neutralized rapidly in the upper airway before infection is fully established when highly potent PT and ACT are impairing the innate and adaptive immune responses. Higgs *et al.* ⁶⁷ proposed that aP vaccines containing antigens that direct a more diverse response, may be more effective. While we have focused on adding RTX to a Th2 inducing aP, it would be very interesting to formulate and test RTX containing vaccines with Th1 adjuvants. Neutralization of ACT along with a cellular response would likely be an ideal protection profile and possibly address the waning protection issue of the aP. Our study was focused on RTX but there are other candidates that should be evaluated as well. Our study highlights the importance of titrating the vaccine dose and we expect this will provide new methodologies for the development of pertussis vaccines. Significant research is still necessary to formulate and evaluate safe and more effective next generation pertussis vaccines, which could include ACT as an antigen.

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Figures

Figure 1. RTX only immunization does not protect against *B. pertussis* challenge in CD-1 mice nor does including RTX with 1/5th aP improve clearance kinetics but ACT neutralizing antibodies are produced.

Fig

Figure 1. RTX only immunization does not protect against *B. pertussis* challenge in CD-1 mice nor does including RTX with 1/5th aP improve clearance kinetics but ACT neutralizing antibodies are produced. (A) CD-1 mice were vaccinated with PBS vehicle, 1/5th human dose of aP, RTX (5.6 μ g) + MPLA (20 μ g), RTX (5.6 μ g) + Alum (125 μ g), or PBS as control vehicle (naïve) (n=4, with 4 technical replicates). Mice were IN challenged with 2×10^7 *Bp* (UT25Sm1). At days 1 and 3 pc the bacterial burdens were determined by culturing of homogenates of the lungs on BG agar. The dashed line represents the lower limit of detection due to plating. Bacterial loads in mice vaccinated with aP were significantly lower than the PBS control group at day 1 ($p = <0.0001$) and day 3 ($p = <0.0001$) in the lung. (B) CD-1 mice were vaccinated with PBS control vehicle, wP, aP, aP + ACT or aP + RTX and challenged intranasally with UT25Sm1 (n=4, with 4 technical replicates). Mice vaccinated with aP, aP+ACT, and aP+RTX had a significantly lower bacteria burden than the PBS control group at day 1 ($p <0.0003$, p

<0.0006, $p < 0.0013$) in the lung. Bacterial loads in all immunized mice were significantly lower than PBS group at day 3 and 8 ($p < 0.0001$; all groups) in the lung. Data in each group were compared to every other group at day 1, 3, or 8 pc using one-way ANOVA with Tukey's post hoc test. (C) Serum from vaccinated/challenged mice was used to determine if ACT neutralizing antibodies were produced following RTX or ACT inclusion in aP vaccine by adenylate cyclase toxin neutralization assay. J774A.1 macrophages were cultured with active ACT, and serum from mice. Neutralization of ACT results in increase of macrophage survival. Serum from vaccinated mice with aP+ACT and aP+RTX resulted in neutralization of ACT, compared to unvaccinated, aP, and wP groups. Results are shown as mean $\pm$ SEM, ****, $p < 0.0001$ *** $p < 0.001$ by one-way ANOVA.

Figure 2. Respiratory tract bacterial burden of CD-1 mice immunized with aP vaccines or aPs supplemented with RTX antigen.

Figure 2. Respiratory tract bacterial burden of CD-1 mice immunized with aP vaccines or aPs supplemented with RTX antigen. Immunization with 1/20th, 1/40th, 1/80th, and 1/160th of human dose aP was used to titrate the minimal effective dose and the maximum non-effective dose. RTX (5.6 µg) was included into 1/40th and 1/80th aP doses. Vaccinated CD-1 mice were challenged with 2×10^7 *Bp*, and bacterial burden was determined at days 1 and 3. CFU counts were determined from (AB) lung homogenate, (CD) trachea homogenate, and nasal lavage (EF). Results are mean $\pm$ SEM (n=4-8, with 4 technical replicates) from independent experiments, ****, $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ determined by one-way ANOVA with Tukey post hoc test compared to naïve mice (PBS control). The dashed line represents the lower limit of

detection due to plating. The blue boxes indicate aP groups whereas aP+RTX are indicated by gold triangles. Gray squares indicate mock vaccinated, and challenged mice. In E and F there were no statistically significant comparisons between the aP titration doses as determined by ANOVA.

Figure 3: Serological responses from mice immunized with aP titrations or aPs supplemented with RTX.

Figure 3: Serological responses from mice immunized with aP titrations or aPs supplemented with RTX. (AB) ELISA was used to determine the anti-RTX IgG titers in serum of immunized and challenged mice at 1 and 3 days pc. (CD) Anti-PT IgG titers in serum of immunized and challenged mice at indicated time points. Titrations of aP were compared by one-way ANOVA with Tukey's post hoc test, shown below graphs and compared to PBS group. Unpaired two-way T test was used to compare addition of RTX to aP titrations, shown by brackets. Results plotted on graph are mean $\pm$ SEM, *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$.

Figure 4: Analysis of proinflammatory cytokines from lungs of mice immunized with aP titrations or aPs supplemented with RTX.

Figure 4: Analysis of proinflammatory cytokines from lungs of mice immunized with aP titrations or aPs supplemented with RTX. Th1 associated cytokines from supernatant of lung homogenates analyzed at days 1 and 3 pc. Cytokines (AB) IL-6, (CD) IL-17, (EF) IFN- γ , and (GH) IL-1 β . The blue boxes indicate aP groups whereas aP+RTX are indicated by gold triangles. Gray squares indicate mock vaccinated, and challenged mice, while white squares represent non-vaccinated and non-challenged mice (NC). Results are mean $\pm$ SEM, **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$ by one-way ANOVA with Tukey post hoc test compared to PBS (naïve) but challenged mice. Brackets between two points represent unpaired two-way T test. Dashed line indicates upper limits of detection, and dotted line indicates lower limit of detection.

Figure 5: Flow cytometric analysis of blood, pulmonary, and nasal lavage neutrophils from mice immunized with aP titrations or aPs supplemented with RTX.

Figure 5: Flow cytometric analysis of blood, pulmonary, and nasal lavage neutrophils from mice immunized with aP titrations or aPs supplemented with RTX. Quantification of the percentage of live, single cells that were classified as neutrophils (GR-1^{hi}CD-11b⁺) from (AB) nasal lavage, (CD) lung homogenate, or (EF) blood. Results are mean $\pm$ SEM, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ by one-way ANOVA with Tukey post hoc test compared to PBS (naïve) but challenged mice. Brackets between two points represent unpaired two-way T test.

Figure 6: Flow cytometric analysis of macrophages from lung and nasal lavage of mice immunized with aP titrations or aPs supplemented with RTX.

Figure 6: Flow cytometric analysis of macrophages from lung and nasal lavage of mice immunized with aP titrations or aPs supplemented with RTX. Percentage of macrophages (defined as CD-11b⁺F4/80⁺) from live, single cells from (AB) nasal lavage and (CD) single cell suspension prepared from lung homogenate. Results shown are mean $\pm$ SEM, * p <0.05 by one-way ANOVA with Tukey post hoc test compared to PBS (naïve) but challenged mice. Brackets between two points represent unpaired two-way T test.

Tables

Table 1: Composition of vaccines for Fig. 1

TABLE 1 Compositions of vaccines used for Fig. 1

Vaccine component	Value for vaccine group ^a :					
	RTX + alum	RTX + MPLA	aP (1/5 human dose)	aP + ACT	aP + RTX	wP ^b
Pertussis toxoid ^c	0	0	5	5	5	0.4
Filamentous hemagglutinin	0	0	5	5	5	3.5
Pertactin	0	0	1.6	1.6	1.6	0.3
Adenylate cyclase toxin or RTX	5.6	5.6	0	10	5.6	0.2
Aluminum hydroxide	0	125	125	125	125	0
Other antigens/adjuvants	0	20	0	0	0	62

^aAll masses of antigens or adjuvant are indicated in μg .

^bThis estimate is based on the number of peptides identified by mass spectrometry for each antigen in the wP (data not shown). The percentage is then used to estimate the potential mass based on the fact that the wP dose used in this study contained 66 μg of total protein.

^cFor total pertussis toxin, the sum of PtxA, -B, -C, and -D peptides was combined.

Table 2: Composition of vaccines used in the titration of aP and RTX supplementation studies

TABLE 2 Compositions of vaccines used in the titration of aP and RTX supplementation studies for [Fig. 2 to 6](#)

Vaccine component	Value for vaccine group ^a :					
	1/20 aP	1/40 aP	1/40 aP + RTX	1/80 aP	1/80 aP + RTX	1/160 aP
Pertussis toxoid	1.25	0.63	0.63	0.33	0.33	0.16
Filamentous hemagglutinin	1.25	0.63	0.63	0.33	0.33	0.16
Pertactin	0.4	0.2	0.2	0.1	0.1	0.05
Adenylate cyclase toxin or RTX	0	0	5.6	0	5.6	0
Aluminum hydroxide	31.25	15.63	15.63	7.81	7.81	3.91

^aAll masses of antigens or adjuvant are indicated in μg .

Supplemental Figures

Figure S1. RTX only nor RTX supplemented aP immunizations alter *B. pertussis* clearance in CD1 mice.

Figure S1. RTX only nor RTX supplemented aP immunizations alter *B. pertussis* clearance in CD1 mice. (A-B) CD1 mice were vaccinated with PBS vehicle, aP, RTX adjuvanted with alum or MPLA and challenged intranasally with *B. pertussis* strain UT25. At days 1 and 3 post challenge the bacterial burdens were determined by culturing of nasal wash, homogenates of trachea on Bordet Gengou (BG) agar. Experiments were performed with 4 mice per group with 4 technical replicates. The dashed line represents the lower limit of detection. (CD) CD1 mice were vaccinated with PBS control vehicle, wP, aP, aP + ACT or aP + RTX and challenged intranasally with UT25. At days 1, 3, and 8 post challenge the bacterial burdens were determined. Experiments were performed with 4 mice per group with 4 technical replicates.

Figure S2. Mouse inflammatory cytokine array of lung homogenate supernatant.

Figure S2. Mouse inflammatory cytokine array of lung homogenate supernatant. Lung homogenate supernatant from 4 mice in each group were pooled and used to probe membranes labelled with antibodies for 40 inflammatory cytokines. Pseudocolor image of day 3 cytokine production are shown in duplicate. The control group is not challenged with *B. pertussis*.

Figure S3. Cytokine analysis of lung homogenates of CD1 mice immunized with various aP doses and supplemented with RTX antigen.

Figure S3. Cytokine analysis of lung homogenates of CD1 mice immunized with various aP doses and supplemented with RTX antigen. Lung homogenates were collected from mice of each of the groups indicated and the concentration of each cytokine indicated was determined. Minimal detection limits are indicated. Error bars show standard error of the mean. The blue boxes indicate aP groups whereas aP+RTX are indicated in gold.

Figure S4. Cytokine analysis of lung homogenates of CD1 mice immunized with various aP doses and supplemented with RTX antigen.

Figure S4. Cytokine analysis of lung homogenates of CD1 mice immunized with various aP doses and supplemented with RTX antigen. Lung homogenates were collected from mice of each of the groups indicated and the concentration of each cytokine indicated was determined. Minimal detection limits are indicated. Error bars

show standard error of the mean. The blue boxes indicate aP groups whereas aP+RTX are indicated in gold.

Figure S5: Serological responses from mice immunized with aP titrations or aPs supplemented with RTX.

Figure S5: Serological responses from mice immunized with aP titrations or aPs supplemented with RTX. (AB) ELISA was used to determine the anti-RTX IgG levels in serum of immunized and challenged mice at 1 and 3 days pc. (CD) Anti-PT IgG levels in serum of immunized and challenged mice at indicated time points. Antibody concentration (µg/ml) represented were determined using relative EC50. Serum absorbance values were logarithmically plotted to known antibody standards using a four-parameter logistic curve. Relative EC50 was then determined by fitting serum concentration to dilutions of samples using a four-parameter logistic curve. Titrations of aP were compared by one-way ANOVA with Tukey's post hoc test, shown below graphs. Unpaired two-way T test was used to compare addition of RTX to aP titrations, shown by brackets. Results plotted on graph are mean $\pm$ SEM, *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$.

Figure S6

Figure S6. Representative images and gating strategy for neutrophils from blood of CD1 mice immunized with various aP doses and supplemented with RTX antigen. (A) Cells were first gated on live, single cells. Then classified as either (B) neutrophils (CD11b⁺Gr-1^{Hi}) or (C) macrophages (CD11b⁺F4/80⁺). Quadrants demonstrate positively labeled cells determined by single label controls marker flow cytometry controls. Region of interest shows neutrophil population (Gr-1⁺CD11b⁺).

Figure S7. Heat map modeling of the effects of base aP dose and supplementation of RTX antigen on correlates of protection in mice.

Figure S7. Heat map modeling of the effects of base aP dose and supplementation of RTX antigen on correlates of protection in mice. In order to understand the key variables collected in this study, in relation to base aP dose and the effects of adding RTX, we developed a heat map that shows the relative increase or decrease of each variable. The color scale indicates relative changes in bacterial burden, cytokines, or phagocytes whereas the black-grey-white scale indicates relative toxin specific antibodies in the serum of each group.

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Chapter 4- Intranasal acellular pertussis vaccine provides mucosal immunity and protects mice from *Bordetella pertussis*

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Abstract

Current acellular pertussis vaccines fall short of optimal protection against the human respiratory pathogen *Bordetella pertussis* resulting in increased incidence of a previously controlled vaccine preventable disease. Natural infection is known to induce a protective mucosal immunity. Therefore, in this study, we aimed to use acellular pertussis vaccines to recapitulate these mucosal immune responses. We utilized a murine immunization and challenge model to characterize the efficacy of intranasal immunization (IN) with DTaP vaccine or DTaP vaccine supplemented with curdlan, a known Th1/Th17 promoting adjuvant. Protection from IN delivered DTaP was compared to protection mediated by intraperitoneal injection of DTaP and whole-cell pertussis vaccines. We tracked fluorescently labeled DTaP after immunization and detected that DTaP localized preferentially in the lungs while DTaP with curdlan was predominantly in the nasal turbinates. IN immunization with DTaP, with or without curdlan adjuvant, resulted in anti-*B. pertussis* and anti-pertussis toxin IgG titers at the same level as intraperitoneally administered DTaP. IN immunization was able to protect against *B. pertussis* challenge and we observed decreased pulmonary pro-inflammatory cytokines, macrophage and neutrophil infiltrates in the lung, and bacterial burden in the upper and lower respiratory tract at day 3 post challenge. Furthermore, IN immunization with DTaP triggered mucosal immune responses such as production of *B. pertussis*-specific IgA, and increased IL-17A. Together, the induction of a mucosal immune response and humoral antibody-mediated protection associated with an IN administered DTaP and curdlan adjuvant warrant further exploration as a pertussis vaccine candidate formulation.

Introduction

Pertussis is a human upper respiratory disease, primarily caused by the Gram-negative pathogen *Bordetella pertussis*. The disease is most severe in unvaccinated infants, where it manifests as fits of paroxysmal coughs followed by the classical whoop as air re-enters the respiratory system, leading to the more commonly known name: whooping cough.¹ Additional complications include vomiting, increased mucus production, and apneic episodes. In severe cases, these symptoms can lead to elevated leukocytosis, pulmonary hypertension, hypoxia, and in some cases death.¹⁻³ Furthermore there is speculation that pertussis may enhance development of neurodegenerative diseases later in life.⁴

In the pre-vaccine era, pertussis was responsible for 200,000 deaths annually in the United States.⁵ Widespread use of diphtheria tetanus whole-cell pertussis (DTP; wP) vaccines controlled the incidence of pertussis.⁶ However, adverse side effects associated with wP immunization¹ led to the development of an acellular pertussis vaccine (DTaP; aP) that was introduced into the schedule in 1996. In the United States the number of pertussis cases has been increasing since the 1990s, despite high vaccine coverage.⁷ Additionally, increasing numbers of cases have been documented in older children, adolescents and adults.⁸ The increase in incidence of pertussis cases suggests that current aP vaccines do not offer complete protection from *B. pertussis* infection. Multiple hypotheses have been proposed explaining the resurgence of pertussis cases including: (1) waning of protective immunity from DTaP/tdap⁹⁻¹¹, (2) vaccine driven evolution of *B. pertussis* strains¹², (3) increased carriage and transmission through asymptomatic carriers¹³, and (4) increased surveillance and more accurate diagnoses technology.

Vaccine-induced protection in aP immunized individuals has been associated with a robust antigen-specific IgG response to the components of the aP vaccines.¹⁴⁻¹⁷ Likewise, wP immunization also resulted in antigen-specific IgG responses; with the addition of a shift to a more diverse T cell response, inducing cell-mediated immunity.¹⁸ In the murine model, immunization through intramuscular (IM) and intraperitoneal (IP) administration has been well characterized demonstrating a Th1/Th17 response from

wP immunized mice, and a Th2 with weak Th17 mediated response in aP immunized mice following *B. pertussis* challenge.¹⁹ However, these immunizations fail to induce the mucosal immune responses elicited from natural infection. Recent studies have revealed that protection correlates with tissue resident memory T (T_{RM}) cells in the lung and nasal cavity of convalescent mice, that produce interleukin-17 (IL-17) and interferon-gamma (IFN- γ).²⁰ T_{RM} cells have been shown to persist in the respiratory tissue and expand upon re-challenge of a convalescent mouse with *B. pertussis*, as well as decrease bacterial burden upon adoptive transfer to naïve mouse.²¹ The study of this population has renewed interest in the induction of a mucosal immune response capable of decreasing bacterial burden at the site of infection.

The induction of a mucosal immune response to *B. pertussis* is associated with the production of secretory IgA antibodies (sIgA) in the nasal cavity. In humans previously infected with *B. pertussis*, IgA antibodies have been isolated from nasal secretions.²² *B. pertussis*-specific IgA antibodies isolated from convalescent patients have been shown to inhibit adherence of *B. pertussis* to respiratory epithelial cells *in vitro*²³, suggesting a protective role of IgA antibodies in mucosal immunity. Initial studies using IgA-deficient mice did not show strong support for a critical role in bacterial clearance of the respiratory tract.²⁴ However, work with a live-attenuated IN pertussis vaccine (BPZE1) recently demonstrated protective role of sIgA antibodies in the respiratory tract.²⁵

DTaP vaccine does not contain a strong pro-inflammatory adjuvant such as endotoxin of wP vaccine or BPZE1. We aimed to investigate IN DTaP immunization alone or with an additional pro-inflammatory adjuvant. We formulated vaccines containing the adjuvant curdlan, a 1,3 β -glucan, derived from *Alcaligenes faecalis*. This polysaccharide has immunostimulatory properties and forms a “sticky” gel at a neutral pH. Importantly, curdlan has been shown to bind to dendritic cells through the ligand Dectin-1, thereby inducing expression of NF- κ B²⁶ leading to a Th1/Th17 mediated immune response as well as production of antigen-specific respiratory IgA antibodies and serum IgG antibodies.^{26–29} Our objective was to determine whether IN DTaP immunization would induce a protective immune response in a murine model. We observed that the gel properties of curdlan facilitated DTaP localization in the upper respiratory tract. We

measured serum and respiratory antibody responses that correlated with protection from bacterial challenge. This study suggests mucosal vaccination with acellular vaccine may be a strategy for decreasing incidence of pertussis.

Results

Acellular pertussis vaccine was retained in the upper and lower respiratory tract when administered by intranasal administration.

Pertussis toxin facilitates infection by disrupting innate immune responses in the airway.³⁰ Therefore, we hypothesized that neutralization of pertussis toxin at the site of infection is essential for protection against *B. pertussis*. We propose that intranasal immunization would prime a protective mucosal immune response. Furthermore, the gel-like properties of curdlan may have a beneficial role in increasing antigen uptake. To determine if use of curdlan would increase vaccine retention in the respiratory system, we IN vaccinated CD-1 mice with commercially available DTaP (IN-aP), DTaP with curdlan (IN-caP), or PBS (mock vaccinated) and tracked the vaccine up to 48 h after vaccination (Fig. 1a). Using *in vivo* animal imaging, we observed fluorescently labeled DTaP particles in the nasal cavity at 6, 12 and 24 h post-vaccination (Fig. 1b). At 12 h post-vaccination, we detected significantly higher levels of fluorescence in IN-caP vaccinated mice compared to IN-aP mice (Fig. 1c), suggesting more DTaP particles were retained in the nasal cavity. This method resulted in the quantification of total particles in nasal cavity. To quantify DTaP particles that were bound to innate immune cells we utilized flow cytometry. Single-cell suspensions were prepared from homogenized lung tissue and antigen presenting cells (APCs) bound to DTaP were quantified as live, single cells positive for CD11b⁺DTaP⁺ (Fig. 1d). We observed a significant increase in CD11b⁺ cells that were bound to or contained DTaP particles in IN-aP mice compared to IN-caP (Fig. 1e). Together, these data suggest a higher deposition of DTaP in the lung with IN-aP when compared to IN-caP. Conversely, in the nasal cavity we measured higher levels of DTaP when mice were vaccinated with IN-caP, compared to IN-aP. These findings suggest that addition of curdlan to the DTaP vaccine causes retention in the nasal cavity, but without it, the vaccine components are more readily detected in the lung.

To visualize the deposition of DTaP particles, sections from the lung and nasal cavity were imaged using confocal microscopy. Vaccinated mice were euthanized after 6 h, lung tissue was flash frozen and skulls were embedded in paraffin for sectioning. Sections from the lung and nasal cavity were counterstained with NucBlue and ActinGreen to visualize epithelial tissue and fluorescent DTaP particles (Fig. 2ac). Vaccine particles were quantified by measuring the percentage of total image field emitting DTaP fluorescence. We detected a significant increase of fluorescent particles in the lungs of mice that were vaccinated with IN-aP compared to IN-caP (Fig. 2b). Using microscopy, there was no significant difference in the number of particles detected in the nares comparing IN-aP to IN-caP (Fig. 2d). Interestingly, we observed that DTaP particles from the IN-aP vaccinated mice were localized in the lumen of the nasal passages, while particles from IN-caP vaccinated mice were deposited into the epithelial cells (Fig. 2c). Overall these data suggest curdlan impacts localization of DTaP in the airway.

Intranasal immunization induces production of anti-PT and anti-FHA IgG in serum

To determine vaccine induced protection, we utilized an established vaccine and challenge protocol.³¹ CD-1 mice were immunized IN with either IN-aP or IN-caP. These groups were compared to pertussis immunizations that are known to be protective: DTaP (IP-aP) and whole-cell pertussis (IP-wP) administered intraperitoneally (IP). Additionally, we aimed to determine if the effects of curdlan were dependent by route, thus we included mice immunized with IP delivered DTaP and curdlan (IP-caP). Lastly, we compared the findings to mock vaccinated mice, with IN delivered PBS, or mice IN immunized with curdlan only (IN-curdan). All mice were immunized with 1/12th of the antigen load contained in the human vaccine dose. Mice were vaccinated at day 0, then received a booster vaccine at day 21. Mice were challenged on day 35 with *B. pertussis*.

We first sought to determine if an IN delivered DTaP vaccine would induce a systemic immune response. To answer this, we performed enzyme-linked immunosorbent assays (ELISAs) with serum from vaccinated and challenged mice against *B. pertussis* antigens found in the vaccine: pertussis toxin (PT) and filamentous hemagglutinin (FHA). We did

not detect a significant difference in serum IgG titers between collection timepoints at days 1 and 3 post challenge (pc) (data not shown). Serum anti-PT IgG titers were similar between mice immunized with IP-aP and those immunized through IN administration, as no significant differences were determined between IP-aP, IN-aP or IN-caP (Fig. 3a). Similarly, we observed a robust titer response to the bacterial adhesin FHA in IN vaccinated mice; however, IN-aP serum anti-FHA titer was 7-fold higher than IN-caP (Fig. 3b). We next determined if IN administration of DTaP (with or without curdlan) would lead to an increased Th1 immune response resulting in a higher ratio of IgG2a compared to IgG1 antibodies. Neither route nor adjuvant impacted the ratio of IgG2a to IgG1.

Intranasal immunization induces production of anti-*B. pertussis* IgA in lung

In the murine model, recent data has pointed to the importance of IgA and a local mucosal immune response in the lung and nasal cavity.^{32–36} We aimed to measure the presence of IgA antibodies in the murine respiratory tissue due to IN immunization. Using ELISAs we measured *B. pertussis* specific IgA titers in homogenized lung tissue supernatant and nasal lavage fluid. Interestingly, we detected a robust IgA response in the lung only when mice were immunized through the IN route (Fig. 4a). Antigen-specific IgA response was not observed in mice immunized with curdlan alone. We observed detectable IgA *B. pertussis*-titer in the nasal lavage fluid from both IN-aP and IN-caP vaccinated groups, although only IN-aP resulted in a significant increase compared to baseline levels (Fig. 4b). The presence of *B. pertussis* binding IgA in the lungs and nasal cavity suggests that IN DTaP is capable of priming a mucosal immune response in the upper and lower murine respiratory systems.

Intranasal immunization decreased pulmonary pro-inflammatory environment

It has been well established that neutralization of PT and inhibition of bacterial adhesins associated with DTaP protection leads to a markedly reduced pro-inflammatory environment at the site of infection when compared to a natural infection.¹⁷ Conversely, challenge in whole-cell protected animals resulted in a severe pro-inflammatory response, similar to the natural infection of *B. pertussis*.³⁷ We hypothesized that the immunostimulatory properties of curdlan would induce a more pro-inflammatory

response following challenge with *B. pertussis* compared to IP-aP. Cytokine concentrations were determined from supernatant of lung homogenate at day 3 pc. The pro-inflammatory interleukin-6 (IL-6) has been shown to be increased following *B. pertussis* infection of a naïve mouse, while DTaP induced protection drastically decreases IL-6 production.^{31,38} Our results demonstrate a significant reduction of IL-6 in the lungs of either IN-aP or IN-caP immunized mice when compared to mock vaccinated or IN-curdan control mice, and are comparable to levels observed in IP immunized groups (Fig. 5a). We measured a similar reduction in the Th1 associated cytokine IFN- γ in the IN administered groups, IN-aP and IN-caP; although, these levels were higher in IN-caP immunized mice (Fig. 5b). We only observed a significant increase in the Th2 associated interleukin-5 in IN-wP immunized mice compared to mock vaccinated mice (Fig. 5c).

IN administration of curdlan, and moreover, vaccine administration through the IN route regardless of adjuvant has been shown to induce an increased IL-17 response.^{27,28} Thus, we hypothesized that we would observe increased levels of IL-17 in mice immunized with IN-caP compared to IN-aP or IP-aP. We quantified IL-17A in lung supernatant and observed significant increases of IL-17A with the addition of curdlan in IP-caP and IN-caP immunized mice supporting our hypothesis (Fig. 5d). Furthermore, we observed that IN administration induced a significant increase in IL-17A compared to IP immunization. This IL-17A response was lower than the robust IL-17A induced by IP-wP (Fig. 5d).

Natural infection with *B. pertussis* is known to cause severe leukocytosis in the murine model³⁹, which can be measured by elevated neutrophils in the peripheral blood. Following *B. pertussis* challenge, we observed that all vaccinated groups ameliorated symptoms of leukocytosis; however, only the administration of DTaP either by IP or IN administration significantly reduced CD11b⁺Gr-1^{hi} neutrophils in the peripheral blood (Fig. 6a). In the lungs, neutrophils were decreased in IN-aP, IN-caP, and IP-aP immunized groups compared to mock vaccinated mice. We observed a similar finding in pulmonary macrophages, although we only determined a significant decrease in mice immunized with IN-caP compared to mock vaccinated mice (Fig. 6c). Taken together

these data suggest that immunization with DTaP through the IN route reduces the pro-inflammatory environment of the murine lung during *B. pertussis* challenge.

Intranasal immunization reduced respiratory *B. pertussis* bacterial burden.

Lastly, we examined the clearance of *B. pertussis* from the respiratory tract following IN immunization. At days 1 and 3 pc, viable bacterial burden was quantified by counting of CFU in the lung, trachea, and nasal lavage fluid. We observed a significant reduction in viable bacteria recovered from the lung in all immunized groups by day 3 pc; however, these changes were not observed at day 1 pc (Fig. 7ab). IN-aP and IN-caP immunized mice bacterial burdens were reduced by 99.4% and 99.7%, respectively, compared to mock vaccinated mice. This reduction in viable bacterial burden was similar to that of mice immunized by IP-aP, an immunization that is known to be effective (Fig. 7b). Importantly, this reduction in bacterial burden was not observed following immunization with curdlan alone, further suggesting an antigen-specific response. Similar trends were observed in the trachea homogenate (Fig. 7cd), and nasal lavage fluid (Fig. 7ef), as all immunized groups regardless of IP or IN delivery cleared *B. pertussis* to the limits of detection. In summary, we observed similar clearance of *B. pertussis* from the respiratory tract of mice immunized IN compared to mice immunized with vaccines known to be protection delivered by the IP route.

Discussion

The natural immune response to *B. pertussis* is initiated by the colonization of the respiratory tract, which leads to a cascade of inflammatory cytokines such as; IL-6, IFN- γ , TNF- α , and IL-1 β leading to increased inflammatory infiltrate consisting of: dendritic cells, macrophages, neutrophils, and lymphocytes.^{31,40} Numerous studies suggest the infection is cleared through opsonization of bacteria by the humoral immune response, combined with a Th1/Th17 cell-mediated response.^{15,19,41,42} Additionally, factors associated with a local immune responses have been observed in convalescent mice following challenge with *B. pertussis*, such as antigen-specific IgA titers and T_{RM} cells, which were shown to decrease bacterial burden when adoptively transferred to naïve mice.²¹

The induction of a protective immune response also has been observed through mucosal immunizations against *B. pertussis*. Oral vaccination with heat-killed *B. pertussis* in newborn infants resulted in the production of *B. pertussis* specific serum and mucosal antibodies; although, this protection was not long lived.⁴³ In the mouse model, a live-attenuated IN pertussis vaccine, BPZE1, has shown protection up to one year after immunization⁴⁴, and is in clinical trials.⁴⁵ Furthermore, BPZE1 vaccine produced antigen-specific IgA antibodies in the baboon model, indicative of elicitation of mucosal immune response.⁴⁶ The mucosal immune response was further explored in a murine model where BPZE1 induced a protective secretory IgA immune response, and the generation of long lasting IL-17 secreting T_{RM} cells in the respiratory system.²⁵ Similarly, this population of antigen-specific memory cells was found to be expanded by an acellular pertussis vaccine (LP-GMP) consisting of a TLR2 agonist and intracellular receptor stimulator of interferon genes (STING) agonist, which correlated with the IL-17 secreting T_{RM} population.⁴⁷ Promisingly, generation of the IL-17 secreting T_{RM} cell population was observed following whole-cell immunization through IP administration; however, Tdap administration through the same route did not generate this population.²⁰

In our study, we demonstrate that by altering the administration route of DTaP immunization from IP to IN delivery, the vaccine still induced a protective immune response. Using fluorescent labeled DTaP particles we visualized that IN-aP was readily detectable (Fig. 1). *In vivo* imaging analysis demonstrated significantly more labeled IN-caP present in the nasal cavity of mice. We detected a higher proportion of IN-caP particles compared to IN-aP particles that embedded in the epithelium of the nasal turbinates (Fig. 2). These particles had a smaller diameter, which may suggest that when the curdlan adjuvant was included DTaP particles were more readily phagocytosed by APCs compared to IN-aP particles. Further work examining the lymph nodes responsible for antigen priming would be beneficial to determine the role of nasal associated lymphoid tissue compared to vaccination of the lung, and trafficking to mediastinal lymph nodes. The location of antigen priming following IN immunization may play a critical role in the optimization of future human IN vaccines. Determining if antigen uptake in the nasal cavity is essential to induce a protective immune response would play a key role in the development of a human IN pertussis vaccines.

We determined that while IN-caP vaccinated mice had significantly lower detection of DTaP in the lungs compared to IN-aP immunized mice that the bacterial clearance was similar between the two groups (Fig. 7), suggesting that antigen priming still occurred. This was further demonstrated through seroconversion as robust antigen-specific serum IgG antibody titers were detected from the two IN immunized groups (Fig. 3). The IgG subclass data (Fig. 3) suggest that immunization with IN-aP most likely induces a Th2 response. Other studies with BPZE1 or LP-GMP adjuvants have observed shifts to Th1 response using IN route.^{20,25}

A key finding of our current study was the production of antigen-specific IgA in the respiratory system only in mice that were administered DTaP through IN administration: IN-aP and IN-caP (Fig. 4). Another difference between the IN-aP and IN-caP groups was the level of IL-17A present in the lung homogenate (Fig. 5). We found that inclusion of curdlan in DTaP resulted in higher levels of IL-17A whether administered through IN or IP route although IL-17A was highest in IN-caP. These findings correlate with results from others suggesting a role of curdlan and IN administration in IL-17 production.^{27,28} Together these data demonstrate induction of mucosal immune response specific to *B. pertussis* in the respiratory system.

In our model, addition of curdlan to DTaP increased IL-17 responses but did not improve clearance (Fig. 7). A caveat of the current work is role of aluminum hydroxide was not addressed. All IN immunizations consisted of DTaP, which contains alum, observations from IN-caP may be affected by the presence of alum. Understanding the role of aluminum hydroxide, along with other potential adjuvants in the mucosal immune response needs to be further studied. Our work does not address long-term protection mediated by IN administration of DTaP or the inclusion of curdlan adjuvant. Future studies will focus on the longevity of IN immunity.

In conclusion, we observed that by altering the route of administration from IP to IN a protective response was reproduced, resulting in clearance of the pathogen to limits of detection. Furthermore, we determined that IN delivery of DTaP was sufficient to prime a local mucosal immune response at site of infection. The inclusion of the adjuvant curdlan in DTaP immunization did increase IL-17 production in the lung; however, we

did not observe a shift in the type of immune response compared to DTaP alone. In humans, DTaP and tdap do not provide perfect protection against pertussis; however, there is no clear path forward to the next generation vaccine. It is clear that developing a new vaccine will take considerable efforts. Here in our study we observed a surprising result, that IN DTaP vaccine could protect mice against *B. pertussis*. These data cause us to wonder if a change in the route of DTaP immunization would be a promising booster vaccine strategy.

Materials and Methods

Composition and administration of vaccines used in study

The vaccines administered in the study were prepared no longer than 1 h before administration. INFANRIX (GSK) human vaccine (DTaP), and the National Institute for Biological Standards and Control WHO whole-cell pertussis vaccine (NIBSC code 94/532) were used in the study. Vaccines were diluted with PBS to 1/12th of the human dose (based on total antigen content). Curdlan (Invivogen, tlr-curd) was prepared by dissolving 50 mg in 2.5 ml sterile purified water. Curdlan preparation was brought into solution by adding 100 μ l 1N NaOH and vortexing. The curdlan suspension (20 mg/ml) was then sonicated for 10 mins and placed in 37°C water bath until administration. Curdlan adjuvant was administered at 200 μ g per mouse. These experiments were conducted in accordance with the National Institutes of Health Guide for the care and use of laboratory animals. The protocols used were approved by West Virginia University Institutional Animal Care and Use Committees (WVU-ACUC protocol 1602000797).

Vaccination of mice for vaccine particle tracking

CD-1 (outbred; strain code 022) mice aged four weeks were obtained from Charles River Laboratories. At five weeks mice were anesthetized with 77 mg/kg ketamine and 7.7 mg/kg xylazine. Mice were administered 50 μ l of vaccine or control, with 25 μ l into each nostril (IN).

Tracking of DTaP in respiratory system

DTaP vaccine particles were labeled using the Alexa Fluor 660 Protein Labeling Kit (Molecular Probes). Briefly, 0.5 ml of DTaP vaccine was added to 50 μ l of 1 M sodium bicarbonate, then added to Alexa Fluor 660 dye stock. The mixture was incubated for 1 h at room temperature with agitation. The solution was concentrated by dialysis in phosphate-buffered saline overnight to remove unlabeled dye. Labeled vaccine was used to immunize mice. At 0, 6, 12 and 24 h post vaccination fluorescent signal was measured using an IVIS Spectrum (Xenogen). Mice were anesthetized using 3% isoflurane, mixed with oxygen prior to and throughout imaging. The following parameters were used: 1) A binning setting of 4 was kept constant for all images, 2) each image was quantified using the automatic image setting, 3) fluorescence photons were measured using total radiant efficiency of a common region of interest placed on the nasal cavity, and 4) measurements were normalized using Living Image (Xenogen ver. 2.5). Use of this quantification method accounted for variations in exposure between images.

At 6, 12, 24, and 48 h post vaccination, animals were euthanized to quantify DTaP in lungs by flow cytometry analysis. Lungs were removed, and homogenized using gentleMACS C tubes (Miltenyi Biotec) with enzymatic lung dissociation kit (Miltenyi Biotec, 130-095-927). All samples were blocked using Fc Block (BD), then labelled with Alexa Fluor 700 – conjugated CD11b (Biolegend, 101222), DTaP particles were detected with Cy5 channel. Following a 1 h dark incubation labeled samples were washed, then fixed using 0.4% w/v paraformaldehyde overnight. Samples were resuspended in PBS and analyzed on a LSR Fortessa flow cytometer (BD). DTaP containing myeloid cells were classified as CD11b⁺DTaP⁺ single, live cells.

Detection of DTaP particles in lung and nasal cavity

Detection of DTaP particles in the lung and nasal cavity were confirmed using confocal imaging. Mock vaccinated and challenged mice were euthanized at 6 h post vaccination. Prior to homogenization, the post-caval lobe of the mouse lung was

removed. The post-caval lobe was flash frozen in OCT medium (Tissue Plus, Fisher Healthcare), using liquid nitrogen. The samples were stored at -80°C until sectioning. Sectioned samples (6 µm) were fixed in acetone, then stained with ActinGreen Ready Probes (Invitrogen) and NucBlue Ready Probes (Invitrogen), using manufacturer protocols.

Skulls were removed from mouse, and the lower jawbone discarded. The skulls were fixed in formalin for 12 h at 4°C, then de-calcified at room temperature for 24 h, before samples were embedded in paraffin. Sectioned samples were de-paraffinized and rehydrated using xylene, and washes with decreasing ethanol concentrations (100 to 70%). An antigen retrieval step was performed using citrate buffer, where samples were heated to 98°C for 20 mins. Samples were then stained as mentioned above. Samples were analyzed for DTaP particles in tissue and airway mucus using a Nikon A1R confocal microscope. DTaP particles were quantified using ImageJ (version 1.52a). Briefly, the threshold tool was used to select only the fluorescent particles above background levels. Then, the threshold adjusted area was quantified using the analyze particles tool. Thus, the data is represented as the percentage of fluorescent particles per area of the total image field. Three image fields per sample were quantified and averaged per mouse.

B. pertussis strains and growth conditions

B. pertussis strain UT25Sm1⁴⁸ was used for murine challenge in all experiments. UT25Sm1 was cultured as described previously.³¹ Briefly, UT25Sm1 was cultured on Bordet Gengou agar plus 15% defibrinated sheep's blood (Remel) with streptomycin 100 mg/ml. *B. pertussis* was incubated at 36°C for 48h, then transferred to modified Stainer-Scholte liquid medium⁴⁹, without the cyclodextrin, heptakis. Liquid cultures were incubated for 24 h at 36°C, with shaking until reaching an OD₆₀₀ of ~0.6, at which time cultures were diluted for challenge dose.

Vaccination and *B. pertussis* challenge

IN immunized mice received 50 µl of vaccine as described above. IP immunized mice received 200 µl of vaccine injected into the peritoneal cavity. IN and IP immunized mice received the same antigen dose of $1/12^{\text{th}}$. Mice received a boost of the vaccines with the same concentrations twenty-one days after initial immunization. At thirty-five days post initial vaccination, mice were challenged with 2×10^7 CFU *B. pertussis* administered in 20 µl through nostrils. At days 1 and 3 pc mice were euthanized, blood and respiratory tissue were isolated as previously described.³¹

Serological analysis of *B. pertussis* specific antibodies

Serological responses specific to *B. pertussis* antigens were quantified by ELISA. High-binding microtiter plates were coated with PT (50ng/well)(LIST Biologicals) and FHA (50ng/well)(Enzo Life Sciences), as described in Boehm *et al.*³¹ Serological responses against UT25Sm1 were cultured to an OD₆₀₀ of 0.24 and microtiter plates coated with 50µl of bacteria per well. Bound antibodies were detected using goat anti-mouse IgG, IgA, IgG2a, and IgG1 antibody conjugated to alkaline phosphatase (Southern Biotech). Positive antibody titers were determined using a baseline set at two times the average of blanks.

Quantification of pulmonary and blood APCs

To determine cell types infiltrating the lung and leukocytes present in peripheral blood, single cell suspensions from the tissues mentioned above were prepared, as described previously.³¹ Briefly, lung tissue was homogenized by Dounce homogenizers, filtered with a 100 µm filter, and red blood cells were lysed for 2 min at 37°C (Pharmlyse). Cell populations were determined using antibodies to cell surface markers: PE-conjugated GR-1 (BD, 553128), Alexa Fluor 700-conjugated CD11b (Biolegend, 101222), PerCP/Cy5.5-conjugated F4/80 (Biolegend, 123128). Neutrophils and macrophages were classified as CD11b⁺Gr-1^{hi} single, live cells or CD11b⁺F4/80⁺ single, live cells, respectively.

Lung homogenate cytokine analysis

To quantify inflammatory cytokines at the site of infection, lung homogenate supernatant was prepared and stored at -80°C, as described in prior work.³¹ Quantitative analysis of cytokines was performed using Meso Scale Discovery cytokine kits: V-PLEX pro-inflammatory panel (K15048D) and IL-17A V-PLEX (K152RFD), per the manufacturer's instructions.

Statistical Analysis

Experiments in the study were performed with 3 to 8 biological replicates. Data were analyzed using GraphPad Prism 7. Comparisons between groups were performed using one-way analysis of variance (ANOVA) with Dunnett's and Tukey's *post hoc* tests. Comparisons between groups with or without curdlan were analyzed by two-tailed unpaired t-test, when applicable multiple T-tests with Holm-Sidak *post hoc* test were applied to curdlan inclusion groups.

Data Availability

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

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Author Contributions

D.T.B., F.H.D., M.B. designed experiments, performed experiments and composed manuscript. M.A.W., J.M.H., T.Y.W., E.S.K., M.P.G., C.B.B., and W.T.W. performed experiments and reviewed manuscript. S.D.B., and K.A.B. were undergraduate researchers who completed experiments and reviewed the manuscript.

Competing Interests

The authors declare that there are no competing interests.

Figures

Figure 1: Localization of acellular pertussis vaccine in the upper and lower respiratory system after IN vaccination.

Figure 1: Localization of acellular pertussis vaccine in the upper and lower respiratory system after IN vaccination. (a) Schematic of vaccine tracking protocol. CD-1 mice were intranasally vaccinated with either fluorescent DTaP alone (IN-aP) or fluorescent DTaP with curdlan (IN-caP). Vaccine particle deposition in the lungs and nasal cavity was measured at 0, 6, 12, 24, and 48 h after immunization. (b) Representative images of nasal cavity fluorescence at 6, 12, and 24 h. The region of interest used for fluorescence quantification is shown in blue. (c) Fluorescence measurements normalized to PBS control at 6, 12, and 24 h. Results shown as mean ±

SEM of total radiant efficiency, $*P < 0.05$. P values were determined by multiple T-tests with Holm-Sidak *post hoc* test between IN-aP and IN-caP vaccinated mice. (d) Representative plots showing live, single cells that are CD11b⁺DTaP⁺. (e) Flow cytometric analysis of CD11b⁺ cells from the lung that contain or are bound to DTaP particles at 6, 12, 24, and 48 h post immunization. Results shown as mean $\pm$ SEM, $*P < 0.05$, $*** P < 0.001$, $****P < 0.0001$. P values were determined by one-way ANOVA with Dunnett's *post hoc* test comparing IN-aP immunized mice to control mock vaccinated mice.

Figure 2: Acellular pertussis vaccine particle localization altered by curdlan adjuvant.

Figure 2: Acellular pertussis vaccine particle localization altered by curdlan adjuvant. (a) Representative images of flash frozen lung sections 6 h after immunization with IN-aP or IN-caP. Fluorescent particles were detected using a 660 laser, samples were counter-stained with NucBlue (blue) and ActinGreen (green). (b) Fluorescent DTaP particles were quantified by determining the percentage area of particles per field of view. ($n=3-4$, with averages of 3 images per lung). Results are shown as mean $\pm$ SEM, $*P < 0.05$. (c) Representative images of paraffin embedded nasal cavity sections 6 h after immunization with IN-aP or IN-caP. (d) Fluorescent DTaP particles quantified by determining the percentage area of particles per field of view. ($n=3-4$, with averages of 3 images per lung). P values were determined by one-way ANOVA with Tukey's *post hoc* test.

Figure 3: Intranasal immunization induces production of anti-PT and anti-FHA IgG in serum.

Figure 3: Intranasal immunization induces production of anti-PT and anti-FHA IgG in serum. ELISAs were used to compare serological responses from mice immunized through IN or IP routes to mock vaccinated mice. Total IgG serum antibody titers from immunized and challenged mice were quantified against (a) PT and (b) FHA at day 3 pc. Serum (c) IgG1 and (d) IgG2 antibody titers against *B. pertussis* were compared to mock vaccinated mice at day 3. Results shown as mean $\pm$ SEM, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. P values were determined by one-way ANOVA with Dunnett's *post hoc* test compared to mock vaccinated mice.

Figure 4: Intranasal immunization induces production of anti-*B. pertussis* IgA in respiratory system.

Figure 4: Intranasal immunization induces production of anti-*B. pertussis* IgA in respiratory system. ELISAs were performed using (a) lung homogenate supernatant and (b) nasal lavage fluid from vaccinated and challenged mice at day 3 pc. IgA titers were determined against whole-cell *B. pertussis*. Results are shown as averages of two independent experiments, represented on a log₁₀ scale for lung and linear scale of nasal lavage with mean $\pm$ SEM (n = 4-8). *****P* < 0.0001. *P* values were determined by one-way ANOVA with Dunnett's *post hoc* test compared to mock vaccinated mice.

Figure 5: Intranasal immunization decreased pulmonary pro-inflammatory cytokines during challenge.

Figure 5: Intranasal immunization decreased pulmonary pro-inflammatory cytokines during challenge. Analysis of cytokines from supernatant of lung homogenate at day 3 pc. Cytokines (a) IL-6, (b) IFN- γ , (c) IL-5, (d) IL-17A were quantified by electrochemiluminescence assay. Results shown as mean $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. P values were determined by one-way ANOVA with Dunnett's *post hoc* test compared to mock vaccinated mice. Bars connecting groups indicate values determined by two-tailed un-paired *t*-test. Upper and lower limits of detection shown as dash or dotted lines, respectively if data points reached these limits.

Figure 6: Intranasal immunization reduced neutrophil and macrophage accumulation in the lung and circulating neutrophils after *B. pertussis* challenge.

Figure 6: Intranasal immunization reduced neutrophil and macrophage accumulation in the lung and circulating neutrophils after *B. pertussis* challenge.

(a) The percentage of live, CD11b⁺Gr-1^{hi} neutrophils from a single cell suspension of the peripheral blood. Percentages of (b) CD11b⁺Gr-1^{hi} neutrophils and (c) CD11b⁺F4/80⁺ macrophages in single cell lung homogenates. Results shown as means $\pm$ SEM, * P < 0.05 ** P < 0.01, *** P < 0.001, **** P < 0.0001. P values were determined by one-way ANOVA with Dunnett's *post hoc* test compared to mock vaccinated mice.

Figure 7: Intranasal immunization reduced respiratory *B. pertussis* bacterial burden

Figure 7: Intranasal immunization reduced respiratory *B. pertussis* bacterial burden. Analysis of bacterial burden was determined at days 1 and 3 pc. Bacteria were quantified by counting of serially diluted CFUs following immunization and challenge.

CFU counts were determined from lung homogenate (A and B), trachea homogenate (C and D), and nasal lavage fluid (E and F). Results are mean $\pm$ SEM ($n = 4 - 8$, with 4 averaged technical replicates) from two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. P values were determined by one-way ANOVA with Tukey's *post hoc* test compared to mock vaccinated mice, or between connected columns. The dashed line represents the lower limits of detection due to plating.

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Chapter 5 - Discussion

Pertussis is a bacterial disease that only exists in the human population. Thousands of years of evolution have optimized *B. pertussis* to the microenvironment of the human respiratory system. This is understandably so, *Homo sapiens* are a species that fit the niche of *B. pertussis*. We are a social species that live in close quarters thereby permitting the dissemination of bacteria that spreads through respiratory droplets. Human offspring are born without fully developed immune symptoms, leaving them more susceptible compared to their adult counterparts. When one individual becomes sick others congregate to care for that person, more so in the case of an infant. Thus, giving rise for bacteria to spread to a new host and continue the infectious cycle.

However, the same forces of evolution that lead to the specialized niche of *B. pertussis* in the human respiratory system, confined the bacteria to life in only one host reservoir. As, scientists this is a flaw we can exploit, and have done so in the past. When Edward Jenner developed the smallpox vaccine, he initiated a mechanism of eradication of the disease by protecting the only species capable of spreading the smallpox virus. In 1980, natural occurring smallpox was declared eradicated through years of widespread whole-cell vaccination ¹. It may now be surprising to some that pertussis, now classified as a re-emerging infectious disease by the CDC in the United States, was once a proposed target for eradication in the 1970s ^{2,3}. Like smallpox, pertussis incidence was well-controlled through widespread immunization with a wP vaccine ⁴. The resurgence of pertussis began to occur in the US in the mid-1980s, and cases have remained well above that level since. This increase in cases coincides with the switch to aP in developed countries. One of the hypotheses on why this increase is occurring is that aP immunization inhibits the toxin-mediated symptoms the disease, but not colonization of the bacteria. This realization has renewed interest in the development of a vaccine that can inhibit colonization of *B. pertussis*, with the ultimate goal of reducing the number of

cases to levels seen in the wP era, thereby re-opening the door the possible eradication of pertussis.

A major long-term goal of this work was to make progress on developing a vaccine that improved upon the shortcomings of current acellular pertussis vaccines covered in chapter 1. In this work we demonstrate the protective effects of neutralizing ACT, a key virulence factor of *B. pertussis*, through an IP administered vaccine. We then explored another route of administration, finding that IN administration of DTaP induced a systemic protective response and a mucosal immune response in the respiratory tissue. This work demonstrates that the neutralization of toxins achieved through parenteral vaccination can be reciprocated by vaccinating the nasal cavity directly, thereby inhibiting PT before it disseminates beyond the respiratory system. This work suggests that the mechanisms of protection in either of the vaccine strategies should be further elucidated and incorporated into future pertussis vaccines.

Lessons learned from Ne Cre luc mouse model

The mouse model has been used for vaccine optimization since the development of wP vaccines⁵. Naturally, along the way, improvements to the mouse model have enabled a better understanding of vaccine-induced protection. In setting out to improve the current vaccines, we aimed to design experiments that were capable of showing this improvement. In a landmark study, Guiso *et al.* developed the intranasal pertussis challenge murine model⁶. In that study, the authors found similar protection from a dose of 1/4th and 1/10th of the human antigen concentration compared to a group receiving the full human dose⁶. We based our initial experiments on these vaccine concentrations.

One of the aims of chapter 2, was to use the NeCre luc, neutrophil reporter mouse model to trace the real-time kinetics of the neutrophil response between DTaP and wP immunized mice. In these experiments, we immunized the reporter mice with 1/5th the human dose of DTaP and wP. At this high vaccine dose, we observed an aggressive pro-inflammatory response following *B. pertussis* challenge of wP immunized compared to DTaP immunized mice (Chap. 2, Figure 4,5). Transcriptional analysis of the lung during early and late phases of an acute infection revealed the highest pro-inflammatory

associated gene changes in the wP-primed mice. Strikingly, we observed the highest levels of neutrophil recruitment occurred after the bacterial burden was cleared to limits of detection, while in comparison to the DTaP-primed group the percentage of neutrophils in the lung had returned to baseline levels following clearance of the infection (Chap. 2, Figure 3,7). The intensity of the neutrophil response may have overwhelmed this group, as we observed severe morbidity in wP-primed mice at day 6, which required euthanasia. The clearance of the pathogen prior to the recruitment of neutrophils suggests that morbidity was due to the immune response elicited. The NeCre luc model provided a novel method of visualizing the spatiotemporal localization of neutrophils in immunized mice. In these studies, we compared the quantification of neutrophils using the luminescence of the luciferase-induced reporter to the quantification of neutrophils through flow cytometry. We determined that using flow cytometry we could accurately depict the neutrophil response over time, with the benefit of using a more standard mouse strain. The NeCre luc reporter mouse may be the most beneficial to those study the neutrophils recruited to a concentrated site, such as those studying the role of innate immune cells in the tumor microenvironment, or acute topical infections. In completing this aim of the study, we concluded that a wP vaccine concentration of 1/5th the human dose may be too high to properly characterize the response of neutrophils overtime.

A second aim of the NeCre luc study was to determine the protective role of a purified toxoid of ACT, RTX, as a single antigen vaccine administered with aluminum hydroxide. We hypothesized that antibodies generated against the antigen would neutralize the toxin activity of ACT, thereby inhibiting the ACT mediated suppression of neutrophils discussed in chapter 1. RTX immunization generated a strong IgG antibody response specific to the RTX antigen; however, RTX as a single antigen vaccine alone was not protective because we did not observe a reduction of bacterial burden in these experimental conditions. Immunization with only RTX would not address PT, thus in these experiments, PT would still be active. Thereby, inhibiting neutrophil and macrophage chemotaxis to the site of infection. We reasoned that while RTX as a single antigen did not induce protection the benefits of neutralizing ACT may be more evident when PT is neutralized as well. In this scenario when macrophages and neutrophils

were recruited to the lung, their bactericidal activity would not be inhibited. This realization caused us to re-craft our approach to a vaccine where we could study the synergistic effects of neutralizing ACT, along with the protective activity of DTaP vaccines.

Optimization of the mouse model

In chapter 3, we built upon our observations learned in the NeCre luc immunization and challenge model. In these experiments we aimed to determine the protective effects of incorporated RTX in a DTaP vaccine. In initial experiments, we included the RTX toxoid discussed in chapter 2, into a 1/5th human dose of DTaP. In these results, we measured an antigen-specific immune response but did not observe an increase in protection compared to DTaP immunized mice. It occurred to us, that the use of such a high dose of vaccine in the mouse model is not relevant to the dose that is administered to humans. This reflection led us to the idea that to establish a relevant model, we needed to determine the lowest vaccine dose that induced protection. We proposed performing a vaccine dose titration to find this dose in mice. This would lead to a vaccine dose that would: 1) more accurately depict the vaccine concentration administered to humans, and 2) establish a sub-protective dose to incorporate future vaccine antigens, such as RTX to determine protection. In chapter 3, we determined that 1/40th of the human dose of DTaP vaccine protected mice while, the 1/80th dose did not. We were then able to add RTX to 1/80th human dose DTaP vaccine. This finding does correlate with others suggesting that 1/50th the human dose of DTaP was protective in mice, as we identified 1/40th the human dose to be the lowest-protective dose by our 2-fold titration ^{7,8}. Defining this sub-optimal dose is crucial in re-thinking the design of DTaP/TdaP immunizations. DTaP/TdaP are safe vaccines which protect individuals from classical pertussis. Therefore, if a potential vaccine is to replace either of these standards, the candidate vaccine would need to perform better, in addition to protecting an individual through a different mechanism. Defining a sub-optimal vaccine dose from which, synergistic effects of potential vaccine antigens can be observed, gives researchers a mechanism to demonstrate increased vaccine potency.

Incorporation of RTX with DTaP

In chapter 1, we discussed the immunomodulatory activity of ACT. ACT increases intracellular cAMP of macrophages and neutrophils recruited to the site of infection. The levels of cAMP suppress the bactericidal activity of the macrophages and neutrophils including phagocytosis, oxidative burst and NET formation in neutrophils⁹⁻¹¹. We, along with others hypothesized that neutralizing ACT through vaccination would increase vaccine efficacy of current acellular vaccines^{12,13}. In chapter 3, we discussed the protective effects of including RTX into a DTaP vaccine. In these studies, we immunized mice using the outbred CD-1 mouse line. Incorporation of RTX into the DTaP vaccine resulted in the production of anti-ACT binding antibodies, that were capable of neutralizing ACT activity against murine macrophages (Chap. 3, Fig. 1,3). These *in vitro* observations translated to *in vivo* vaccine-mediated protection, as we observed decreased bacterial burden and pro-inflammatory responses with the inclusion of RTX compared to DTaP alone (Chap. 3, Fig. 2). Defining the sub-optimal vaccine dose was critical to this experiment. We observed synergic effects of RTX at 1/80th human vaccine dose; while those effects were masked at the higher doses of DTaP (Chap. 3, Fig. 1).

We observed that the inclusion of RTX into the DTaP vaccine increased vaccine-induced protection when compared to DTaP alone. We did not observe an increase in Th1 associated cytokines, suggesting that DTaP with RTX did not alter the vaccine-induced immune response from the Th2 promoting DTaP. Overall, the immune response to *B. pertussis* was similar to DTaP induced protection. We observed strong IgG responses to the *B. pertussis* toxins, and a reduction in the pro-inflammatory response evident by a reduction in pro-inflammatory cytokines, macrophages, and neutrophils in the lung when RTX was included in DTaP compared to DTaP alone. The data suggests that the enhanced protection was due to the production of anti-ACT neutralizing antibodies. However, due to the nature of the Th2 response observed from DTaP with RTX, it seems unlikely that the inclusion of an additional antigen alone would increase the duration of protection compared to DTaP. We hypothesized that to alter the T-helper cell response and increase the duration of protection, we needed to continue investigating ways to promote a cell-mediated immune response.

The work performed in this dissertation was completed in the mouse model of pertussis. The use of animal models has vastly contributed to the understanding of the pathogenesis and the immune response elicited against *B. pertussis* and other *Bordetella* species. Mice and humans generate similar pro-inflammatory T-cell responses and antibody profiles upon infection with *B. pertussis*¹⁴. In chapters 3 and 4, we utilized only female CD-1 outbred mice, compared to inbred mouse strains. The use of inbred mouse strains increases the reproducibility of experiments compared to experiments using outbred mice strains. Outbred strains were used in our experiments specifically to increase the genetic variability, to model the genetic variability seen in the human population. As a result, we observed increased variation in experiments performed using CD-1 mouse lines compared to inbred strains such as BALB/c (data not shown). In addition to genetic variability between mouse strains, future experiments should utilize both male and female sexes. Epidemiology pertussis studies have found a higher proportion of pertussis in female patients compared to male counterparts¹⁵. Classically, females are more susceptible to upper respiratory infection compared to males¹⁶. However, females are known to induce greater innate immune cell activation and stronger humoral responses than males in both human and mice¹⁷. It is possible that male mice may not reciprocate the strength of the immune response observed following immunization and challenge of female mice. In future experiments the incorporation of both male and female mice can address this concern.

Importance of a Mucosal Response against *B. pertussis*

In our studies, we found it interesting that bacteria were cleared earliest in the lungs compared to nares and trachea (Chap 3., Fig. 2). We began to investigate the possibility of a mucosal pertussis vaccine that could prime the respiratory tract directly. We hypothesized that by administering the vaccine intranasally, the primed local immune response could neutralize the pathogen before the impairment of the innate immune response by toxins of *B. pertussis*.

Mucosal-vaccine induced immunity poses the benefit of preventing the establishment of an infection prior to PT entering the circulatory system. Conversely, parenteral pertussis vaccine-induced immunity requires circulating PT to encounter memory T and B cells

present in the draining lymph nodes, then mount a secondary immune response to the infection. It is well documented that natural infection of a human patient confers long-term protection. While the human mucosal immune response is yet to be characterized, *B. pertussis*-specific IgA antibodies suggest a role for mucosal protection ¹⁸. Evidence in support of a role of a mucosal immune response against *B. pertussis* from the baboon model find that convalescent baboons and mice clear the infection significantly earlier than any parental vaccine, wP or aP ^{19,20}. The natural response to pertussis was discussed in chapter 1, but is known that natural infection with *B. pertussis* induces a strong mucosal response, which correlates to sterilizing immunity ^{21,22}. Wendelboe *et al.*, reviewed published data comparing the duration of protection from acellular and wP pertussis vaccines to convalescent protected individuals ²³. The authors determined that humans who were previously infected with *B. pertussis* were protected up to 20 years, compared to up to 12 years following wP immunization, and 4 years from those immunized with various DTaP vaccines ²³. We posed the question, “If we can mimic an infection with an acellular vaccine, could we induce a protective response?” Now, there are clear differences between an infection with pathogenic bacteria and a vaccine composed of purified protein. For one, not only does the pathogen express thousands of potential PAMPS, but the colonization of the respiratory tract produces additional damage-associated molecular patterns (DAMPS) that would be not produced following intramuscular acellular immunization. PAMPS/DAMPS associated with the natural infection recruit cells of the innate immune system to the infection, leading to phagocytosis of the bacteria, and ultimately antigen presentation of the many potential antigens to naïve T cells ^{24,25}. This is unlikely to occur with a parenterally administered subunit vaccine, where a humoral response is generated against the specific antigens contained in the vaccine.

As discussed previously, there has been promising pre-clinical success with a live-attenuated *B. pertussis* intranasal vaccine, BPZE1 ^{26–29}. BPZE1 is a highly attenuated strain, lacking production of three functional virulence factors of *B. pertussis*: PT, dermonecrotic toxin, and tracheal cytotoxin ²⁹. The strain is not pathogenic but is able to colonize the respiratory tract, before eventually cleared. The vaccine strain is protective in mice and baboons, and in mice has been shown to induce a Th1 mediated response

^{26,29,30}. Recently, it has shown that immunization with this strain resulted in production of IL-17, IgA antibodies, and the T_{RM} population in the nares of mice, each of these factors was shown to play a role in protection ²⁸. The work performed by Solans *et al.* further demonstrates the importance of a local mucosal immune response in reducing colonization of the respiratory tract, and of generating a pro-inflammatory cell-mediated response in long-term protection against *B. pertussis*.

Intranasal DTaP induced a protective immune response

In chapter 4, we changed the route of vaccine administration from an intraperitoneally administered vaccine to an intranasally administered vaccine. In doing so, going from a vaccine response that was well characterized to the relatively unexplored vaccine route: mucosal vaccination ^{8,22,31–33}. One of the challenges we were faced with was the number of potential antigens that encounter the nasal-associated lymphoid tissue (NALT) on a daily basis ³⁴. Data from orally administered vaccines demonstrates that gut-associated lymphoid tissue promotes tolerance over immunogenicity ³⁵. We reasoned that because the alum adjuvant is not optimal for mucosal vaccination, that IN administered DTaP may not induce enough inflammation to induce a protective immune response ³⁶. It may be that in order to induce a systemic response, the vaccine would have to stimulate a pro-inflammatory response upon vaccination, as was the case in the BPZE1 vaccine discussed above. To increase the pro-inflammatory properties of DTaP, we supplemented DTaP with the β -glucan curdlan. Curdlan was an attractive candidate as it had been previously tested as an intranasal adjuvant, and has been shown to promote a Th1/Th17 immune response *in vitro* and *in vivo* ^{37–40}. Specifically, curdlan binds to the fungal receptor dectin-1 expressed on the surface of DCs, found at high concentrations in the NALT, along with macrophages ^{41,42}. Also, lining the mucosal surface are microfold (M) cells, which specialize in phagocytosis of macromolecules, viruses, and bacteria. On the basal surface of the M cells are intraepithelial pockets, which can contain DCs, T and B cells ^{34,41}. APCs from the mucosal tissue can then track to the draining lymph nodes, where they activate B cells, ideally inducing a systemic and mucosal immune response ³⁴.

Mucosal immunizations provide a unique vaccination strategy because antigen priming can occur in multiple lymphoid organs and tissues. Furthermore, the appropriate tissue may change based on the type of vaccine and/or depending on the pathogen. In the case of pertussis, the appropriate tissue for vaccination is yet to be determined and all human vaccines are provided by IM injection. Since we aimed to vaccinate the airway, we thought it was necessary to track where the vaccine is going during IN immunization. In chapter 4, we accomplished this by fluorescently labeling the DTaP vaccine particles. In Chap. 4, Fig 1,2, we observed that the majority of DTaP alone (IN-aP) was deposited in the lung while DTaP with curdlan (IN-caP) was found mostly in the nares. Ultimately, we did not determine a difference in *B. pertussis*-specific IgG antibody titers present in the serum or IgA antibodies in the lung supernatant. Suggesting that whether antigen uptake occurred in the lungs or the nasal cavity, IN immunization elicited a systemic response not

Figure 1: Proposed model of IN-caP induced protection.

The fungal polysaccharide curdlan is administered as a semi-gel state at a neutral pH. The immunostimulant properties of the β -glucan elicit a Th1/Th17 response. Curdlan binds to DCs through the dectin-1 receptor. The dectin-1 signaling pathway induces the non-canonical expression of NF- κ B, promoting a Th1/Th17 immune response. Curdlan as an adjuvant induced antigen-specific serum IgG antibodies, and IgA antibodies in the lungs of vaccinated mice.

significantly different than IP. Furthermore, the inclusion did not further reduce the bacterial burden of the respiratory system (Chap. 4, Fig. 7). In these experiments we demonstrated that the IN administration of DTaP could induce the production of IgA antibodies. It is then possible that antibodies found in the lung homogenate and nasal lavage fluid could be neutralizing PT at the site of infection. Thus, leading the lower levels of pro-inflammatory cytokines found in the lung homogenate. These data suggest that IN-aP immunization alone is capable of generating a systemic and mucosal immune response.

Role of curdlan in a mucosal immune response

Curdlan has been shown to induce Th17 responses when administered through the intranasal route ³⁷. In this study, we determined the highest amount of IL-17A in the lungs of groups that were IN immunized, with the DTaP with curdlan inducing the highest levels (Chap. 4, Fig. 5d). However, while significant, this response is not as strong as was observed following wP-induced IL-17A response as we demonstrated in this and other studies. What is interesting is that when we measured DTaP vaccine particles in the nares of the mice, more appeared to be embedded in the nasal cavity epithelium with IN-caP than compared to more particles in the lumen with IN-aP (Chap. 4, Fig. 2c). Furthermore, the particles from IN-caP had a smaller, more, particulate appearance. It may be that curdlan played a role in antigen uptake in the nasal cavity. In this study, we only observe a correlation between the smaller particles in the mucosal tissue and the increased IL-17A production found in DTaP with curdlan. Future experiments will be needed to further elucidate this mechanism. One of the caveats of this experiment is that we did not identify the cell types that contained or bound vaccine particles in microscopy imaging. It is possible that when DTaP was administered with curdlan, the vaccine particles were more likely to be phagocytosed by M cells of the mucosa, which are known to endocytose particulate antigens ⁴³. Thereby, it may be possible that antigen uptake is more efficient through this mechanism.

Future directions in pertussis vaccine development

In this work, we demonstrated the induction of a protective response from an intranasally administered vaccine. This work suggests that mucosal immunization with the current DTaP vaccine alone produces a systemic immune response and the production of IgA antibodies and IL-17A in the respiratory tract. The next question to answer is, what is the mechanism of protection elicited from DTaP and DTaP with curdlan? In order to develop effective mucosal pertussis vaccines, a better understanding of the mucosal immune response to *B. pertussis* is needed.

Identification of target vaccine tissue

The use of fluorescent vaccine particles gave us the ability to track the vaccine in the nasal cavity of mice. Presumably, antigen uptake and presentation could be explored using a similar experimental design that could identify M cells and continued tracking of the vaccine particles. An experiment of this nature would also help to answer the question of appropriate tissue targeting. In our experiments, a volume of 50 μ l of the vaccine was administered intranasally. This volume is known to reach the lungs in the mice, while the use of lower volumes results in the majority of detection of bacteria or labeled protein accumulating in the upper respiratory system ^{44,45}. Thus, in the mouse model, this administration route has the potential of antigen priming occurring in the upper and lower respiratory system, as the dose is exposed to the two tissues. In performing the experiment mentioned above, one could elucidate whether either of the tissues plays a role in establishing the mucosal immune response. Additionally, upon antigen presentation either to T_{RM} in the respiratory tissue or draining lymph node one would expect to measure a spike in pro-inflammatory cytokines following the immunization. Using a *B. pertussis* outer membrane vesicle vaccine (OMV) Raeven *et al.*, measured the induction of a moderate pro-inflammatory response compared to wP immunization ⁴⁶. This response was measured in serum 4 hours post-boost immunization, resulting in significant increases of G-CSF, KC, CXCL10, IL-6, IL-1 β , IL-5, MCP-1. Similarly, Warfel *et al.*, measured increases of IL-6, IL-1 β , IL-23, G-CSF, MCP-1, and MIP-1x from nasopharyngeal washes from baboons following challenge with *B. pertussis* ⁴⁷. As early as 48 hours after challenge levels of IL-6 and G-CSF were

significantly increased compared to negative control baboons. It may be beneficial to study the production of cytokines and chemokines produced early in the immune response to *B. pertussis* infection in the context of immunizations. The rationale being, that the production of cytokines associated with antigen presentation measured in NALT tissue or the draining lymph nodes could further elucidate the generation of the *B. pertussis*-specific immune response. In the mouse model it may not seem to be of consequence, but in human applications targeting of the appropriate tissue depends on the delivery method used in vaccination. In humans, nasal droppers, nasal sprays, nebulizers, and dry powders have been used for delivery of vaccines to the upper respiratory tract ^{34,48}. Vaccination of the lower respiratory system requires the use of a nebulizer-generated small-particle liquid or powder vaccines that are capable of reaching the lungs ^{34,49}. Understanding the differences in the immune response elicited between antigen priming occurring in either tissue will be crucial for the translation to non-human primate and ultimately clinical trials.

Mucosal Response to *B. pertussis*

Figure 3: Summary of proposed mucosal immune response to *B. pertussis*.

During the primary infection with *B. pertussis* innate immune cells are recruited nasal cavity. Bacteria and potential antigens can be phagocytosed by DC cells embedded in the epithelium layers, once activated DCs migrate to the draining lymph nodes where they present antigen to the naïve T cells. Specialized lymphoid tissue in the nasal cavity, NALT, line the nasal cavity. These NALT tissues contain M cells that endocytosis bacteria or particulate antigen and translocate the organism or macromolecule to DCs, B and T cells present on the basal surface of the cells. Antigen priming of naïve T cells differentiate to Th1, and Th17 effector cells in the draining lymph nodes or NALT tissue. T_{FH} cells activate B cells which promote *B. pertussis* antigen specific IgG and IgA antibodies. Effector Th1 and Th17 migrate to respiratory tract, a subset of CD4 memory effector cells are retained in the tissue as T_{RM}. Effector Th1/Th17 cells recruit macrophages and neutrophils phagocytose the now opsonized bacteria. Upon secondary infection by *B. pertussis* T_{RM} recognized the pathogen proliferating to effector Th1 and Th17 T cells, secreting IL-17 and IFN-γ. IL-17-secreting T cells have also been shown to play a critical role in the secretion of SIgA antibodies. The activation of the mucosal memory response immediately recruits macrophages and neutrophils to the infection, which with help from SIgA antibodies can efficiently phagocytosis *B. pertussis*. Thus, limiting the extent of the secondary infection. Figure adapted from Wilk

et al.⁵⁰ Solans and Locht⁵¹ and Neutra and Kozlowski⁴¹. PMN-polymorphonuclear neutrophils, Mφ-macrophage, B- Naïve B cells, T-Naïve T cell, NALT-Nasal associated lymphoid tissue, Th17-Th17 T helper cell, Th1-Th1 T helper cell, DC-Dendritic cell, T_{FH} -T follicular helper cell, T^{RM} TH17- Tissue resident memory Th17 cell, T^{RM} TH1- Tissue resident memory Th1 cell, Mem B- Antigen-specific Memory B cell

Importance of nasal cavity specific analysis

The findings presented in chapter 4 suggest that intranasal immunization is a viable strategy that needs to be further investigated. We and other groups are studying intranasal immunization against *B. pertussis*^{28,52}. It is clear that to continue down this avenue of research, the correlates of protection of the local mucosal immune response need to be fully characterized. Until recently, the majority of pertussis research has focused on characterizing the immune response in the lower respiratory tract in the mouse model; however, except in rare cases, *B. pertussis* infects the upper respiratory system⁵³. Wilk *et al.*, have begun to focus on the immune response and bacterial clearance of the nasal cavity, specifically⁸. This approach involves the intricate process of removal of the epithelial lining for analysis by flow cytometry and bacterial burden. Through this method, they have identified the role of IL-17 in promoting nasal cavity SIgA and IL-17-secreting T_{RM} cells in vaccine-induced protection^{8,28}. The role of T_{RM} in protection against *B. pertussis* is yet to be fully understood. Data exists that correlates the presence of the T_{RM} cell population in the nasal cavity to long term protection, specifically in the nasal cavity of mice⁵². Currently, it is suggested that the production of IFN-γ and IL-17 are essential to clearance in the nasal cavity, and data have shown the production of IFN-γ and IL-17 from T_{RM} and γδ T cells in the nares^{8,28,54}. However, the mechanism of clearance remains to be discovered.

A closer examination of the nasal cavity, the bacterial burden in the nares can be more accurately depicted. The nasal cavity clearance data included in this dissertation, along with the work of others, quantify bacteria burden present in nasal lavage fluid¹⁴. This method quantifies bacteria that were removed from the upper respiratory system by the lavage; however, it is apparent that a significant portion of the bacteria remain⁵². In moving forward, techniques to more accurately quantify the bacterial burden in the nares will be necessary to establish true sterilizing immunity. The importance of clearance in the nares is emphasized by our findings that the infection in the lung was cleared, but not nasal cavity. The different anatomy and microenvironments of the lung

and of the upper respiratory tract may play a role in bacterial adherence and colonization ⁵⁵. Findings by Conover *et al.* suggest that *B. pertussis* can potentially form biofilms in the mouse nose, while they did not in the mouse lungs; however, this is yet to be shown by other groups ⁵⁶. The authors found that the BPS polysaccharide of *B. pertussis* was responsible for colonization and promoted biofilm formation in the nasal cavity; but not in the lungs. Additionally, FHA has also been shown to promote biofilm formation and is critical for biofilm formation in the mouse nose and trachea ⁵⁷. Understanding of differences between the lower and upper respiratory system will be essential in determining why *B. pertussis* infection persists in the nasal cavity compared to the lower respiratory system. To observe these differences, experiments capable of addressing the mechanisms behind the immune responses between the tissues are needed.

In wP immunized and naïve mice we have measured robust recruitment of macrophages and neutrophils to the lungs (Chap. 2, Fig. 4, Chap. 3, Fig. 5,6, Chap. 4, Fig. 6). In these experiments, we determined the numbers of cells recruited to the infection; we did not address their functionality. It is possible that due to the Th1 response induced in convalescent or wP vaccine-induced protection APCs may more efficiently phagocytose bacteria. The experiments performed by Wilk *et al.* and Solans *et al.* have shown that IL-17 is critical for *B. pertussis* clearance from the nasal cavity. The mechanism; however, is yet to be determined. It would make the most sense to start with the roles of neutrophils in the nasal cavity, as IL-17 is known to induce recruitment of neutrophils.

Neutrophils in the nasal cavity, the unsung hero?

The question we are faced with is what changes between neutrophils in the mucosal response of an immune individual and that of a naïve individual? We have already discussed the first answer, that neutrophils have only been found to play a role in the clearance when bacteria are opsonized. We now know that IgA secreted through the mucosa can opsonize *B. pertussis*, presumingly leading to increased phagocytosis ²⁸. Another reason may be the timing of the neutrophil response. In a primary infection, there is a slight increase in neutrophils at day 1; however, the neutrophil response does

not peak until days 5 and 7 post-infection ⁵⁸⁻⁶¹. It could be hypothesized that the IL-17 produced by T_{RM} and $\gamma\delta$ T cells present in the nasal cavity, would recruit neutrophils to the nasal cavity earlier. Thus, decreasing the ability of *B. pertussis* toxins to inhibit phagocytosis of the bacteria. A third reason could be the neutralization of the toxins themselves. Secreted IgA and IgG present in the mucosa of a protected host could be inhibiting dysregulation of the innate immune response. The neutralization of PT alone has been shown in numerous studies to provide protection ^{62,63}. Neutralizing PT in the mucosa would increase neutrophil and macrophage recruitment to the site of infection, PT inhibits both macrophage and neutrophil recruitment to the infection ^{64,65}. Lastly, it was previously mentioned that *B. pertussis* biofilm formation could occur in the nasopharynx and trachea of a mouse ⁶⁶. Neutrophils produce NETS that trap bacteria in a “web” of DNA and proteins, destroying them through released enzymatic granules and proteases ¹¹. Recently Thanabalasuriar *et al.*, demonstrating that NETS were able to confine *P. aeruginosa* biofilms in the ocular biofilms and prevent dissemination ⁶⁷. These mechanisms provide potential roles of neutrophil-mediated clearance in the mucosal response to *B. pertussis*; however, as mentioned above, experiments need to be performed on neutrophils of the nasal cavity.

Neutralizing ACT in the nasal cavity

In chapter 3, we demonstrated the neutralization of ACT from serum of aP+RTX immunized mice (Chap. 3, Fig. 1). ACT can inhibit many of the bactericidal actions of macrophages and neutrophils, as discussed in Chapter 1. Determining the effects of neutralizing ACT directly in the respiratory tract, could lead to a more complete inhibition of the “one-two punch” toxin activity of PT and ACT, thus increasing clearance of *B. pertussis* in the nasal cavity⁶⁴. In chapter 3, found that the inclusion of RTX into DTaP administered through IP injection reduced IL-6 production in the lung. (Chap 3, Fig. 4). IL-6 is a pleiotropic pro-inflammatory cytokine known to be increased upon *B. pertussis* infection, as we and others have observed a large increase in IL-6 following challenge of a naïve or wP protected mouse ^{31,68}. In our interpretation, a reduction of IL-6 correlates with DTaP-mediated protection. Interestingly, in chapter 4, we observed the lowest levels of IL-6 in the lung when mice were immunized intranasally compared to intraperitoneally with IN-aP or IN-caP (Chap. 4, Fig 5.). Suggesting that priming the site

of infection, rather than inducing priming in a distal lymph node and generating a systemic response induces a stronger response in the respiratory tract.

It would then appear that by administering RTX in an intranasal DTaP vaccine, one could combine the additive effects of neutralizing RTX, with the enhanced local response generated from an intranasal vaccine. One would suspect that these protective effects would be the most evident in the bactericidal activity of macrophages and neutrophils in the respiratory tract where the cells would encounter active ACT. In particular, we would hypothesize enhanced phagocytosis and bacterial killing in neutrophils and macrophages due to neutralizing ACT mediated inhibition of oxidative burst. Additionally, it is known that ACT can inhibit NET formation due to cAMP levels ¹¹. It would be interesting to measure NET production and bacteria mediated killing in the lungs and nasal cavity, although these fragile structures will require fine-tuning of protocols. Furthermore, there is potential for NET formation to reduce biofilm formation in the upper respiratory system. Again, these experiments will need to focus on the upper respiratory system, but the results could be pivotal in the generation of future pertussis vaccines.

Potential future adjuvants

Our work with curdlan adjuvant demonstrated a modest increase in IL-17A in the lungs of mice immunized with DTaP and curdlan compared to mice immunized with DTaP alone (Chap. 3, Fig. 5). However, we did not observe an increase in macrophages or neutrophils recruited to the lungs. This may be due to the fact that all DTaP groups still contain aluminum hydroxide, promoting a Th2 response that is masking the effects of curdlan. Experiments to further elucidate the effects of β -glucans without aluminum hydroxide are ongoing and have yielded positive initial results. Still, it may be that optimal adjuvant is still yet to be found. Studies examining TLR 2 and 9 agonists from the Kingston Mills group have had success in the mouse model inducing Th1/Th17 responses and increasing IgG2a antibodies ^{14,69}. The same group has combined a TLR2 agonist with c-di-GMP, an agonist for the intracellular receptor stimulator of interferon genes (STING). This parenteral vaccine has been shown to promote Th1/Th17 responses, and particularly a *B. pertussis*-specific T^{RM} response in the lungs.

Importantly, this protective response was measured up to 10 months after immunization. Work isolating outer membrane vesicles (OMV) from *B. pertussis* has proven to be successful. *B. pertussis* OMVs and DT toxoids were shown to induce Th1/Th17 immune responses and increase the IgG2a/IgA ratio compared to a DTaP vaccine ⁷⁰. OMV vaccines are promising because they provide an increased pro-inflammatory response compared to DTaP; but, less reactogenicity than wP immunizations ^{46,71}. Particulate adjuvants are an interesting area of research as the particles may increase phagocytosis in the upper respiratory system, as discussed above. The combination of DTaP, CpG, and microparticles intranasally administered was shown to protect mice similar to levels observed in mice immunized with parenteral administered DTaP ⁷². It is possible that the conjugation of toxoids to the particles could increase vaccine effectiveness in future vaccines. Defining an optimal adjuvant is crucial to the success of a mucosal pertussis vaccine. The problem arises that boosting the immune response with a powerful adjuvant could have adverse effects and ultimately might also prevent licensure due to safety concerns.

Mucosal pertussis booster

It is because of this balancing act that the commercial producers of pertussis vaccines are at an impasse. DTaP/Tdap do prevent a large number of potential cases of whooping cough but these vaccines are not as effective as the wP vaccines in regard to the durability of protection. The use of the DTaP/Tdap vaccine strategy does protect the majority of those who receive the vaccine from clinical pertussis. It is clear that shortcomings exist with DTaP/Tdap vaccines, but the question remains, what is the alternative? DTaP/Tdap vaccines were originally designed to be safer vaccines capable of inducing similar protection to that of their wP predecessors, and they have. After completing the 5th dose DTaP protection ranges from 80-90% effectiveness ². Data from adolescents suggest that the problem exists in years following the completion of DTaP ⁷³. According to ClinicalTrials.gov, there are currently 318 clinical trials either ongoing, recently completed or upcoming studying pertussis, of those 62 are focused on booster immunizations. However, most of these focus on safety and formulation of currently available immunizations. It seems that the vaccine industry is reluctant to replace current vaccines due to effectiveness in preventing disease and their safety. However,

there is potential for replacement of Tdap with a booster vaccine capable of inducing a Th1/Th17 response. The problem, as eluded to in chapter 1, is that vaccine imprinting of the Th1 primary response induced from wP in humans promotes the same response even after DTaP booster immunizations ⁷⁴. This dilemma provides the strongest support for the development of an intranasal vaccine. The hypothesis being that parenteral booster immunizations are recognized by the memory T and B cells generated from the parenteral priming immunization. In altering the route of administration to the intranasal route, and with a different mucosal adjuvant the vaccine will stimulate the differentiation of naive B and T cells and induce a local mucosal immune response.

The work completed in this dissertation highlights the need to re-evaluate a system when the current strategies cannot address the problem. The development of the next generation of *B. pertussis* vaccines must improve upon waning immunity of DTaP/Tdap immunizations. It is the consensus of current literature that the most applicable way to do so is through mimicking the natural infection. To accomplish this goal researchers will need to continue to optimize systems that have reached their potential. To do so will require innovative methods, that are outside the realm of previous strategies. In this work, we demonstrate the optimization of the mouse model of pertussis to implement testing of future *B. pertussis* antigens and demonstrate the incorporation of a protective ACT toxoid into an acellular pertussis vaccine. We then altered the route of administration of the DTaP vaccine to a mucosal delivered vaccine and demonstrated the additive effects of generating a local mucosal immune response, capable of neutralizing *B. pertussis* toxins at the site of infection. This work establishes a foundation for future vaccine development, demonstrating the potential for improvements of DTaP/Tdap vaccines through the inclusion of RTX and intranasal delivery of pertussis vaccines.

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