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Utilizing the coughing rat model of pertussis to improve vaccine efficacy

Jesse M. Hall

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Utilizing the coughing rat model of pertussis to improve vaccine efficacy

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Utilizing the coughing rat model of pertussis to improve vaccine efficacy.

Jesse Michael Hall

**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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2021

**Keywords: *Bordetella pertussis*, pertussis, DTaP, mucosal immunization, whole
body plethysmography, coughing rat model of pertussis**

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Abstract

Utilizing the coughing rat model of pertussis to improve vaccine efficacy.

Jesse Michael Hall

Pertussis, also known as whooping cough, is a respiratory disease caused by the highly contagious, Gram-negative pathogen *Bordetella pertussis* (*Bp*). Infection occurs through inhalation of aerosolized droplets containing *Bp*, which then colonizes ciliated epithelial cells of the respiratory tract. Here, *Bp* expresses toxins and virulence factors that lead to leukocyte recruitment, paroxysmal cough, and impairment of host innate responses. Currently, in developed countries, acellular pertussis vaccines (aP; DTap; Tdap) are used to prevent *Bp* infection and whooping cough disease. However, we currently realize that the aP vaccine efficacy quickly wanes resulting in a reemergence of pertussis. Recent work performed by the CDC illustrates that current strains are genetically divergent from the strains originally used to formulate the aP, which may be partially responsible for the short-lived aP immunity. In an effort to evaluate pathogenesis of recent circulating strains and, in turn, improve vaccine efficacy, an animal model that recapitulates clinical manifestations of pertussis is necessary. First, we reintroduced the coughing rat model of pertussis by comparing two genetically divergent strains, Tohama 1 and recent isolate D420. Seven-week-old Sprague-Dawley rats were intranasally (IN) challenged with 10^8 viable CFUs. We measured the hallmark signs of pertussis disease such as neutrophilia, pulmonary inflammation, paroxysmal cough using whole body plethysmography (WBP), bacterial burden of the respiratory tract and characterized the serological response to known virulence factors. Overall, rats infected with D420 had an increase in bacterial

burden in the lung and nasal cavity, an increase in coughs over the course of infection, and an increase in anti-*Bp* IgM antibody titers compared to Tohama 1 challenged rats. The coughing rat model of pertussis can further be used as a preclinical tool to evaluate vaccine efficacy, as they are more feasible than baboons while still demonstrating classical manifestations of the disease. Next, we evaluated vaccine mediated immunity induced through IN and oral gavage (OG) vaccination of DTaP in the coughing rat model of pertussis, compared to intramuscular (IM)-whole cell pertussis (wP) and IM-aP immunized rats. Following vaccination, rats were similarly IN infected with 10^8 CFUs of *Bp* strain D420, cough, bacterial burden, respiratory distress, and both systemic and mucosal serological responses were analyzed over the nine-day infection. Our data show that both IN and OG vaccination protected against *Bp* colonization in the respiratory tract and *Bp* induced cough, similarly to IM-wP and IM-aP vaccinated rats. IN-aP and OG-aP vaccination also resulted in the production of anti-*Bp* IgG antibody titers at day 9 post-challenge; additionally, IN-aP vaccination induced mucosal anti-*Bp* IgA antibodies in the nasal cavity. Histology confirmed that both IN-aP and OG-aP immunization protected against acute inflammation in the lung. Altogether, these data further support that mucosal vaccination can generate a protective immune response against pertussis. Furthermore, the coughing rat model of pertussis can be used to improve our understanding of *Bp* pathogenesis and can be used to evaluate the “next generation” of pertussis vaccines.

Dedication

The work presented here is dedicated to my parents John and Dianne Hall. To the endless sacrifices you have made for me, I am forever grateful.

To my wife Samantha, Felix, and God who has provided me with unwavering support and love.

To my great grandfather John P. Howard

To anyone who believes that they too can make a difference.

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Table of Contents

Abstract.....	ii
Dedication	iv
Acknowledgements	v
Table of Contents	vi
Chapter 1. Introduction.....	1
1.1 <i>Bordetella pertussis</i> overview	2
1.2 Pathogenesis.....	3
1.3 Pertussis vaccines	8
1.4 Waning efficacy of the current acellular vaccine.....	10
1.5 Recent Evolution of <i>Bp</i>	11
1.6 Animal models of pertussis.....	11
1.7 Coughing rat model of pertussis	14
1.7.1 Intranasal challenge	17
1.7.2 Intrabronchial challenge	18
1.8 Natural immunity against <i>Bp</i>	22
1.9 Vaccine mediated immunity	25
1.10 Mucosal immunization of DTaP	28
1.11 Overall Objective	29
1.12 References	31
Chapter 2. Re-investigating the coughing rat model of pertussis to understand Bordetella pertussis pathogenesis	51
2.1 Abstract.....	53
2.2 Introduction	54

2.3 Results.....	58
2.4 Discussion	67
2.5 Materials and methods.....	71
2.6 Acknowledgements.....	78
2.7 Figures.....	79
2.8 Supplemental Figures	90
2.9 References	102
Chapter 3. Mucosal immunization with DTaP confers protection against <i>Bordetella pertussis</i> infection and cough in Sprague-Dawley rats.....	113
3.1 Abstract.....	115
3.2 Introduction	116
3.3 Results.....	120
3.4 Discussion	131
3.5 Materials and methods.....	136
3.6 Acknowledgements.....	143
3.7 Figures.....	145
3.8 Supplemental Figures	155
3.9 References	164
Chapter 4. Discussion	176
4.1 Overview	177
4.2 Lessons learned from the coughing rat model of pertussis.....	178
4.3 Investigation into the mechanism of <i>Bp</i> induced cough.....	181
4.4 <i>Bordetella bronchiseptica</i> and <i>Bordetella parapertussis</i> challenge experiments in rats	182
4.5 Lessons learned from mucosal immunity	183
4.6 Mucosal immunity	187

4.7 Improvements to DTaP/Tdap.....	188
4.8 Improvement of oral vaccination against pertussis	191
4.9 Experimental oral vaccines of potential.....	192
4.10 In summary	193
4.11 References	194

List of Figures

Figure 1. Reported pertussis incidence in the United States from 1922-2019.....	9
Figure 2. Percent death of young <i>Bp</i> infected rats.	17
Figure 3. Summary of the immunity generated from natural infection.	25
Figure 4. Summary of the DTP and DTaP vaccine mediated immunity.....	27
Figure 5. Experimental design of intranasal infection of <i>Bp</i>	80
Figure 6. Infection of <i>Bordetella pertussis</i> induces cough in Sprague-Dawley rats.	81
Figure 7. <i>Bordetella pertussis</i> infection impairs respiratory capacity.	82
Figure 8. <i>Bp</i> infection induces acute and chronic inflammation in the lung of Sprague-Dawley rats.	83
Figure 9. <i>Bp</i> infection induces inflammation of the lung and decrease in weight gain...	84
Figure 10. Analysis of the nasal associated lymphoid tissue.	85
Figure 11. Analysis of bacterial burden over the course of <i>Bp</i> infection.	86
Figure 12. Immunofluorescence staining of <i>Bp</i> localization in the respiratory tract.	87
Figure 13. Neutrophil recruitment and proinflammatory cytokine production after challenge determined by flow cytometry and multiplex immunoassay analysis.....	88
Figure 14. Measurement of serum antibody titers over the course of <i>B. pertussis</i> infection.	89

Figure 15. IN booster vaccination induces systemic anti-Bp and anti-PT antibody titers.	145
Figure 16. Intranasal and oral vaccination of acellular pertussis vaccine decreases cough of <i>B. pertussis</i> infected rats.	146
Figure 17. Intranasal vaccination decreases pulmonary restriction of <i>Bordetella pertussis</i> infected rats.	147
Figure 18. Mucosal vaccination induces production of anti- <i>Bp</i> IgG, while IN immunization also induces both anti-PT IgM and IgG antibodies.	148
Figure 19. Intranasal immunization elicits the production of anti- <i>Bp</i> IgA in the respiratory tract.	149
Figure 20. Mucosal vaccination protects against acute and total inflammation in the lung of <i>Bp</i> infected Sprague-Dawley rats.	150
Figure 21. Oral and intranasal immunization decreased <i>B. pertussis</i> bacterial burden in the respiratory tract.	152
Figure 22. Measurement of cytokines in the lung and serum at days 1 and 9 post infection.	153
Figure 23. Systemic and mucosal anti- <i>Bp</i> and anti-PT antibodies correlate with observed protection.	154
Figure 24. Vaccine generated immune responses from intramuscular and mucosal immunization of DTaP, and booster immunization of DTaP	189

List of tables

Table 1. <i>Bordetella</i> virulence associated genes. +/- annotates expression in select strains	7
Table 2. Summary of literature and results using the coughing rat model of pertussis..	16
Table 3. Summary of vaccine mediated immune responses following <i>Bp</i> challenge in the coughing rat model of pertussis.	185

List of Supplemental figures

Figure S 1. Set up for the whole-body plethysmograph	90
Figure S 2. Diagram illustrating Penh during <i>Bp</i> infection.....	91
Figure S 3. <i>Bordetella pertussis</i> infection induces cellular recruitment in the lungs	92
Figure S 4. Bacterial colonization comparisons between CD1 mice and Sprague-Dawley rats	93
Figure S 5. Hematoxylin and eosin staining of the nasal cavity of Sprague-Dawley rat.	94
Figure S 6. Immunofluorescence (IF) staining of control rat's respiratory tract.....	95
Figure S 7. Serum cytokine response during twelve-day <i>Bordetella pertussis</i> infection	96
Figure S 8. Lung cytokine response during twelve-day <i>Bordetella pertussis</i> infection. .	97
Figure S 9. Serum chemokine during twelve-day <i>Bordetella pertussis</i> infection	98
Figure S 10. Lung chemokine during twelve-day <i>Bordetella pertussis</i> infection	99
Figure S 11. Measurement of serum antibody titers over the course of <i>B. pertussis</i> infection.....	100
Figure S 12. Area under the curve analysis of IgM and IgG serum antibody titers against <i>Bordetella pertussis</i> over the course of infection	101
Figure S 13. Experimental design of vaccination and challenge.....	155
Figure S 14. Respiratory profile of Sprague-Dawley rats vaccinated and challenged with <i>B. pertussis</i>	156
Figure S 15. ELISpot assay was performed to measure the number of <i>Bp</i> specific IgG cells from the bone marrow at day 9 post challenge.....	158
Figure S 16. Measurement of lung and body weight at days 1 and 9 post challenge..	159
Figure S 17. Measurement of cytokines in the lung at days 1 and 9 post infection	160
Figure S 18. Measurement of cytokines in the serum at days 1 and 9 post infection..	161
Figure S 19. Characterizing circulating cell populations in the blood and lung post infection.	162

Figure S 20. Proposed mucosal immune response induced through oral vaccination. 163

List of abbreviations

ACIP	Advisory Committee on Immunization Practices
ACT	Adenylate cyclase toxin
ANOVA	Analysis of variance
aP, DTaP ,Tdap	Acellular pertussis vaccine
AUC	Area under the curve
<i>Bb</i>	<i>Bordetella bronchiceptica</i>
BG	Bordet-Gengou
<i>Bp</i>	<i>Bordetella pertussis</i>
BrkA	Bordetella resistance to killing A
BSA	Bovine Serum Albumin
CDC	Centers of Disease Control
CoP	Correlates of protection
DTP	Diphtheria Tetanus Pertussis vaccine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot assay
F	Frequency
FHA	Filamentous hemagglutinin
Fim	Fimbriae
GALT	Gut-associated lymphoid tissue
H&E	Hematoxylin and eosin

IACUC	Institutional Animal Care and Use Committee
IB	Intrabronchial
IC	Intracerebral challenge
IF	Immunofluorescence
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MPLA	Monophosphoryl lipid-A
MVb	Minute Volume
MVC	Mock vaccinated challenge
NALT	Nasal-associated lymphoid tissue
NETs	Neutrophil extracellular traps
OG	Oral Gavage
PAU	Pause
PBS	Phosphate buffer Saline
PEF	Peak expiratory height
PenH	Enhanced pause
PIF	Peak inspiratory height
PMNs	Polymorphonuclear cells
PRN	Pertactin
PT	Pertussis toxin

PTd	Detoxified pertussis toxin
RTX	Repeat in toxin domain
SSM	Stainer-Scholte liquid media
STING	Stimulator of interferon genes
Te	Expiratory time
Ti	Inspiratory time
Tr	Relaxation time
TVb	Tidal volume
vags	Virulence associated genes
vrgs	Virulence repressed genes
WBP	Whole-body plethysmography
wP	Whole-cell pertussis vaccines
WT	Wild-type

Chapter 1. Introduction

1.1 **Bordetella pertussis overview**

Pertussis is a respiratory disease caused by the Gram-negative pathogen *Bordetella pertussis* (*Bp*) (1). Pertussis, also known as whooping cough, was originally reported during the 15th century in Persia (2). Patients with pertussis were often described as if they were being strangled without the capacity to expel the irritants in the lung causing the infection, simultaneously being incapable of inspiration (2). Paroxysmal coughing occurs so violently that blood can be expelled from the nose and mouth (2). It was not until 1906 that Jules Bordet and Octave Gengou were able to culture *Bp in vitro* (3). Bordet-Gengou (BG) agar was created as a defined medium for the isolation of *Bp*, and it consists of glycerine, potato starch, blood, and agar (4). Potato starch is the critical component of BG, as fatty acids have been shown to inhibit *Bp* growth, and the starch works to sequester the fatty acids not only produced by *Bp*, but from the sputum and nasal secretion of patients (3).

Bp is a respiratory pathogen that exclusively infects humans, particularly infants (5). Transmission of *Bp* occurs through the aerosolization of respiratory droplets containing *Bp* (6). Once inhaled, *Bp* adheres to the epithelial cells of the respiratory tract (7–9). In humans, incubation of *Bp* is on average is approximately 7-21 days, and *Bp* infection can be broken down into three separate phases: catarrhal, paroxysmal, and convalescent (10). During the catarrhal phase of pertussis, mild symptoms ensue reflective of a common upper respiratory tract infection (11). Symptoms include rhinorrhea, sneezing, anorexia, and a cough that increases in severity and frequency as the infection progresses (11). One week following the first signs of illness, the patient then enters the paroxysmal phase, which is characterized by paroxysmal coughing lasting upward of one

to four weeks (11). The Centers for Disease Control (CDC) defines paroxysms as multiple rapid, violent coughs followed by the classical whoop sound associated with pertussis, as a result of hurried and forced inspiration of air into the lungs (12). Along with the paroxysms; vomiting, exhaustion, uncontrolled mucus production, increase in white blood cell count (lymphocytosis and leukocytosis), bronchopneumonia, hypoxia, and choking can occur in infants (13). Four to six weeks following the onset of pertussis, the patient will enter the convalescent phase, where paroxysms begin to diminish; however, nonparoxysmal coughing can persist several months following infection (11). Macrolides are the standard treatment currently used to treat patients with pertussis. A fourteen-day round of erythromycin is recommended, azithromycin and clarithromycin have also been used (14). However, recently in China macrolide resistant *Bp* has emerged (15). In severe cases, pertussis can be fatal (0.1% of pertussis cases from 2000-2016) especially in unvaccinated infants (16). It is estimated there are 24.1 million cases of pertussis worldwide contributing to about 160,700 deaths every year in children < 5 years of age (17).

1.2 Pathogenesis

Animal models of pertussis have been instrumental in our limited understanding of *Bp* pathogenesis. As highlighted above, *Bp* is transmitted via the inhalation of respiratory droplets containing *Bp* from an infected host (6, 18). Upon entering the host, regulation of virulence factors is controlled via the master virulence regulator, *bvgAS*, which is a two-component signal transduction system (19–21). The sensor kinase *bvgS* is activated resulting in the phosphorylation of the response regulator, *bvgA* (22). Phosphorylation of *bvgA* and *bvgS* activation, commonly referred to as the *bvg*⁺ phase, leads to the

expression of virulence associated genes (vags) such as pertussis toxin (PT), filamentous hemagglutinin (FHA), fimbriae (Fim), and adenylate cyclase toxin (ACT) as well as the repression of virulence repressed genes (vrgs) (22–26).

Upon inhalation, *Bp* uses adhesins such as FHA and Fim to attached to the ciliated epithelium of the respiratory tract (22). The autotransporter pertactin (PRN) has been suggested to play a role in initial attachment of *Bp*, as PRN contains Arg-Gly-Asp motifs, which have been shown to play a role in epithelial binding (27). FHA is 232-kDa hairpin-shaped protein that is expressed on the outer membrane of all *Bordetella* spp. Data from animal models have shown that FHA is crucial for *Bp* attachment and is highly immunogenic (13, 28). Proteomic analysis has suggested that FHA is one of the most abundant proteins of *Bp* (29). *Bp* lacking FHA have shown to have diminished capacity for colonization of the trachea (30, 31). Fim, which are filamentous in structure, are expressed on the cell surface and have been shown to be required for tracheal colonization (32). Fim negative strains have shown decreased capacity for bacterial attachment *in vitro*, and *in vivo*; additionally, Fim negative strains had reduced growth in the upper respiratory tract in mice (32, 33). *Bp* has 6 serotypes of Fim, with Fim1 being common amongst all *Bp* strains while Fim2 has been found on numerous *Bp* isolates (34).

Once *Bp* colonization occurs and infection is established, toxins are then released, the most notable of which is PT. PT is an AB₅ ADP-ribosylating toxin that is released and disseminated systemically (35). PT consist of 6 polypeptides from S1 to S5 subunits; S1 designates the enzymatically active A subunit of PT, while S2, S3, S4, and S5 comprise

the B subunit (36–39). The S2-S5 subunits form a pentamer that binds to glycoproteins on eukaryotic cell membrane surfaces delivering the A subunit (36–39). In the cytoplasm, ADP-ribosylation of G_i proteins occur rendering them inert (40, 41). This leads to an increase in cytosolic cAMP and dysregulation of G_i protein signaling. The resulting effect leads to the dysregulation of innate immune cells particularly neutrophil and macrophage recruitment to the site of infection (40, 41). PT has also been shown to aid in adherence during colonization. Morse *et al* (1976) demonstrate that PT also causes leukocytosis during infection (42). Other studies have shown that PT can increase sensitivity to histamines, bradykinin, and serotonin (43).

ACT is also regulated by *bvgAS* and is secreted during infection. ACT is comprised of an adenylate cyclase (AC) domain and a repeat in toxin domain (RTX) linked with a hydrophobic domain responsible for pore formation (44–48). ACT remains localized, as most of the ACT remains associated with FHA on the bacterial surface, and secreted ACT quickly forms aggregates in solution rendering it inactive (49, 50). Upon secretion, ACT binds phagocytic cells via the $\alpha M\beta 2$ integrin or CR3 receptor (51). Once bound, the AC domain is translocated into the cell where it can bind calmodulin to catalyze cAMP from ATP, resulting in the dysregulation of cellular signaling pathways (52). Most notably, cAMP formation results in the inhibition of antigen presentation by antigen presenting cells and induces apoptosis (53–55). ACT activity in macrophages results in membrane ruffling, cytoskeleton rearrangement, and loss of complement-mediated phagocytosis (56).

Other virulence factors associated with *Bp* include dermonecrotic toxin, tracheal cytotoxin, and lipopolysaccharide (LPS) (57). Dermonecrotic toxin is a heat-labile toxin that causes necrotic lesions, and intravenous administration of dermonecrotic toxin is lethal in mice (58, 59). Tracheal cytotoxin is a disaccharide-tetrapeptide and during infection, tracheal cytotoxin leads to damage of the ciliated epithelial cells of the respiratory mucosa (57). LPS of *Bp* lacks the O antigen and it is referred to as lipooligosaccharide (LOS), which is extremely immunogenic; and O antigen negative mutants are attenuated during infection (22).

Virulence factor	Gene	<i>B. pertussis</i>	<i>B. bronchiseptica</i>	<i>B. paraptussis</i>	Function	Secreted	Component of aP
Toxins							
Adenylate cyclase toxin	<i>cya</i>	+	+	+	hemolysis, disregulate cell signalling, inhibition of macrophage function	+	-
Dermonecrotic toxin	<i>dnt</i>	+	+	+	Dermal necrosis	+	-
Pertussis toxin	<i>ptx, ptxI</i>	+	-	-	ADP-ribosyltransferase, disregulate cell signaling, leukocytosis, lymphocytosis	+	+
Tracheal cytotoxin	<i>tct</i>	+	+	+	Impairment to ciliated epithelial cells	+	-
Other virulence factors							
Autotransporter subtilisin-like protease	<i>sphB1</i>	+	+	+	Autotransporter	-	-
<i>Bordetella</i> resistance to killing	<i>brk</i>	+	+/-	+	Resistance to complement	-	-
Filamentous hemagglutinin	<i>fha</i>	+	+	+	Adhesion/immunosuppressive	+/-	+
Flagella	<i>flg, fli</i>	+	+	-	Motility	-	-
Fimbriae	<i>fim2, fim3</i>	+	+	+	Adhesion	-	+
Lipooligosaccharide	<i>wlb, wbm</i>	+	+	+	Endotoxin	-	-
Pertactin	<i>prn</i>	+	+	+	Adhesion	+	+
Tracheal colonization factor	<i>tcf</i>	+	-	-	Damage to ciliated epithelial cells	+	-
Type III secretion system	<i>bsc, btr</i>	+	+	+	Protein-export apparatus	+/-	-
Virulence associated gene 8	<i>vag8</i>	+	+	+	Autotransporter	-	-

Table 1. *Bordetella* virulence associated genes. +/- annotates expression in select strains

1.3 Pertussis vaccines

In the pre-vaccine, era the number of pertussis cases ranged from 150,000-200,000 cases in the United States annually (**Fig. 1**) (59). After the *in vitro* culture of *Bp*, the development of whole-cell pertussis vaccines (wP) immediately ensued. The first licensed wP was made from aliquots of killed *Bp* in 1914 (60). It was not until 1948 that wP was combined with diphtheria and tetanus toxoids creating the Diphtheria Tetanus Pertussis vaccine (DTP) (11, 61, 62). Widespread use of DTP in the United States led to a 90% decrease in the number of reported *Bp* infections (63). After implementation of the DTP, the number of pertussis cases in the United States was < 1,000-5,000 cases between 1970-1980 (**Fig. 1**) (59). With the decrease in number of pertussis cases, it was hypothesized the disease was eradicated (64). Despite the efficacy of this vaccine, serious adverse side-effects ensued including swelling and redness at the injection site, fever, and uncontrollable crying of infants (65). In rare instances, vaccine-induced encephalopathy was reported following DTP immunization (66). Due to these side-effects, patient compliance regarding DTP vaccination decreased, particularly in Britain, Japan, and United States (11). After two infant mortalities were reported following DTP immunization, the Japanese Ministry of Health and Welfare formed the Pertussis Vaccine Study Group for the development of a safer and effective acellular pertussis vaccine (aP, DTaP, Tdap) in 1981 (67). Low to middle income countries still use DTP vaccines today to protect against pertussis.

It was not until 1984 that Sato and Sato developed the first aP in Japan, which consisted of formaldehyde treated FHA and formalin treated PT (67). Vaccination with the newly developed aP was protective in children over the age of 2 years old (68). A subsequent

Figure 1: Reported pertussis incidence in the United States from 1922-2019. Adapted from the CDC.

vaccination study also confirmed that the aP was less reactogenic than the wP, despite being protective and generating a serological response to the vaccine antigens (69). During the development of the aP, 13 antigens were originally selected for testing (70). The antigens that were selected to be included in the aPs were FHA, Fim, PT, and PRN, as each one of these antigens were immunogenic, providing a serological response after vaccination and playing a role in *Bp* pathogenesis (70). ACT was thoughtfully considered to be included, but with the limited research surrounding ACT at the time and the limited availability of a purified product, ACT was left out of aP formulations. It was not until 1996

that DTaP was licensed for use in infants in the United States. One year later, the Advisory Committee on Immunization Practices (ACIP) recommended DTaP vaccination over DTP vaccination in infants. It was not until 2006, that the ACIP recommended that a Tdap booster vaccine be administered during adolescence.

1.4 Waning efficacy of the current acellular vaccine

After the switch from the DTP to DTaP, there has been a significant increase in the number of pertussis cases, especially in the United States (71) (**Fig. 1**). While nationwide vaccine coverage is 95% in the United States, the incidence of pertussis has risen in the past 10 years as the number of only DTaP vaccinated individuals has increased in size. (72). Studies suggest that four years after a patient's last DTaP vaccination, the induced vaccine efficacy is only nine percent, which is unacceptable vaccine protection (5). While vaccine coverage remains high, the population dynamics are changing, and more people are DTaP immunized as new generations are born and the DTP immunized only generations age. Numerous studies have demonstrated waning efficacy following DTaP vaccination in parallel with the emergence of genetically divergent strains of *Bp* (73–79). Increased surveillance of *Bp* has led to the identification of clinical isolates that do not express PRN, FIM, and even PT, the hallmark toxin of the organism (80). Research has shown that PRN mutant strains have been present since the 1990s, while only 15% of isolates express PRN currently (81). *Bp* is evolving to not express the proteins that are found in the current aP (80). One plausible hypothesis to explain these observations is that circulating *Bp* strains have evolved due to aP vaccine pressure. Vaccine-driven evolution of *Bp* could contribute to the waning efficacy of the aP. While we know the current strains are genetically different, we do not know if this genetic variability affects

virulence, disease burden, toxicity, or fitness of the pathogen (79). With that said, it is imperative to find a means to increase the efficacy of the current pertussis vaccines.

1.5 Recent Evolution of *Bp*

Since the introduction of DTaP in 1996, the *Bordetella* research community has observed cyclic increases in the reported number of pertussis cases caused by the bacterium *Bp* (82). Current surveillance of *Bp* in the United States has identified genetic changes alongside the reemergence of pertussis (83, 84). This is highly alarming as *Bp* is naturally considered to be a monomorphic pathogen (85). Recent clinical isolates have been identified that are deficient in either FHA, PRN, and PT, which are components of the aP, despite adequate vaccine coverage (86–89). New data generated by the CDC highlights the genetic divergence of current circulating strains of pertussis from the reference strains that originally helped to produce the vaccine (83). The clinical isolates differ due the presence of SNPs and chromosomal rearrangements which could contribute to the pathogen's overall fitness and ability to infect. It is essential to critically evaluate the significance of the evolution of these strains and their potential disease burden.

1.6 Animal models of pertussis

Bp is exclusively a human respiratory pathogen, and as a result, a diverse repertoire of animal models have been used. The evolution of animal models of pertussis has been fundamental in our understanding of *Bp* pathogenesis and the formulation of vaccines against this pathogen. Robust efforts have been made to develop animal models capable of recapitulating pertussis as seen in humans. Currently, mice have been widely accepted and are used to study pertussis, in addition to non-human primates, piglets, and rats.

Cost, accessibility of reagents, and genetic manipulation also contribute to the feasibility of mice as a model of pertussis.

Mice infected with *Bp* develop leukocytosis, hyperinsulinemia, histamine sensitization, and comparable respiratory infection to humans (90). In mice, three different routes of *Bp* challenge have been explored: intracerebral (IC), intranasal (IN), and through aerosol exposure. These routes of *Bp* challenge were selected as *Bp* can only grow in the respiratory tract or the brain of mice (11). In 1947, Pearl Kendrick and Grace Eldering developed the IC mouse model to assess wP vaccine efficacy, as the pertussis community at the time needed a way to test pertussis vaccine potency (91). IC *Bp* challenge in mice was considered the gold standard for testing wP vaccine potency at the time, as results correlated to similar vaccine potency tests in field trials by the Medical Research Council (92). In short, mice were intraperitoneally (IP) vaccinated with wP, followed by a subsequent 10^5 CFU IC *Bp* challenge at 14 days post-vaccination (91). Vaccine efficacy was determined by the survival of the mouse (91). After development of the aP, IC challenge model in mice failed to predict aP vaccine efficacy; however, the IN *Bp* challenge model was successful at evaluating aP vaccine efficacy (93). Vaccine efficacy was then determined not solely on survival of the mouse, but also the serological response to vaccine antigens and bacterial clearance in the respiratory tract (93). Aerosol challenge of *Bp* was investigated as IN *Bp* challenges rely on depositing large bacterial loads into the lungs of mice rather than uniform bacterial placement in the respiratory tract (22). Aerosol challenge resulted in a more uniform respiratory tract infection compared to IN *Bp* challenge (94). Intraperitoneal challenge of *Bp* in mice was also investigated as a

potential method for challenge; however, *Bp* did not readily colonize in the peritoneal cavity (95). Limitations of the mouse model of pertussis include the absence of a paroxysmal cough, reproducible pertussis pneumonia, transmission, and limited nasal colonization (22).

Piglets have also been used as an animal model to study pertussis (96–99). In 2005, Elahi *et al* demonstrated that 4-week-old piglets can be infected from intrapulmonary inoculation of *Bp* (96). *Bp* challenged piglets display nonparoxysmal coughs, labored breathing, nasal discharge, and fever (96). Following challenge, all piglets developed bronchopneumonia, and histology confirmed pulmonary inflammation of neutrophils and macrophages in the airway (96). One caveat of the pig model of pertussis is that pigs older than five weeks of age are not readily infected following *Bp* challenge (97). This phenomenon is observed as the peptide beta-defensin 1 is present in the upper respiratory tract after five weeks of age and protects against *Bp* challenge (98). The pig model of pertussis has demonstrated that maternal immunization confers protection to offspring following vaccination (99). Pregnant sows were vaccinated with heat inactivated *Bp* and their newborn piglets fed from their mothers for approximately 5 days followed by subsequent *Bp* challenge (99). Newborn piglets were protected against marked pathological changes and bacterial burden in the respiratory tract (99).

Besides rats, nonhuman primates are the only other known model of pertussis that share similar coughing manifestation following *Bp* challenge (100, 101). IN inoculation of *Bp* in nonhuman primates results in lymphocytosis, leukocytosis, and transmission of *Bp* (101–

106). Recently, Warfel *et al* (2012) evaluated baboons as a potential pertussis animal model. Olive baboons were selected as they have similar body temperatures to humans (101). Baboons IN challenged with 10^{10} CFU of *Bp* resulted in cough, bacterial colonization, and leukocytosis (101). Warfel *et al* (2014) also showed that aP vaccinated baboons were protected against pertussis disease following subsequent *Bp* challenge; however, they were not protected against bacterial colonization and transmitted *Bp* to unvaccinated baboons (107). wP immunized and convalescent baboons rapidly cleared the infection following *Bp* challenge (107). Protection afforded by the aP was suggested to be a more Th2-skewed immune response, while wP and convalescent immunity induces a mix of Th1 and Th17 immune responses (107–109). These data illustrate that the immune response generated by aP vaccination protects against pertussis disease but fails at protecting against *Bp* transmission to an unvaccinated host; however, the Th1 and Th17 immune response generated by wP and prior infection prevents colonization and the subsequent transmission of *Bp* (107). More recently, rhesus macaques have been used as a nonhuman primate model of pertussis (110). While the nonhuman primate model of pertussis shares many clinical manifestations of pertussis similar to humans, the expensive animal cost, limited and specialized facilities, and ease of use limit the use and availability of this model.

1.7 Coughing rat model of pertussis

The coughing rat model of pertussis was first introduced in 1939, and nine studies in total have been published using the coughing rat model of pertussis to study *Bp* pathogenesis and vaccine-mediated immunity (**Table 1**). Two separate *Bp* challenge methods have been used in the development of the coughing rat model of pertussis. In 1939, Hornibrook

and Ashburn IN challenged rats with *Bp*, as mice were readily infected following IN challenge of *Bp* (111). IB inoculation of *Bp* was not utilized until 1989, which was developed in part as IB inoculation of *Pseudomonas aeruginosa* resulted in an respiratory infection (112). During this time, Hornibrook was also working on developing a semisynthetic liquid medium for the isolation of soluble antigens (113).

Publications			Results							
Abbr. Title	Date	Inf. Method	<i>Bp</i> induced Cough	Histology of the lung	Leukocytosis/ lymphocytosis	<i>Bp</i> induced Antibody titers	<i>Bp</i> recovered from lung	Body wt. change	Vaccines studied	
Study of Experimental Pertussis in the Rat	1939	Intranasal	Yes	Acute and chronic inf.			Yes			
Development of a Rat Model for Respiratory Infection of <i>Bp</i>	1989	Intrabronchial	Yes	Acute and chronic inf.	Yes		Yes			
Coughing Rat Model of Pertussis* (Pertussis report)	1993	Intrabronchial	Yes		Yes		Yes	Loss of body wt.		
Cough production, leucocytosis, and serology of rats	1994	Intrabronchial	Yes		Yes	IgG:PT, <i>Bp</i> , FHA				
Responses to <i>Bp</i> mutant strains and to vaccination	1994	Intrabronchial	Yes			PT, <i>Bp</i> , FHA			DTP	
Differences in Coughing and other responses to <i>Bp</i> infection	1997	Intrabronchial	Yes		Yes	IgG:PT, <i>Bp</i> , FHA		Loss of body wt.		
Responses to acellular pertussis vaccine coughing rat model	1998	Intrabronchial	Yes		Yes	IgG: PT, <i>Bp</i> , FHA, PRN		Loss of body wt.	2-aP, 3-aP, DTP	
Time-course of infection and responses in a rat	1999	Intrabronchial	Yes		Yes	IgG:PT, FHA	IgA: PT, FHA	Lung Wt.		
Bspr/BtA and Anti factor regulates <i>Bb</i> to cause cough	2019	Intranasal	Yes				Yes			

Table 2. Summary of literature and results using the coughing rat model of pertussis (111-120).

1.7.1 Intranasal challenge

It was first reported in 1939 that young rats developed paroxysmal cough after IN inoculation of *Bp* (111). Hornibrook and Ashburn hypothesized that rats could be a suitable animal model to study pertussis, as mice infected with *Bp* produced pneumonia and death following IN challenge (111). To test this hypothesis, 3-week old rats were intranasally infected with *Bp* and survival was monitored for up to 10 days post-challenge (111). Following challenge, approximately 12 percent of *Bp* infected rats died after two days post-challenge (**Fig. 2**) (111). By day 9 post-challenge, 72.5 percent of *Bp* infected rats died (**Fig. 2**) (111). Coughing was recorded for 12 of the 51 *Bp* infected rats, and it was noted that on average, coughing did not appear until approximately 8 days post-challenge (111). Coughing continued in some rats for up to 60 days post-challenge (111).

Figure 2. Percent death of young *Bp* infected rats. 3-week old Wistar albino and hooded rats were intranasally infected with 1.7×10^8 CFUs of *Bp*. Survival was monitored for up to 9 days post-challenge. Adapted from (Hornibrook and Ashburn, 1939).

Pathological examination revealed that *Bp* colonization of the lung resulted in interstitial bronchopneumonia combined with exudate infiltration into the alveoli (111). By day 3 post-challenge, infiltrating neutrophils accumulated in the lung, followed by monocyte infiltration by day 6 post-challenge (111). It was not until 2019, that IN inoculation of *Bordetella bronchiceptica* (*Bb*) was used to evaluate mechanisms responsible for *Bb* elicited cough (114). In summary, these data revealed that rats can be readily infected with *Bp* and infected rats developed cough-like paroxysms similar to humans.

1.7.2 Intrabronchial challenge

To the best of our knowledge, the rat model of pertussis was not used again for almost 50 years. It can be hypothesized that the lack of studies using the coughing rat model of pertussis was due to the development and implementation and success of DTP (112). As safety concerns surrounding the DTP emerged, so too did the need for an adequate animal model to understand the pathogenesis of *Bp*. Significant progress was made, in parallel, utilizing mice as a challenge model to identify protective aP antigens; however, mice lacked several aspects of disease as seen in humans: Most notably, *Bp* induced cough combined with an upper respiratory tract infection and a repeatable pertussis elicited pneumonia. Woods *et al* (1989) decided to utilize IB challenge Sprague-Dawley rats with *Bp* encased in agar beads, as this challenge method was successful at establishing *Pseudomonas aeruginosa* infection in rats (112). In brief, 10^7 CFUs of *Bp* was added to a mixture of melted 2% agar and heated mineral oil, and rapidly cooled creating *Bp* encased agar beads (112). Two hundred adult (~200 gram) Sprague-Dawley rats were challenged with either Tohama I (virulent) or Tohama III (avirulent) (112). Tohama I was recovered from the lung for up to 7 days post-challenge, while no infection

was observed in Tohama III infected rats (112). No morbidity was observed following challenge (112). Pathological analysis revealed lymphocyte infiltration into the bronchi at day 3 post-challenge, followed by inflammation of the mucous membranes and bronchioles by monocytes and polymorphonuclear cells (PMNs) by days 7 and 21 post-challenge (112). It was also noted that intrabronchial challenge of *Bp* induced a “paroxysmal like” cough as seen in humans (112). A following study performed by Wardlaw *et al* (1993) utilized sound activated tape recorders to detect cough for up to 21 days post challenge (115). Isolated coughs along with multi-cough events, similar to a paroxysmal cough, were detected (115). Peak numbers of cough were recorded at day 10 post-challenge and an increase in white blood cell counts were also observed over the 21-day infection (115). Hall *et al* (1994) further confirmed that *Bp* infected rats developed paroxysmal cough and leukocytosis (116). The serological response to *Bp* challenge was also investigated: Intrabronchial challenged rats developed IgG antibody titers to *Bp* sonicate, PT, and FHA at day 28 post-challenge (116). It was not until 1999, that Hall *et al* illustrated the unique inverse relationship between bacterial burden and cough (117). By day 10 post-challenge, as cough counts were the highest, bacterial burden in the lung was the lowest (117).

With the development of a pertussis model that shared similar coughing manifestations as humans’, mechanisms responsible for the *Bp* induced cough were explored. Rats challenged with Phase 1 (virulent) strains of *Bp* such as 18-323, Tohama I, and L-84 induced coughing at days 9-14 post-challenge (118). Phase 4 (avirulent) strain L-84 induced no coughing, highlighting that virulence factors are responsible for *Bp* induced

cough (118). *Bp* strain 357 harboring a transposon-insertion in the region encoding for PT induced no coughing following challenge (118). Similar results were observed in rats challenged with *Bordetella parapertussis*, which does not produce PT (118). Although debated, these results suggest that PT plays a role in the induction of cough during pertussis infection. Further studies are needed to accurately depict the factor(s) inducing cough following infection.

Strains of rats were also evaluated in the development of the rat model of pertussis. While past studies relied on Sprague-Dawley rats to evaluate *Bp* pathogenesis, Brown Norway, Lewis, and Hooded Lister rats were also tested (119). Brown Norway, Lewis, Hooded Lister, and Sprague-Dawley rats all coughed following IB challenge of *Bp*, although Sprague-Dawley rats coughed more consistently compared to the other strains (119). Leukocytosis was observed in all four strains of rats, as high as 4-fold compared to the non-infected controls (119). Younger, 4-week-old rats had an increased propensity toward infection as a decrease in weight gain and increase in mortality were observed compared to 6-week-old rats, regardless of the rat strain (119). Hall et al (1997) further illustrated that total IgE in the serum of Sprague-Dawley, Brown Norway, Lewis, and Hooded Lister rats were elevated following *Bp* challenge (119). Anti-*Bp* and anti-FHA IgG antibodies were detected in all rat strains; however, Hooded Lister and Lewis rats did not develop anti-PT IgG antibody titers post challenge, suggesting host genetics could possibly play a role (119).

Limited studies have investigated the vaccine efficacy of DTP and DTaP in the coughing rat model of pertussis. To measure vaccine efficacy of the DTP, one single human dose was administered IP to 3-week-old Sprague-Dawley rats, followed by subsequent intrabronchial *Bp* challenge 3 weeks later (118). DTP vaccination provided protection against *Bp* elicited cough compared to mock vaccinated challenged rats (118). DTP vaccinated rats also developed serum anti-FHA and anti-*Bp* antibody titers at day 28 post challenge (118). Hall *et al* (1998) also confirmed that two 1.5 human doses of Evans' DTP administered subcutaneously at 21 and 7 days before challenge protected against cough upon intrabronchial *Bp* challenge (120). SmithKline Beecham's 3-component aP vaccine containing detoxified PT (PTd), FHA, and a 69kDa antigen (presumably pertactin (PRN)) as well as Connaught's 5-component containing PTd, FHA, agglutinins 2+3 (fimbriae), and PRN administered subcutaneously 21 and 7 days before challenge were also protective against *Bp* induced cough (120). Both aP vaccines and Evans' DTP protected against *Bp* induced leukocytosis (120). Vaccination with PT, FHA, PRN, and fimbriae alone with no adjuvant did not significantly protect against cough compared to mock vaccinated challenged rats (120). These data suggest that vaccination against pertussis needs to be multi-valent and adjuvanted. Overall, aP and wP vaccines were protective against *Bp* induced cough and leukocytosis (120). Protection against bacterial burden in the coughing rat model of pertussis following vaccination has yet to be established.

In summary, the coughing rat model of pertussis can be used as a tool to not only evaluate *Bp* pathogenesis, but also as a preclinical model to investigate vaccine efficacy. The coughing rat model of pertussis can be utilized to investigate mechanisms responsible for

coughing during *Bp* infection, as well as in the validation of new vaccine formulations or routes of immunization to determine their ability to protect against *Bp*.

1.8 Natural immunity against *Bp*

As described above, *Bp* is a respiratory pathogen. During infection, *Bp* colonization remains strictly within respiratory tract, and dissemination is rarely observed during pertussis infection (13, 121). Both the innate and adaptive immune responses are vital to recognition and defense against *Bp*. Only in mice with immunodeficiencies does *Bp* disseminate to other organs such as the spleen and liver (122). Recognition of *Bp* in the airway induces the infiltration of macrophages, dendritic cells, neutrophils, and NK cells (123–125).

Macrophages have been shown to play a role in immunity against *Bp*. In mice, depletion of macrophages by liposome-encapsulated clodronate in the airway led to an increase in bacterial burden in the respiratory tract up to 2 weeks post-challenge (126). Macrophage killing of *Bp* by nitric oxide has been shown to be increased with supplementation of IFN- γ (127, 128). *Bp* challenge in IFN- γ $-/-$ mice results in dissemination of *Bp* and mortality (122, 129). IFN- γ secreting Th1 cells were associated with recovery in children with pertussis (130, 131). Altogether, these data show that macrophages are essential in clearance and protection against *Bp*.

The role of neutrophils in pertussis immunity has been quite a contrast to that of macrophages. Studies in mice have shown that infiltration of neutrophils occurs as early as 5 days post-challenge and as late as 14 days post-challenge (132, 133). During naïve

infection, neutrophils seem to not play a role in the clearance of *Bp*, presumptuously, due to the inhibitory function of both PT and ACT (41, 134). PT works by delaying the overall neutrophil response during the initial stages of infection, and ACT works by dysregulating neutrophil phagocytic propensity (41, 135). Neutrophils of convalescent mice or mice receiving sera with opsonizing antibodies toward *Bp* are capable of antibody-mediated phagocytosis; suggesting they play a role in *Bp* clearance in an immune competent host (41). Neutrophil extracellular traps (NETs) have also been observed during *Bp* infection (136).

Induction of adaptive immunity towards *Bp* is necessary for protection against pertussis. Both mice and patients previously infected with *Bp* have measurable amounts of IgA in their nasal fluids, and *Bp* specific IgG antibodies in their serum (137). In humans, passive immunization of *Bp* specific sera resulted in a decrease in overall disease severity (138). Patients with an increase in anti-PT and anti-PRN antibody titers had a lower incidence in pertussis than members of the same house-hold after *Bp* exposure (139). In mice, the generation of antibodies following *Bp* challenge does not result in significant levels of antibody titers until approximately 1 month post infection, suggesting antibody responses are not a major player in immunity to a primary infection (137). Immunization with *Bp* specific antigens results in the induction of antibody titers to *Bp*, and passive immunization results in protection against pertussis (140). Ig -/- mice fail to clear pertussis infection, eventually developing a chronic respiratory infection; however, the transfer of *Bp* immune competent mature B cells was capable of restoring protection against pertussis (140, 141).

Cell mediated immune responses have also been shown to play a role in immunity to *Bp*. CD4⁺ T cells specific for PT, FHA, and PRN were isolated from infants following pertussis infection. The isolated T cells were found to secrete IFN- γ , with limited secretion of IL-4 and IL-5, demonstrating that *Bp* infection induces a Th1 rather than Th2 immune response (130, 131). Athymic *nu/nu* mice challenged with *Bp* resulted in chronic infection and even death; however, this phenotype was rescued by adoptive transfer of splenic CD4⁺ cells but not CD8⁺ cells (137). CD4⁺ positive mice isolated from *Bp* infected mice secrete Th1/Th17 associated cytokines: IFN- γ and IL-17 (122, 129, 142). IFN- γ ^{-/-} and IL-17 ^{-/-} mice challenge with *Bp* result in an increase in disease severity and bacterial colonization (142). Additional studies in the nonhuman primate model of pertussis illustrate that convalescent baboons develop protective Th1 and Th17 immune responses (108). Taken together, these data conclude that *Bp* infection induces a protective Th1/Th17 immune responses.

In summary, protection against pertussis requires both innate and adaptive immune responses (**Fig. 3**). Natural infection induces protective Th1/Th17 T cell responses as well as protective humoral responses. As a result, innate immune cells recruited to the site of infection can become activated and opsonization of *Bp* results in antibody-mediated phagocytosis.

Figure 3. Summary of the immunity generated from natural infection. Natural *Bp* infection induces both a humoral and effector Th1/Th17 immune response. Adapted from (Kapil and Merkel, 2019). Created with Biorender.com

1.9 Vaccine mediated immunity

After the resurgence of pertussis, considerable efforts have been made to dissect the different immune responses induced from either wP or aP vaccination. Recent work in humans has shown that although DTaP vaccination induces higher IgG antibody titers compared to DTP vaccinated individuals, vaccine-mediated protection from DTP immunization lasts considerably longer (143, 144). Further evidence supporting variation in vaccine-mediated immune response was identified when characterizing the T cell responses following DTaP and DTP vaccination. Patients receiving DTP vaccination resulted in a robust Th1 phenotype, while DTaP vaccinated patients presented with a Th2 immune response (145, 146). Studies in mice illustrate that DTP vaccination resembles similar immune responses to natural infection. DTP immunization induces a robust pro

inflammatory response, further activating neutrophils' and macrophages' ability to clear *Bp* from the airway (142, 147, 148). Mice receiving DTP vaccination cleared *Bp* infection more readily compared to DTP immunized mice deficient in IFN- γ and IL-17, suggesting that vaccine-mediated immunity induced by DTP is driven by IFN- γ and IL-17 (140, 142). Vaccination with DTaP in mice results in a predominant Th2 immune response but also a Th17 response, likely due to the adjuvant effect of alum. IL-4 $-/-$ mice vaccinated with DTaP had similar protection to wild-type (WT) mice also vaccinated with DTaP and the observed protection was mediated by IL-17 (1). In humans, IL-17 mediated protection induced from DTaP vaccination is absent (142, 149, 150). The antibody responses following DTP and DTaP vaccination are also different. DTP consists of a diverse repertoire of antigens, while DTaP consists specifically of 3-5 antigens: PT, FHA, PRN, Fim2, and Fim3. Primarily the antibody type generated from DTP immunization is IgG2a while DTaP vaccination results in IgG1 antibodies (1, 142, 149). Warfel et al (2014) illustrated that DTaP vaccinated baboons were protected from pertussis; however, they were not protected against *Bp* colonization (149). Furthermore, DTaP vaccinated baboons were capable of transmitting *Bp* to unvaccinated baboons (149). DTP vaccinated baboons were protected against *Bp* burden in the airway (149). These data demonstrate the clear differences in the generated vaccine-mediated immune responses (**Fig. 4**). Taken together with our understanding of convalescent immunity, these data can be used as a roadmap to an improved DTaP vaccine.

Figure 4. Summary of the DTP and DTaP vaccine mediated immunity. DTP immunization induces both a Th1/Th17 immune response similarly to natural infection, while DTaP immunization induces more of a Th2 driven immune response. Adapted from (Higgs, 2012). Created with Biorender.com

1.10 Mucosal immunization of DTaP

With *Bp* strictly being a respiratory pathogen, stimulating local immunity at the respiratory mucosa is critical for the defense against pertussis. Humans previously infected with *Bp* develop longer lasting immunity (approximately up to 20 years) (151). On average, the protective immune response generated from DTaP vaccination is approximately 4-12 years (151). In the nonhuman primate model, convalescent baboons cleared *Bp* from the respiratory tract following challenge, while DTaP vaccinated baboons failed to protect against *Bp* colonization (149). DTaP immunization induced protection against pertussis disease, but not bacterial burden, allowing for asymptomatic transmission of *Bp* to naïve baboons (149). These data suggest that mucosal immunity is necessary for generating longer lasting protection against *Bp* but also in protecting against asymptomatic transmission of *Bp* to a naïve host.

Both intranasal (IN) and oral gavage (OG) immunizations have been explored to stimulate a protective mucosal immune response. In mice, IN administration of DTaP has resulted in protection against bacterial burden in the respiratory tract (152, 153). As a result, both anti-*Bp* and anti-PT IgG antibody titers were induced following IN DTaP vaccination in the serum, in addition to IgA antibody titers specific to *Bp* in the lung (152, 153). Wolf *et al* (2021) illustrated that the humoral responses elicited from IN DTaP immunization remained up to 6 months post-vaccination and were protective against bacterial burden post challenge (153). BPZE1, which is a live attenuated vaccine strain of *Bp*, has also been administered IN and has shown to be protective in mice (154). In clinical trials, IN vaccination of BPZE1 elicits both systemic and mucosal antibody titers specific to *Bp* (155). In infants, oral vaccination of wP resulted in the production of mucosal antibody

titers in the saliva, as well as systemic *Bp* specific antibody titers in their sera (156). In a following study, the frequency of pertussis was lower in newborns for up to one year following oral vaccination of 10^{12} CFUs of killed *Bp* (157). Oral vaccination resulted in both IgG antibody titers in the serum and IgA titers in the saliva. Studies in mice have shown that oral vaccination of bacterial vectors (*Salmonella typhimurium* and *Escherichia coli*) expressing FHA elicited anti-FHA titers in the lung (158). These data suggest that mucosal vaccination can stimulate a protective immune response at the respiratory mucosa.

1.11 Overall Objective

The objective of this body of work is to build upon the already established coughing rat model of pertussis and use this model as both a tool and preclinical model to investigate the efficacy of mucosal immunization with DTaP. As highlighted above, with the implementation of DTaP vaccines we have seen a resurgence of pertussis, especially in the United States. Improved animal models combined with improved vaccination strategies are a necessity to combat pertussis. In chapter 2, the overarching goal was to further develop the rat model of pertussis that recapitulates the same clinical manifestations of pertussis in humans. We intranasally challenged rats with reference strain Tohama 1, which was isolated in 1954 and is one of the most commonly used lab strains, and strain D420 which is a current representative clinical isolate that was isolated in 2002 (159, 160). To count coughs and the respiratory profile following challenge, we utilized whole body plethysmography (WBP). WBP revealed that challenge with D420 resulted in an increase in cough over the twelve-day infection compared to Tohama 1 infected rats. In chapter 3, we utilized the rat model of pertussis to evaluate IN and OG

immunization of DTaP as a potential mucosal vaccination strategy compared to IM administration of DTP and DTaP. Our results show that mucosal vaccination of DTaP through oral and intranasal immunization protects against both bacterial burden and *Bp* induced cough. Mucosal vaccination also induced both systemic (IgG) and mucosal (IgA) antibody titers. In chapter 4, the lessons learned from the coughing rat model of pertussis and implications/uses of the model are explored, as well as avenues to improve vaccine efficacy. The work presented here carefully details a preclinical model that can be used to not only evaluate pathogenesis of *Bp*, but can be used to test vaccine efficacy. The data presented further supports that mucosal immunization of DTaP could potentially be a viable option to improve the current DTaP vaccine efficacy.

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Chapter 2. Re-investigating the coughing rat model of pertussis to understand *Bordetella pertussis* pathogenesis

Re-investigating the coughing rat model of pertussis to understand *Bordetella pertussis* pathogenesis

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2.1 **Abstract**

Bordetella pertussis (*Bp*) is a highly contagious bacterium that is the causative agent of whooping cough (pertussis). Currently, acellular pertussis vaccines (aP; DTaP; Tdap) are used to prevent pertussis disease. However, it is clear that the aP vaccine efficacy quickly wanes, resulting in the re-emergence of pertussis. Furthermore, recent work performed by the CDC suggest that current circulating strains are genetically distinct from strains of the past. Emergence of genetically diverging strains combined with waning aP vaccine efficacy call for re-evaluation of current animal models of pertussis. In this study, we used the rat model of pertussis to compare two genetically divergent strains Tohama 1 and D420. We intranasally challenged seven-week-old Sprague-Dawley rats with 10^8 viable Tohama 1 and D420 and measured the hallmark signs/symptoms of *Bp* infection such as neutrophilia, pulmonary inflammation, and paroxysmal cough using whole body plethysmography. Onset of cough occurred between 2-4 days after *Bp* challenge averaging five coughs per fifteen minutes, with peak coughing occurring at day eight post infection averaging upward of thirteen coughs per fifteen minutes. However, we observed an increase of coughs in rats infected with clinical isolate D420 through 12 days post challenge. The rats exhibited increased bronchial restriction following *Bp* infection. Histology of the lung and flow cytometry confirm both cellular infiltration and pulmonary inflammation. D420 infection induced higher production of anti-*Bp* IgM antibodies compared to Tohama 1 infection. The coughing rat model provides a way of characterizing disease manifestation differences between *Bp* strains.

2.2 Introduction

Whooping cough (pertussis) is a respiratory disease that is caused by the bacterium *Bordetella pertussis* (*Bp*). Pertussis is characterized by severe leukocytosis, bronchopneumonia, hypoglycemia, and paroxysmal cough (1, 2). During the catarrhal stage, *Bp* colonizes the upper respiratory epithelium. Once colonization occurs, *Bp* releases toxins such as pertussis toxin (PT), adenylate cyclase toxin (ACT) as well as others. Following the catarrhal stage is the paroxysmal stage that is characterized by severe coughing episodes. Innate and adaptive immune responses result in clearance of the bacterium allowing for the patient or host to convalesce. Despite extensive research, a full understanding of *Bp* pathogenesis remains elusive for reasons such as: vaccine pressure, lack of predictive models, and strain evolution (3). One of the major reasons for this gap of knowledge is the lack of a suitable animal model that shares common disease symptoms as seen in humans. Besides non-human primates, rats are the only other known model of pertussis that share similar coughing manifestation as seen in humans (4). As of now, the origin or the trigger of cough has yet to be determined (5). The rat model of pertussis offers a potential animal model that can be used to evaluate *Bp* pathogenesis.

In 1938, Hornibrook and Ashburn were the first to report that rats infected with *Bp* induced cough-like paroxysms and bronchopneumonia as seen in humans (6). Hornibrook and Ashburn found that young rats were capable of being infected and the bacterium could be cultured from the lungs (6). Infected rats produced coughs that could be heard from a distance of 20 feet (6). Twenty-six of the thirty-one infected rats that did not succumb to

the infection had pathology indicative of inflammation in the lungs, most notably early neutrophil infiltration followed by recruitment of mononuclear cells (6). The subsequent studies utilized intrabronchial inoculation of *Bp* encased in agar beads and confirmed leukocytosis and paroxysmal cough by sound activated tape recorders from 5 to 21 days post challenge (7, 8). Further development of the rat model led to the evaluation of PT negative strains and the evaluation of coughs (9). Strain BP357, which is deficient in PT resulted in low cough induction (9). Further evaluation of the rat model demonstrated leukocytosis, weight loss, and paroxysmal cough in Sprague-Dawley rats during the course of *Bp* infection (10). During the development phase of the acellular pertussis vaccine (aP, DTaP), the rat model was used to test vaccine efficacy of various acellular pertussis formulations and was used to validate protection against the onset of leukocytosis and cough (11). Recently, an intranasal rat model has been used to evaluate bacterial factors responsible for cough in *Bordetella bronchiseptica* (*Bb*) (12). BspR, which is an anti-sigma factor of *Bb*, plays some role in cough induction upon infection (12). Collectively, these studies show that the pertussis rat model can be used to critically evaluate *Bp* pathogenesis and disease progression.

In the 1940s, whole-cell pertussis (wP and DTP) vaccines were introduced to protect against *Bp* infection (13). Widespread use of DTP in the United States led to a 90% decrease in the number of reported *Bp* infections (13). Despite the efficacy of this vaccine, serious adverse side-effects ensued, leading to the development of acellular pertussis vaccines. The antigens that are included in the current aPs contain: filamentous hemagglutinin (FHA), fimbriae (FIM), PT, and pertactin (PRN). After the switch from the

wP to the aPs, there has been a significant increase in the number of pertussis cases in the US and Europe (14). While nationwide vaccine coverage is 95% in the United States, the incidence of *Bp* infections rose in the past 10 years as the number of aP vaccinated only population has increased in size. (15). While vaccine coverage remains high, the population dynamics are changing, and more people are aP immunized as the new generations are born and as the wP only generations age. Numerous studies have demonstrated waning efficacy of aPs in parallel with the emergence of genetically divergent strains of *Bp* (3, 16–21). Increased surveillance of *Bp* has led to the identification of clinical isolates that do not express PRN, FIM, and even PT the hallmark toxin of the organism (22). One plausible hypothesis to explain these observations is that circulating *Bp* strains have evolved due to acellular vaccine pressure. While we know the current strains are genetically different, we do not know if this genetic variability affects virulence, disease burden, toxicity, or fitness of the pathogen (3).

Early rat studies were performed with intranasal administration of *Bp* for infection (6). Subsequent rat challenge studies used *Bp* encased in agar beads for intrabronchial instillation (7–11, 23). While the agar bead infection method was successful at establishing infection, we aimed to utilize the simplicity of intranasal administration. We sought to re-investigate the coughing rat model of pertussis to compare the pathogenesis of reference strain Tohama 1 to the recent clinical isolate D420, which has been extensively studied in the baboon model of pertussis (24–30). Tohama 1 was first isolated from a case of whooping cough in 1954, while D420 was isolated in 2002 from a critically ill infant in Texas (25, 31). Although Tohama 1 is now a reference strain that has been

widely used since Sato and Sato developed the aP but recent data has shown that this strain is an considered an outlier (32, 33). Tohama 1 does expresses PRN but has lower expression of PT and ACT (34). When Tohama 1 was used as a challenge strain in baboons, the baboons did not exhibit symptoms of pertussis despite being infected (26). This led to the selection of recent clinical isolate D420 as the baboon challenge strain, which readily infected and caused disease (26). With 48 years of potential genetic divergence, we sought to understand the differences in pathogenesis between these two commonly studied strains. D420 is known to infect mice and baboons and it belongs to the clade (CDC013) of strains that represented 50% of isolates recovered in the US in 2000 (3). However, it is important to note that D420 has an intact pertactin gene and it does express the PRN protein as confirmed by shotgun proteomic analysis (data not shown).

In our current study, we aimed to re-establish an upper respiratory tract infection model in rats following intranasal challenge with Tohama I and D420. We hypothesized that recent isolate D420 would induce a more severe disease profile compared to Tohama 1, as previous research investigating pathogenesis in rhesus macaques noted that Tohama 1 infected animals did not exhibit overt disease symptoms (26). Sprague-Dawley rats were intranasally challenged with *Bp* and we characterized their disease progression profile over a twelve-day infection. Cough was critically assessed utilizing whole body plethysmography (WBP). Bacterial colonization, leukocytosis, and serological responses were measured as a result of infection. Rats challenged with D420 had increased coughing, greater bacterial burden in the respiratory tract, and a more robust IgM antibody

response compared to Tohama 1. The coughing rat model of pertussis can shed light on the pathogenesis of *Bp* and will likely be useful tool for pertussis vaccine evaluation.

2.3 Results

D420 infected rats have increase number of coughs compared to strain Tohama 1 over the entire course of infection.

Over the past decade it is became clear that improvements to the acellular pertussis vaccine strategy are needed due to the rise of PRN mutants, genomic divergence, and epidemiological data. We believe in order to improve pertussis vaccines we need to better understand the pathogenesis of *Bp* in an animal model that shares similar clinical manifestation of pertussis as seen in humans. Here, we intranasally infected seven-week-old Sprague Dawley rats with 10^8 CFU of *Bp* strain D420 or Tohama 1 (**Fig. 5**) in an effort establish infection and observe *Bp* induced cough. For negative controls (no bacterial challenge), rats were intranasally administered sterile phosphate buffer saline (PBS). Paroxysmal cough is the hallmark symptom of pertussis and is thought to play a major role in transmission of the organism to a new host. To quantify respiratory function during infection, we utilized whole body plethysmography (WBP). WBP instrumentation consists of specialized containment chambers which monitor box flow, temperature, and airflow changes to measure respiratory function. Counting coughs via WBP provides an extremely accurate and unbiased way of counting coughs based on cough waveforms. Rat containment chambers were placed inside a laminar flow hood and connected to a computer that would monitor the rats breathing (**Fig. S1**). Early studies reported that *Bp* infection in young rats induced coughs that were audible by ear (6) and in our preliminary

studies this was apparent to us as well (data not shown). Studies in the 1990s, of paroxysmal cough in rats were quantified with an analog sound recording device (7). We hypothesized that rats infected with recent isolate D420 would induce more coughs than reference strain Tohama 1, because rhesus macaques infected with Tohama 1 did not exhibit overt disease symptoms but D420 induced robust coughing in baboons (26). *Bp* infected rats developed cough at days 2-11 (**Fig. 6B-C**), while the mock infected rats (**Fig. 6A**) only had a few isolated coughs, unrelated to infection. Days 1-3 post infection, the average cough count of rats infected with Tohama 1 or D420 was less than 5 coughs per fifteen minutes. The average cough count doubled by day 7 post infection with peak coughing occurring at day 8 post infection with an average of 13 coughs per fifteen minutes of monitoring. After day 8, the average cough count for rats infected with D420 remained above 10 coughs per fifteen minutes, while the rats infected with Tohama 1 averaged less than 5 coughs. To summarize the cough data, the average cough count over the course of infection is shown (**Fig. 6D**). We observed a significant increase in the number of coughs at day eight post infection of *Bp* infected rats compared to the mock challenge control. Rats infected with D420 coughed a total of 949 times, whereas rats infected with Tohama 1 coughed a total of 724 times over the entire twelve-day infection. To analyze differences in cough count between strains, we utilized Area Under the Curve (AUC) analysis (**Fig. 6E**). AUC analysis allows us to quantify the number of coughs over the entire course of infection per rat. Upon this analysis, we note a significant increase in the number of coughs for rats infected with D420 compared to the mock challenged rats over the twelve-day infection, while there was no significant difference observed in Tohama 1 infected rats compared to mock challenge (non-infected).

***Bp* infection causes pulmonary distress.**

Infection with *Bp* leads to mucus production, lung damage, and invasion of cellular infiltrates into the bronchioles of the lung (35). The lungs of infants with *Bp* exhibit edema, necrotizing bronchiolitis, and inflammation in the lung leading to respiratory distress (35). WBP was used to quantify the pulmonary distress over the course of infection. Pulmonary distress was measured by calculating the enhanced pause (PenH) of the animal (**Fig. 7**). The higher the PenH value, the increased respiratory distress of the animal. PenH corresponds with the resistance associated with peak inspiratory height (PIF) and peak expiratory height (PEF) and taking into account time between early and late expiration per breath (**Fig. S2**). With increased constriction, the expiratory peak becomes prominent and the area of the (PEF) becomes a larger percentage of the complete expiratory area. Others factors that contribute to the increased PenH is a decrease in relaxation time (Tr) in relation to expiratory time (Te). Previous research has shown that severe inflammation in the lung of mice directly correlated with an increase in PenH (36). More recently, SARS-CoV-2 infected mice were observed to have subtle but significant increase in PenH post challenge (37, 38). Compared to the mock challenged rats both D420 and Tohama 1 infected rats had an increase in PenH (**Fig. 7**). We observed a significant increase at day 9 with rats infected with D420 and at day 6 with rats infected with Tohama 1 compared to mock challenge.

Histological assessment of the lung and nasal cavity

To further evaluate the inflammation in the lung, we utilized histology to confirm both acute and chronic inflammation in response to *Bp* infection. At days 1, 3, 6, 9, and 12 post

challenge the left lobes were collected, sectioned, and stained with hematoxylin and eosin (H&E) (**Fig. 8A&C**). Whole-lung images were taken to assess cellular infiltration (**Fig. S3**). Once stained, the blinded slides were then scored by a board-certified pathologist. Mock challenge animals exhibited minimal inflammatory infiltrates consisting of focal accumulations of mononuclear cells in the parenchyma and occasional infiltrates of neutrophils surrounding blood vessels (**Fig. 8A**). Rats infected with Tohama 1 and D420 had significant increase in their acute inflammation scores compared to mock challenge animals at day 1 and 3 post infection (**Fig. 8B**). Day 1 post infection had the highest acute inflammation score (**Fig. 8B**). Rats exhibited mild to moderate neutrophil infiltration of the parenchyma, blood vessels, and the bronchioles (**Fig. 8A**). Markers of acute inflammation resolved after day 6 post challenge; however, infected rats at day 9 exhibited obvious differences in the lung associated with mild to moderate infiltration of mononuclear cells and higher chronic inflammation compared to mock challenge animals (**Fig. 8C**). At day 12 post infection, we notice moderate resolution of inflammation in both D420 and Tohama 1 infected rats (**Fig. 8B&D**). We also observed a significant increase in lung weight, which can also be associated with elevated inflammation, at days 1 and 9 post challenge in D420 infected rats compared to the mock challenge control (**Fig. 9A**). At day 12 post challenge rats infected with Tohama 1 had a significant increase in lung weight compared to the mock challenge rats (**Fig. 9A**). These results confirm that *Bp* infection induces inflammation in the lung of coughing Sprague-Dawley rats.

Due to the fact that *Bp* is a mucosal pathogen, we sought out to investigate any phenotypic changes or inflammation in the Nasal-Associated Lymphoid Tissue (NALT) of

pertussis infected rats via histology. NALT helps elicit immunity against airborne and mucosal pathogens (39). The NALT is located above the hard palate and studies have also shown that with the introduction of antigen via vaccination or bacterial components, cellular expansion of the NALT ensues (40–44). The NALT was stained with H&E, and we observed an increase in cellular infiltrate and a size enlargement in the NALTs of *Bp* infected rats compared to the mock challenged (**Fig. 10A**). ImageJ analysis was used to measure the area of both the left and right NALT of each animal (**Fig. 10B**). This data could mark the potential cellular expansion of the NALT upon infection with *Bp*. Further analysis of the expanding cell populations in the NALT will warrant more analysis especially in the context of vaccine immunity.

D420 infected rats have increased bacterial burden in the respiratory tract.

We determined bacterial burden in the respiratory tract for Tohama I and D420 challenged rats at days 1, 3, 6, 9, and 12. Over the coughing timeline, we see an overall decrease in viable bacteria in the lung, trachea, and nasal cavity. In the lung, we observed a significant increase in the recovered bacteria in the D420 infected rats compared to the Tohama 1 infected rats at both days 1 and 3 (**Fig. 11A**). The same trend is also seen in the nasal cavity at day 1 post infection (**Fig. 11C**). We observed no difference in the viable bacteria recovered in the trachea (**Fig. 11B**). Previous pertussis studies utilizing the rat model of pertussis noted weight loss from intrabronchial infection of *Bp* strain 18-323 (10). *Bp* infection is sufficient to cause weight loss in rats (10). To confirm this using intranasal infection, body weight was measured before challenge and upon euthanasia to calculate percent weight change. We observed a significant decrease in weight gain in rats infected

with *Bp* strain D420 and Tohama 1 compared to the mock challenged at day 12 post infection (**Fig. 9B-D**).

Next, we aimed to compare the bacterial colonization between outbred mice and rats in an effort to relatively compare the bacterial burden between models. We utilized the bacterial burden data in CD1 mice and compared them to our data in rats (45). To address the body weight differences between the mice and rats, we used the CFUs/organ and divided them by their respective body weight for a crude comparison. Our data suggests that despite having a higher initial challenge dose in the rat, the bacterial burden remaining in the respiratory tract is higher in a mouse compared to the rat later during infection (**Fig. S4**). In general, the rat model is likely a lower challenge dose per body weight model of pertussis compared to mouse models.

Immunofluorescence confirms colonization of *Bp* in the lungs and nasal cavity.

We next wanted to assess the location of bacterial colonization in the nasal cavity and visualize any bacteria left behind following flushing of the nares. We sectioned the skulls after flushing the nasal cavity and utilized Immunofluorescence (IF). After visually scanning the nasal cavity with confocal microscopy, we found bacteria in the nasal turbinates and the NALT (**Fig. 12A&C**). We also confirmed that the bacteria were found between the epithelial cells (**Fig. 12B**). The relative locations of where the bacteria were found in the nasal cavity can be seen in (**Fig. S5A**). Our results show that *Bp* remained in the nasal cavity over the entire course of infection even after flushing the nares (**Fig. S5B**). The flushing of the nasal cavity underrepresents the total number of *Bp* in the nasal cavity. In the lung, *Bp* colonizes the bronchioles, while the alveolar spaces remain non-

colonized (**Fig. 12D&F**). IF was performed on the nasal cavity and lung of mock challenge animals to access non-specific binding (**Fig. S6**). To assess any potential differences between the strains we blindly manually counted the labeled microcolonies in the lung and nasal cavity. It is also worthy to note, we found no differences in microcolony counts in the lung or nasal cavity between the strains (**Fig. S5 C-D**) despite our observed differences in bacterial burden by CFU enumeration. CFU is likely a better indicator of overall bacterial burden, but IF revealed that some *Bp* remains in the airway following flushing of the nares.

Measurement of recruitment of neutrophils in response to *Bp* infection

Previous studies utilizing the rat model of pertussis noted leukocytosis and an increase in the amount of total white blood cells using agar-encased *Bp* for infection (8). Neutrophils circulating through the blood were measured during intranasal infection by antibody staining and flow cytometry, as *Bp* infection results in varying amounts of neutrophilia (35, 46). One caveat of analyzing cell populations via flow cytometry in the rats is the limited availability of rat specific antibodies compared to mice. Recent research by Barnett-Vanes et al has developed a flow cytometry panel to analyze cell populations in a model of LPS-induced pulmonary inflammation (47). The percentage of CD45+ (CD161- B220- CD43+ His48Hi) neutrophils in the blood were assessed in *Bp* infected rats compared to the mock challenged animals (**Fig. 13A**). Specifically, a significant increase in the number of circulating neutrophils in the blood of rats infected with D420 at days 1 and 9, with Tohama 1 infected rats only having a significant increase to mock challenge at day 9 (**Fig. 13A**). Next, cytokines concentrations in the lung and serum were measured to identify factors that could contribute to the recruitment of neutrophils,

specifically IL-6 and IL-17. IL-6 is a potent inducer of Th17 polarization and been found elevated during murine infection with *Bp* (45, 48–50). Surprisingly, we did not observe any significant changes in IL-6 due to *Bp* infection (**Fig. 13B&C**). It has been well documented in the mouse model that the production of IL-17 in pertussis plays a role in the increase of circulating neutrophils (51). There were no significant changes in IL-17a in the serum or lungs of *Bp* infected rats compared to mock challenge (**Fig. 13D&E**). Th1 and Th2 cytokines and chemokines in the lung and serum were also measured (**Fig. S7-Fig. S10**). We did not observe an increase in the induction of cytokines by infection overall; however, in the serum, rats infected with D420 had a significant increase in IL-5 compared to Tohama 1 infected rats at day 12 post infection (**Fig. S7**). There was a significant increase in IL-5 and TNF-alpha at day 6 post infection in the lung in rats infected with Tohama 1 compared to D420 (**Fig. S8**). We also observed significant increases in chemokines MIP-1a, RANTES, MCP-3, MIP-2, and IP10 in rats infected with D420 at day 12 post challenge in the serum compared to rats infected with Tohama 1 (**Fig. S19**). In the lung, MIP-2 and IP10 were increased in rats infected with D420 compared to mock challenge control (**Fig. S10**). Despite slight differences highlighted above we did not observe a massive difference in the cytokine and chemokine responses during infection.

Serological analysis of *Bp* specific antibodies

Enzyme-linked immunosorbent assays (ELISA) were performed to determine the serological response generated from *Bp* infection. We measured IgM and IgG antibody titers in the serum to whole bacterium and *Bp* associated virulence factors at days 1, 3, 6, 9, and 12 post infection. In previous studies utilizing the rat model, intrabronchial

infection induced the generation of IgG antibody titers to sonicated *Bp* 28 days post infection (8). Intranasal infection of strain D420 has generated significant anti-PT IgG antibody titers in the baboon model at days 17-19 of infection (26). However, analysis of antibody titers generated against an intranasal pertussis infection in the rat has yet to be determined and we wanted to characterize the primary antibody response to *Bp* (**Fig. S11**). We observed a significant 10-fold increase in the anti-*Bp* IgM antibody response at day 9 and an almost 20-fold increase 12 days post challenge in rats infected with D420 compared to mock challenge, while we also saw a significant 6-fold increase in anti-*Bp* IgM at day 12 post infection to the amount of anti-*Bp* IgM from Tohama 1 infection (**Fig. 14A**). At day 12 post infection, we observed that *Bp* infection induced significant production of anti-*Bp* IgG in the serum compared to mock challenge animals (**Fig. 14F**). At day 12 post infection, Tohama 1 infected rats had a significant increase, nearly double anti-*Bp* IgG antibody titers compared to rats infected with D420. AUC analysis revealed a significant increase in anti-*Bp* IgM antibodies from rats infected with D420 compared to Tohama 1 infected rats (**Fig. S12A**). However, no difference was observed in AUC of the anti-*Bp* IgG antibody response over the total course of infection between the bacterial challenge groups (**Fig. S12B**). It is also important to note we also did not see a significant increase in the generation of antibodies titers to PT, ACT, PRN, and FHA in *Bp* infected rats compared to mock challenge infected animals (**Fig. 14B-E, G-J**). We would also like to note that IgA antibodies were not detected to either whole bacteria or PT in the lung and nasal cavity at days 1 and 9 post challenge (data not shown).

2.4 Discussion

To date, the only known reservoir for *Bp* is humans, making the development of a suitable animal model challenging. Multiple models have been studied to recapitulate similar disease symptoms seen in humans, as well as provide insight into the pathogenesis of *Bp*. We set out to re-investigate the rat model of pertussis that was originally introduced in 1938 (6). In our study, we have confirmed that intranasal administration of *Bp* led to bacterial colonization of the upper and lower respiratory tract (**Fig. 11**). Through intranasal instillation, bacteria were able to infect the nasal cavity, trachea, and lung post challenge. This is important to note as infants with pertussis experience pneumonia more frequently than adolescents and adults (35). Mouse models of pertussis also rely on depositing large numbers of bacteria into the lung to establish infection (2). Here, our data shows that through intranasal infection, we establish an infection of both the upper and lower respiratory tract, resembling the infection seen in adolescents and adults with pertussis (52, 53).

Previous reports have shown *Bp* infection of rats induced paroxysmal coughs, and these coughs were counted by analog audio recordings (7). Utilizing WBP we are able to precisely and digitally count coughs and measure respiratory capacity for the first time with rats infected with *Bp* (**Fig. 6&7**). Here, coughs induced from infection appeared at day 1-2 post challenge, with the number of coughs gradually increasing with peak cough occurring at day 8 post challenge. Rats challenged with intrabronchial instillation of *Bp* noted similar peak cough counts approximately 10 days post challenge (54). This is also one of the first studies utilizing WBP to count coughs in rats with bacterial infection. We did observe some infected rats that did not overtly cough. This reflects the frequency of

coughs in adolescents and adults (55). Previous studies using baboons also show that *Bp* infection elicits severe coughs lasting over 2 weeks post challenge with peak coughs per hour being at day 4 post challenge (26). It is also important to note that at peak coughing, the bacterial burden was low, which was also observed in previous *Bp* infected rat research (7). The data suggest that other factors could be playing a role in cough production despite the bacterial infection being resolved. Given that *Bp* infected rats coughed, we also investigated transmission of *Bp* from infected 7-week-old rats (actively coughing) cohoused with more susceptible 3-week-old rats and we have observed no evidence of transmission via negative qPCR (IS481) analysis of swabs from the nasal cavity and no active coughing in the naïve 3-week-old rats. We also performed a challenge study with *Bb* strain KM22 in 7-week-old Sprague Dawley rats, and challenged rats coughed violently and earlier after bacterial challenge compared to *Bp* infected rats. *Bb* infected rats coughed the most when the bacterial loads in the respiratory tract were at their highest, which could potentially lead to transmission of the bacterium. TLR4 deficient rats could be investigated to evaluate transmission of *Bp* (56). We have also observed that the increased bronchi restriction coincides with the increased coughs during days 6-11 post challenge (**Fig. 7**). The decreased lung capacity and increase in cough could be further associated with an increase of chronic inflammation in the lung (**Fig. 8**). Our data suggests that the coughing rat model of pertussis can be used to potentially evaluate the mechanism of cough, which still requires further investigation.

Initial studies characterizing the serum antibody response to rats infected with *Bp* noted an increase antibody response to endotoxin correlated with the low amount of cultivable

organisms in the lung following intrabronchial challenge (7). Hall et al. demonstrated that rats infected with *Bp* become sera positive to whole cell *Bp* lysate, FHA, and PT at 28 days post intrabronchial challenge (8). Anti-FHA antibody titers were not detected until day 16 post challenge, while anti-PT antibody titers were not detected until day 28 post challenge (23). In our study, we characterized the antibody response following intranasal challenge of *Bp* over the whole infection timeline. Using *Bp* coated ELISA plates, we detected anti-*Bp* IgG antibody titers in rats infected with *Bp* at day 9 post challenge with a significant increase compared to mock challenge observed at day 12 (**Fig. 14**). We also measured a significant increase in anti-*Bp* IgM antibody titers in rats infected with D420 compared to the mock challenge and Tohama 1 infected animals (**Fig. 14**). One potential hypothesis that could explain this observation is the genetic differences between the two strains as D420 harbors the *ptxP3* allele, leading to an increase in PT production (25). PT has been shown to suppress *Bp* antibody responses in the serum and limit expression of antigen presenting receptors (57–60).

With the increased interest in developing new pertussis vaccines, it is imperative for the development of animal models to evaluate the pathogenic potential of current emerging strains, as well as identify any potential differences in disease burden between genetically divergent strains. Sato and Sato first used *Bp* strain Tohama 1 in the development of the acellular pertussis vaccine, and Tohama 1 is still being used in pertussis research today, notably due to its genetic malleability (61). D420 has been instrumental in the development of the baboon model and has also been used to study vaccine efficacy in regards to transmission in the baboon model (29). We hypothesize that the observed differences could be due to the increased amount of PT through the *ptxP3* allele of D420

(25). Strains, such as D420, harboring the *ptxP3* allele lead to increase in virulence, PT production, and prevalence (62, 63). A previous study utilizing the intranasal mouse model has shown an increase in bacterial colonization with *ptxP3* strains (64). Parton et al. illustrated that infection with a PT deficient strain was unable to induce cough (9). It is possible that there are other factors beyond PT that can account for the observed virulence differences between Tohama I and D420. In the rat model, unlike the mouse, we also did not measure any significant increases in IL-17 and IL-6 in the lung and serum following challenge. IL-17 and IL-6 are increased following *Bp* infection in mice and baboon (30, 48, 65). Further investigation is needed to explain the observed differences. One potential approach to evaluate differences between strains is the use of a neonatal rat model of pertussis. Studying *Bp* in neonates may be useful in distinguishing subtle differences between strains. Hornibrook et al demonstrated that *Bp* infected young 3 week rats had 65% mortality, while 7 week old rats do not succumb to infection with similar dose (6).

In summary, we have demonstrated that the rat model of pertussis can be used as a tool to further study *Bp* pathogenesis and recapitulate some of the similar symptoms of pertussis as seen in humans. Pertussis is often described as a 100 day cough, because adolescents and adults extended duration of coughing (66). Coughing episodes in infants with pertussis can lead to vomiting, choking, gagging, and apneic episodes that can lead to seizures (67). Mice have not been used to evaluate coughing manifestation in pertussis until recently, and baboons are expensive and limited to specialized facilities (68). The coughing rat of pertussis provides feasibility through low husbandry costs, availability of

animals, and ease of use as a model. The immunological tools available in murine research is vast but as basic research continue to evolve, so will the resources and immunological tools available for rats. Recently flow cytometry markers have been developed to study innate immune cells, and adaptive immune cells such as B and T cells (47). In other studies, we have ELISpots assays to investigate antigen specific immune populations as well. Increasing availability of antibodies has also allowed for investigating systemic and mucosal serological responses. Future studies utilizing the coughing rat model of pertussis will capitalize on the growing immunological toolbox in regard to vaccination against pertussis.

In this study, we carefully compared two established strains that have been used in the pertussis field, Tohama 1 and D420. In future studies, we aim to evaluate whole cell and acellular vaccine mediated immunity against D420. Armed with this model, we can further our understanding of pathogenesis, host response during pertussis, genetic divergence between strains, and vaccine-mediated immunity.

2.5 Materials and methods

***Bordetella pertussis* strains and growth conditions.** *Bp* strain Tohama 1 was graciously provided by Dr. Peter Sebo (Czech Academy of Sciences) and strain D420 was acquired from the CDC Pertussis Lab provided by Drs. Maria L. Tondella and Michael Weigand. *Bp* strain Tohama 1 and D420 were cultured on Bordet Gengou (BG) agar (Remel™ Cat. R45232) that was supplemented with 15% defibrinated sheep blood (Hemostat Laboratories Cat. DSB500) (69). Bacteria were cultured on BG plates for 48 hrs at 36°C. *Bp* was transferred from BG plates with polyester swabs (Puritan Cat. 22-

029-574) and transferred into 20 ml Stainer-Scholte liquid media (SSM) in new 125 ml flasks (Thermo Fisher Scientific Cat. FB500125) (70). The liquid cultures were grown for 24 hrs at 36°C inside a shaking incubator at 180 rpm.

Intranasal challenge with *B. pertussis*. Seven-week-old ~170g female Sprague-Dawley rats (Charles River Cat. 001CD) were used for challenge. *Bp* was grown as described above. The rats were then anesthetized with ketamine and xylazine 50-100/5-10 mg/kg and challenged with 10⁸ CFUs (100µl intranasally), administering two 50µl doses one in each nostril (**Fig. 5**). Body weight of each rat was recorded before challenge and immediately post euthanasia. Rats were euthanized at day 1, 3, 6, 9, and 12 post bacterial challenge. Mock challenge animals (no bacteria) were administered 100µl of sterile endotoxin free phosphate buffer saline (PBS) (Thermo Fisher Scientific Cat. TMS012A) intranasally. Upon euthanasia blood was collected via cardiac puncture and transferred into ethylenediaminetetraacetic acid (EDTA) (BD Cat. 365974) and serum separation (BD Cat. 026897) tubes. Blood collected in the EDTA tube was used for flow cytometric analysis, while blood collected in the serum separation tubes were used to isolate the serum via centrifugation (15,000 x *g* for 3 min) and used for serological and cytokine analysis. Lung and trachea were excised and homogenized to determine bacterial burden. Upon removal, the wet weight of the lungs was recorded. Lungs were then collected in gentleMACS C tubes (Miltenyi Biotec) and homogenized using gentleMACS™ Dissociator (Cat. 130-095-927). The trachea was homogenized using a Polytron homogenizer. To determine the bacterial burden in the nares, 2mls of sterile 1x PBS was flushed through the nares and collected for plating. Serial dilutions of the

homogenates and nasal collection were plated on BG plates supplemented with ceftibuten (Sigma-Aldrich Cat. SML0037) 10 µg/ml to decrease growth of normal rat respiratory tract flora.

Analysis of coughing and bronchiole restriction using whole-body plethysmography. To quantify respiratory function during infection, we utilized a Buxco® FinePointe™ Whole Body Plethysmograph instrument (WBP) (DSI). FinePointe™ software was used for collecting, analyzing, and reporting the breathing data. Everyday post challenge for twelve consecutive days at 5:00PM the rats were placed inside designated chambers to acclimate for 5 min. We chose this time, not only due to rats being nocturnal, but this was the time the animals were most awake and active as confirmed by video camera (data not shown). Following acclimation, the rat's respiratory profile was recorded for 15 min. Each chamber is fitted with a transducer that measures the changes in box flow and airflow of the subject. The chambers are also fitted with a screen pneumotach that allows airflow in and out of the chamber that can be recorded. Coughs were counted during the designated 15 min, and enhanced pause (PenH) was calculated to signify bronchiole restriction. Coughs were counted based on large box flow changes of the subject, and changes in both humidity and temperature of the air flowing in and out of the subject with classical cough-like waveforms. Cough detection algorithm is applied with patented fuzzy logic criteria to determine if the event is a cough (71). When analyzing the number of coughs, each cough in a multi-cough event was counted individually.

Histological assessment of the lung and nasal-associated lymphoid tissue. Upon euthanasia, the rat skull was excised, and the mandible removed. The skulls were fixed in 10% formalin (Fisher Scientific Cat. SF98-4) for 48 hrs at 26 °C. After fixation, formalin was removed, and skulls were frozen at -80 °C until decalcification. Skulls were decalcified with Richard-Allan Scientific decalcifying solution (Thermo Scientific Ref. 8340-1) at room temperature for 24-48 hrs and embedded in paraffin. Samples were sectioned and stained with Hematoxylin-Eosin (H&E). A Biotek Lionheart Fx was used to scan and image the NALT. ImageJ was used to trace and measure the area of both the left and right NALT using the images from the Lionheart Fx. The left lobe of the lung was excised and fixed in 10% formalin 48 hrs at 26 °C. The left lobe was then embedded in paraffin and stained with H&E by the WVU Pathology Department. H&E stained sections were used to characterize and score acute and chronic inflammation of the lung. All scorings were done by a board-certified pathologist (iHisto). Individual scores were based on a standard qualitative scoring criterion: (0 – none, 1 – minimal (rare), 2 – mild (slight), 3 – moderate, 4 – marked, 5 –severe). Chronic inflammation was characterized by mononuclear infiltrates of the parenchyma, blood vessels, and airway. Acute inflammation scores were assigned due to the presence of neutrophils in the parenchyma, blood vessels, and airway. All examination and scoring were performed blindly as no knowledge of treatment groups were known.

Imaging of *Bp* in the lung and nasal cavity. Detection of *Bp* in the nasal cavity and the lung were quantified via immunofluorescence (IF) and confocal imaging. The left lobe of the lung and nasal cavity were preserved and sectioned as described above. Sectioned

samples underwent deparaffination and rehydration using xylene and ethanol (100%-70%). Antigen retrieval was performed by incubating samples in citrate buffer at 98 °C for 20 min. Samples were blocked using 5% Bovine Serum Albumin (BSA) (Fisher Scientific Cat. 159008) for 1 hr and primarily labeled utilizing a polyclonal rabbit FHA antibody (Gift from Dr. Erik Hewlett) diluted in 1x PBS. Secondary labeling occurred utilizing an anti-rabbit IgG conjugated with Texas Red (Fisher Scientific Cat. T2767) diluted in 1x PBS. Samples were then covered in mounting media (Prolong Gold Antifade reagent with DAPI Cat. 8961). Samples were imaged using a Nikon A1R confocal microscope. Images were analyzed on DAPI channel and at wavelength 650nm for Texas-Red acquisition. Images were acquired using 100x oil immersion lens (100x/1.40 Nikon Plan APO). To identify any potential differences in IF between the two strains, all IF images were deidentified and the microcolonies were manually counted blindly by four volunteers with 2-3 fields of view used per sample.

Flow cytometry analysis of phagocytes. Neutrophil recruitment in the blood was evaluated by flow cytometry. Blood samples upon collection were then lysed with Pharmylse buffer (BD Biosciences Cat. 555899) for 20 min at room temperature, with slight vortexing throughout. The remaining cells were resuspended in RPMI + 10% FBS to neutralize the lysis buffer, then centrifuged at 1,000g x 5 and washed with the RPMI +10% FBS again. Blood was then resuspended in 1%FBS+PBS+5mM EDTA. Samples were then block with anti-CD32 (BD Pharmingen Cat. 550270) antibody for 30 min at 4°C. After incubation, the cells were stained with the appropriate antibody markers; CD45 Alexa flour 700 (Biolegend Cat. 202218), CD161 APC (Biolegend Cat. 205606), CD45R PE Cy7 (eBioscience Cat. 25-0460-82), His48 FITC (eBioscience Cat. 11-0570-82),

CD43 PE (Biolegend Cat. 202812), and CD3 VioGreen (Miltenyi Biotec Cat. 130-119-125) (47). After the addition of antibodies, the cells incubated 1 hr at 4°C in the dark. To prepare the lung samples for flow cytometry, the lung homogenate was pushed through a 70 µm cell strainer (BioDesign Cell MicroSives Cat. N70R), creating a single-cell suspension. The suspension was centrifuged at 1,000 x *g* for 5 min. After removal of the supernatant, the pellet was resuspended in Pharmlyse buffer and the cells incubated at 37°C for 2 min. After incubation, the cells were centrifuged at 1,000 x *g* for 5 min, supernatant was removed and blocked and labeled with antibody as described above. The lung and blood samples were centrifuged at 1,000 x *g* for 5 min and the pellets were resuspended in 0.4% paraformaldehyde and stored overnight at 4°C. Samples were washed with 1x PBS and resuspended in 1x PBS for analysis. Cell samples were analyzed on a LSR Fortessa and samples were gated and analyzed using FlowJo v10.

Lung and serum cytokine and chemokine analysis. Lung homogenate samples were centrifuged at 19,000× *g* for 4 min and the supernatant was removed and stored at −80 °C. Quantitative analysis of cytokines in the serum and lung homogenate was performed using ProcartaPlex Multiplex Immunoassay kit: Cytokine & Chemokine 22-Plex Rat ProcartaPlex™ Panel (Cat. EPX220-30122-901) per the manufacturer's instructions.

Serological analysis. Antibody titers of infected rats were measured by ELISA. *Bordetella pertussis* specific ELISA plates were coated with 50 µl of 10⁸ *B. pertussis* grown as mentioned above for infection. For measurement of antibody titers to FHA (Enzo ALX-630-123-0100), PRN (GSK), PT (List Biological Laboratories #180), and ACT (Gift

from Dr. Erik Hewlett), we coated plates with 50 μ l of each antigen at 1 μ g/ml. Once coated, plates incubated over night at 4°C. Plates were washed with 1x PBS-Tween 20 and blocked with 5% skim milk for 2 hours at 37°C. Plates were washed again and the serum from the challenge studies were serially diluted down the ELISA plate and incubated for 2 hours at 37°C. After incubation, the plates were washed and coated with 100 μ l of secondary goat anti-rat IgG (SouthernBiotech Cat. 3030-04) and IgM (SouthernBiotech Cat. 3020-04) antibody at a dilution of 1:2,000 in PBS + 5% milk. Once coated, the plate was incubated for 1 hour at 37°C. Plates were then washed again with PBS-Tween 20 and 100 μ l *p*-nitrophenyl phosphate substrate (Thermo Scientific Cat. 37620) was added, prepared by manufactures instructions, and the plate was developed for 30 min at room temperature for titers to IgG and IgM. Biotek Synergy H1 microplate reader was used to measure the colorimetric signal of the ELISA plate at A_{450} . Positive antibody titers were determined by any values that were higher than the baseline. The baseline is set as double the average value of the blank, no serum added to these wells. Generating Nightingale rose plots in Python: Data from individual mice were averaged in Microsoft Excel and log transformed. Values were formatted in Excel to be compatible with Python. Data were imported from .csv files using the pandas package and plotted using the “Barpolar” representation feature in the plotly.graph_objects module. Samples with a titer less than 50 were assigned a value of 0.

Statistical analysis. All data was analyzed using GraphPad Prism 7. The minimum biological replicates for the challenge studies were four. For statistical comparisons between multiple groups over the entire course of the infection a two-way analysis of

variance (ANOVA) was used One-way ANOVA was used for comparison between groups for each individual day with Tukey's post hoc test. Unpaired Student *t*-tests were used for area under the curve (AUC) analysis. Follow-up statistical tests are annotated in figure legends.

Data availability. Data requests for figures provided can be addressed to the corresponding author.

Ethics statement. All studies were performed in accordance West Virginia University Institutional Animal Care and Use Committee approved protocol 1811019148.6.

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JMH, DTB, and JK performed bacterial challenge. JMH, SMK, and JK monitored rat coughing and respiratory function by whole body plethysmography. All authors

participated in the animal experiments. JMH and ESK contributed to flow cytometry panel design and analysis. JMH and TYW prepared and ran flow cytometry samples. MAW and MAD performed cytokine analysis. JMH analyzed H&E histology slides. JMH and GJB performed and analyzed immunohistochemistry. JMH performed ELISA assays. JMH, MB, and FHD contributed to experimental design. JMH wrote manuscript with critical revisions from all authors.

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The authors would also like to acknowledge that figure 1 was Created with BioRender.com.

2.7 Figures

Figure 5. Experimental design of intranasal infection of *Bp*. Schematic representation of Sprague-Dawley rats intranasally infected with *Bp*. Whole body plethysmography was used to measure cough and analyze lung function over the course of a twelve-day infection. Body weight, Bacterial burden of the respiratory tract, innate lymphoid recruitment, and antibody titers were measured at the days 1, 3, 6, 9, and 12 post challenge. The left lobe of the lung and NALT were sectioned and stained with hematoxylin and eosin for histological analysis and immunohistochemistry was performed to visualize bacteria in the left lobe of the lung and nasal cavity.

Figure 6. Infection of *Bordetella pertussis* induces cough in Sprague-Dawley rats. Coughs were measured each day of the twelve-day infection using whole body plethysmography. Coughs were counted for **(A)** mock challenge rats, **(B)** rats infected with D420, and **(C)** rats infected with Tohama 1. **(D)** Summary of results **(A-C)** shown as mean $\pm$ SEM ($n = 4-8$). **(E)** Area under the curve is represented as averages of coughs per fifteen minutes for each rat. P values were determined by two-way ANOVA followed by a Bonferroni comparison test and unpaired Student t-test for AUC, $*P < 0.05$ D420 compared to the mock challenged control group, $^{\#}P < 0.05$ Tohama 1 compared to the mock challenged control group.

Figure 7. *Bordetella pertussis* infection impairs respiratory capacity. Over the course of infection, Sprague-Dawley rat's respiratory function was analyzed using whole body plethysmography. Each day at 5p.m., rat's bronchial restriction was determined by utilizing WBP by calculating for PenH. Results shown as mean $\pm$ SEM (n = 4-8). P values were determined by two-way ANOVA, * $P < 0.05$, ** $P < 0.01$ compared to the mock challenge group followed by Dunnett's comparison test.

Figure 8. *Bp* infection induces acute and chronic inflammation in the lung of Sprague-Dawley rats. The left lobe of the lung was sectioned and stained with hematoxylin and eosin from rats infected with D420, Tohama 1, or the PBS control. (A) Representative images of acute inflammation demonstrating neutrophil recruitment around surrounding blood vessel (asterisk) and parenchyma (arrows) at 400x magnification. (B) Average acute inflammatory score of the lung by the predominance of neutrophils in the parenchyma, blood vessels, and airways. (C) Representative images of chronic inflammation showing mononuclear cells surrounding the blood vessel (asterisk), lamina propria (arrow), and bronchioles (arrowhead) at 200x magnification. (D) Average chronic inflammatory score of the lung characterized by mononuclear infiltrates in the parenchyma, blood vessels, and airway. Histological assessment was determined blinded with no knowledge of the treatment groups. Results are shown as mean $\pm$ SEM ($n = 4$) P values were determined by one-way ANOVA followed by Dunnett's comparison test, $*P < 0.05$ compared between challenge groups. $\#P < 0.05$, $##P < 0.01$ compared to mock challenge.

Figure 9. *Bp* infection induces inflammation of the lung and decrease in weight gain. (A) Post euthanasia, the lung weight was measured and recorded. To observe any weight changes over the course of infection, we calculated percent weight change for (B) mock challenge, (C) D420, (D) Tohama 1 infected rats. Rats body weights were recorded before infection as well as immediately post euthanasia. Percent weight change was calculated by taking the differences between starting weight and end weight and dividing by the initial weight and multiplying by 100. Results shown as mean $\pm$ SEM ($n = 1-4$). Only one mock challenge rat weight was measured at days 1, 3, 6, and 9. P values were determined by one-way ANOVA, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared to the mock challenge group.

Figure 10. Analysis of the nasal associated lymphoid tissue. The skull was sectioned allowing for staining of the NALT with hematoxylin and eosin. (A) Representative 20x images of the NALT. Arrows indicate the NALT. (B) ImageJ analysis analysis of the area of both the left and right NALT. Dotted line represents the average area of the NALT in the mock challenged group. Results shown as mean $\pm$ SEM ($n = 4$). P values were determined by two-way ANOVA, $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$ compared to the mock challenge group.

Figure 11. Analysis of bacterial burden over the course of *Bp* infection. Bacteria were quantified by serially diluted CFUs following intranasal challenge. CFU counts were determined from (A) lung homogenate (B) trachea and (C) nasal lavage. Results are shown as mean $\pm$ SEM ($n = 4$) P values were determined by two-way ANOVA corrected with Bonferroni comparison test, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ compared between D420 and Tohama 1.

Figure 12. Immunofluorescence (IF) staining of *Bp* localization in the respiratory tract. *Bp* was labeled using a polyclonal antibody to FHA and counter tagged with a fluorescently conjugated antibody (Texas-Red). Sections were counterstained with DAPI. **(A-C)** Representative images of *Bp* in the nasal cavity over the course of infection. *Bp* was found captured in the cilia of the nasal cavity as well as the NALT. **(D-E)** Representative images of the bronchiole and alveoli of infected rats. *Bp* was found localized in the bronchioles over the course of infection and absent in the alveoli.

Figure 13. Neutrophil recruitment and proinflammatory cytokine production after challenge determined by flow cytometry and multiplex immunoassay analysis. (A) Blood neutrophil count of Sprague-Dawley rats infected with *Bordetella pertussis*. At days 1, 3, 6, 9 and 12 post infection, rats were euthanized, and the blood was collected via cardiac puncture. Flow cytometry was used to quantify the amount of neutrophils (CD45⁺ CD161⁻ B220⁻ CD43⁺ His48^{hi}) in the blood. Quantification of the percentage of single, CD45⁺ cells. Dotted line represents the average frequency of CD45⁺ neutrophils in the mock challenge animals over the course of the study. (B-C) Analysis of cytokine IL-6 from supernatant of lung homogenate and serum. (D-E) analysis of cytokine IL-17 in the serum and lung homogenate supernatant. Cytokines analyzed using ProcartaPlex multiplex immunoassay kit. Results shown as mean $\pm$ SEM ($n=4$). Dotted line represents the average mock challenged cytokine levels measured. P values were determined by one-way ANOVA followed by Tukey comparison test, # $P < 0.05$, ## $P < 0.01$, #### $P < 0.0001$ compared to mock challenge.

Figure 14. Measurement of serum antibody titers over the course of *B. pertussis* infection. ELISA was used to compare serological responses from rats IN challenge with *B. pertussis*. Total IgM and IgG serum antibody titers were measured from challenged rats against **(A & F)** whole *B. pertussis* bacterium, **(B & G)** FHA, **(C & H)** PRN, **(D & I)** PT, and **(E & J)** ACT. Dotted line represents the average serum titer for mock challenge rats. *P* values were determined by two-way ANOVA corrected with Bonferroni comparison test, **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 compared between infection groups. For comparison to mock challenged control group one-way ANOVA was used followed by Tukey comparison test, ###*P* < 0.01, ####*P* < 0.001.

2.8 Supplemental Figures

Figure S 1. Set up for the whole-body plethysmograph (WBP). Pictured far left on the table is the WBP and computer. Pictured on the right in the bio safety hood are the chambers where the rats are monitored.

Figure S 2. Diagram illustrating Penh during *Bp* infection. Enhanced pause (Penh) is a calculation that is used as a measurement for respiratory distress. Peak expiratory height (PEF) constitutes as the maximum expiratory flow that occurs in one breath; peak inspiratory flow (PIF) is the maximum inspiratory flow that occurs in one breath; Inspiratory time (Ti) the time it takes from the start to end of the inspiration phase of the breath; expiratory time (Te) the amount of time it takes from the start for the expiration to the end of the respiration; relaxation time (Tr) the time it takes the subject to expel certain amount of volume. Following *Bp* challenge, infected rats have an increase in respiratory distress compared to mock challenge rats.

Figure S 3. *Bordetella pertussis* infection induces cellular recruitment in the lungs. **(A)** The left lobe of the lung was sectioned and stained with hematoxylin and eosin of rats infected with D420, Tohama 1, or the PBS control. Representative pictures shown are multiple 4x magnification images stitched together for each lung.

Figure S 4. Bacterial colonization comparisons between CD1 mice and Sprague-Dawley rats. To assess the effect of body weight on bacterial colonization, we took CFUs for each respective tissue and divided them by the body weight. CFUs/organ for the CD1 mice came from (Boehm 2018). Results are shown as mean $\pm$ SEM ($n = 4$) P values were determined by two-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ compared between groups.

Figure S 5. (A) Hematoxylin and eosin staining of the nasal cavity of Sprague-Dawley rat. Arrows represent the nasal associated lymphoid tissue, NS represents the nasal septum, * represents the nasal turbinates. Red boxes indicate areas where the bacteria were found. (B) Immunohistochemistry (IHC) staining of *Bordetella pertussis* being captured by the cilia of the nasal cavity. Slides were prepared as described in the Material and Methods over the course of infection. *B. pertussis* was labeled using a polyclonal antibody to FHA and probed with a fluorescently conjugated antibody (Texas-Red). Sections were counterstained with DAPI. (C-D) Blinded average microcolony counts of *B. pertussis* in the nasal cavity and the lung. Results shown as mean $\pm$ SEM ($n = 3$).

Figure S 6. Immunofluorescence (IF) staining of control rat's respiratory tract. *Bp* was probed for using a polyclonal antibody to FHA and counter tagged with a fluorescently conjugated antibody (Texas-Red). Sections were counterstained with DAPI. **(A-D)** Representative images of nasal cavity and lung over the course of infection.

Figure S 7. Serum cytokine response during twelve-day *Bordetella pertussis* infection. Cytokines measured include: **(A)** IL-2, **(B)** IL-4, **(C)** IL-5, **(D)** IL-10 **(E)** IL-13, **(F)** TNF- α , and **(G)** IFN- γ . Dotted line represents the average cytokine concentration of the mock challenge control group. Results shown as mean $\pm$ SEM ($n=4$). P values were determined by one-way ANOVA followed by Tukey comparison test, # $P < 0.05$ compared between infection groups.

Figure S 8. Lung cytokine response during twelve-day *Bordetella pertussis* infection. Cytokines measured include: **(A)** IL-2, **(B)** IL-4, **(C)** IL-5, **(D)** IL-10 **(E)** IL-13, **(F)** TNF- α , and **(G)** IFN- γ . Dotted line represents the average cytokine concentration of the mock challenge control group. Results shown as mean $\pm$ SEM ($n=4$). P values were determined by one-way ANOVA followed by Tukey comparison test, # $P < 0.05$ compared between infection groups. * $P < 0.05$ compared to mock challenge.

Figure S 9. Serum chemokine during twelve-day *Bordetella pertussis* infection. Chemokines measured include: **(A)** MCP-1, **(B)** MIP-1a, **(C)** RANTES, **(D)** MCP-3, **(E)** MIP-2, **(F)** Eotaxin, and **(G)** IP10. Dotted line represents the average cytokine concentration of the mock challenge control group. Results shown as mean $\pm$ SEM ($n = 4$). P values were determined by one-way ANOVA followed by Tukey comparison test. $\#P < 0.05$, $##P < 0.01$, $###P < 0.001$ compared between infection groups.

Figure S 10. Lung chemokine during twelve-day *Bordetella pertussis* infection. Chemokines measured include: **(A)** MCP-1, **(B)** MIP-1a, **(C)** RANTES, **(D)** MCP-3, **(E)** MIP-2, **(F)** Eotaxin, and **(G)** IP10. Dotted line represents the average cytokine concentration of the mock challenge control group. Results shown as mean $\pm$ SEM ($n = 4$). P values were determined by one-way ANOVA followed by Tukey comparison test. * $P < 0.05$, *** $P < 0.001$ compared to mock challenge.

Figure S 11. Measurement of serum antibody titers over the course of *B. pertussis* infection. ELISA was used to compare serological responses from rats IN challenge with *B. pertussis*. Total IgM (A-B) and IgG (C-D) serum antibody titers were measured from challenged rats against *Bp*, FHA, PRN, PT, and ACT. ($n = 4$). P values were determined by two-way ANOVA corrected with Bonferroni comparison test, $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$ compared between infection groups. For comparison to mock challenged control group one-way ANOVA was used followed by Tukey comparison test, $\#P < 0.05$, $\##P < 0.01$, $\###P < 0.001$.

Figure S 12. Area under the curve analysis of **(A)** IgM and **(B)** IgG serum antibody titers against *Bordetella pertussis* over the course of infection. Results shown as mean $\pm$ SEM ($n=4$). P values were determined by t-test for area under the curve, *** $P < 0.001$ compared between infection groups.

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**Chapter 3. Mucosal immunization
with DTaP confers protection
against *Bordetella pertussis*
infection and cough in Sprague-
Dawley rats**

Mucosal immunization with DTaP confers protection against *Bordetella pertussis* infection and cough in Sprague-Dawley rats

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3.1 ABSTRACT

Pertussis is a respiratory disease caused by the Gram-negative pathogen, *Bordetella pertussis* (*Bp*). The transition from a whole cell pertussis vaccine (wP; DTP) to an acellular pertussis vaccine (aP; DTaP; Tdap) correlates with an increase in pertussis cases, despite widespread vaccine implementation and coverage, and it is now appreciated that the protection provided by aP rapidly wanes. To recapitulate the localized immunity observed from natural infection, mucosal vaccination with aP was explored using the coughing rat model of pertussis. Immunity induced by both oral gavage (OG) and intranasal (IN) vaccination of aP in *Bp* challenged rats over a nine-day infection was compared to intramuscular (IM)-wP and IM-aP immunized rats that were used as positive controls as IM immunization is the current route for wP and aP vaccination. Our data demonstrate that both IN and OG immunization of aP resulted in production of anti-*Bp* IgG antibody titers similar to IM-wP and IM-aP vaccinated controls post-challenge. IN-aP also induced anti-*Bp* IgA antibodies in the nasal cavity. Immunization with IM-wP, IM-aP, IN-aP, and OG-aP immunization protected against *Bp* induced cough, while OG-aP immunization did not protect against respiratory distress. Mucosal immunization (IN-aP and OG-aP) also protected against acute inflammation and decreased bacterial burden in the lung compared to mock vaccinated challenge (MVC) rats. The data presented in this study suggests that mucosal vaccination with aP can induce a mucosal immune response and provide protection against *Bp* challenge.

3.2 Introduction

Infection of the respiratory mucosa by the Gram-negative bacterium *Bordetella pertussis* (*Bp*) causes the disease known as pertussis (whooping cough) (1). Clinical manifestations of pertussis are characterized by paroxysmal cough, hypertension, leukocytosis and in severe cases death, particularly in infants who have yet to receive their first vaccine dose (2–4). Before pertussis vaccines were introduced in the United States, pertussis led to approximately 200,000 deaths annually (5). Largely, this disease has been under control by the use of diphtheria tetanus whole-cell pertussis (DTP; wP) and acellular pertussis (DTaP; Tdap; aP) vaccines. DTP was first introduced in the 1940s/1950s, and was largely effective in decreasing pertussis incidence (6). Due to the robust immune response and reactogenicity concerns, developed countries converted to the use of DTaP in the 1990s (2).

Since the introduction of the aP vaccine, pertussis cases have been increasing, despite high vaccine coverage. It has been hypothesized that the increase in pertussis cases is attributed to: waning immunity from DTaP and Tdap vaccination, vaccine driven evolution of *Bp* strains, increased surveillance of pertussis, increased asymptomatic transmission, and improved PCR based molecular identification of cases (7–13). Infant baboons vaccinated with DTaP (1 human dose at 2, 4, and 6 months of age) were still colonized after experimental *Bp* challenge in addition to subsequent transmission to naïve baboons (14). In the same study, Warfel *et al* (2014) showed that convalescent baboons that cleared a prior *Bp* infection, were not colonized following re-challenge of *Bp* one month

later (14). In humans, studies suggest that convalescent immunity can confer protection for approximately 4-20 years (15), while DTaP immunity falls short lasting on average 4-12 years (15). Overall, these data demonstrated that immunity induced through natural infection can generate longer lasting protection that also elicits pathogen clearance. As *Bp* is a respiratory pathogen, it can be hypothesized that generating an immune response at the respiratory mucosa is necessary for protection against pertussis.

Infection with *Bp* localizes to respiratory epithelium primes the immune response against subsequent *Bp* infection by recruitment of antibody producing cells and tissue resident memory T cells (Trm) (16). Bacteria invading mucosal surfaces can induce inflammation resulting in subsequent production of IgA antibodies (17). Patients previously infected with *Bp* have developed IgA antibody titers in their nasal secretions (18). In addition, anti-*Bp* IgA antibodies from patients who have convalesced from *Bp* infection inhibit bacterial attachment *in vitro* and increase *Bp* uptake and killing by human polymorphonuclear leukocytes (19, 20). Convalescent humans also generate *Bp* specific IgG antibody titers that have shown to correlate with protection (21, 22). *Bp* infected mice generate *Bp* specific CD4⁺ T cells in the lung that secrete IFN- γ and IL-17 (23, 24). In mice, *Bp* infection induced Trms in the lung and were associated with pathogen clearance (16). Nonhuman primates previously infected with *Bp* generate both Th1 and Th17 memory cells that are still detected two years post infection (25). To induce a similar protective immune response that is observed during natural infection, mucosal vaccination has recently been investigated (26–28)

Mucosal immunization has been scarcely used as a vaccination strategy to generate a protective immune response against pertussis in clinical and pre-clinical models. Oral vaccination with wP induced the production of *Bp* specific antibody titers in both the saliva and serum of newborns (29). In a subsequent study in 1985, oral immunization of 10^{12} CFUs of killed *Bp* led to the induction of antibody titers in the saliva and serum of newborns (30). The frequency of pertussis was lower in orally vaccinated newborns during the first year of life compared to newborns who were unvaccinated although, this difference disappeared by the end of the year (30). In mice, oral administration of attenuated bacterial vectors *Salmonella typhimurium* and *Escherichia coli* expressing *Bp* antigen, filamentous hemagglutinin (FHA), results in the production of anti-FHA IgA antibody titers in the lung (31). Intranasal immunization of a live attenuated vaccine strain of *Bp*, BPZE1 was protective both in preclinical and clinical studies (32–35). Previously, our lab has shown that IN immunization of DTaP can induce a protective immune response in mice (27, 28). Boehm *et al* (2019) illustrated that IN vaccination of DTaP with and without the addition of the adjuvant curdlan induces both anti-*Bp* and anti-pertussis toxin (PT) IgG antibody titers, as well as *Bp* specific IgA in the lung (27). A subsequent study performed by Wolf *et al* (2021) suggested that IN-aP vaccination is protective through the induction of humoral responses 6 months after booster vaccination and challenge (28). Mounting evidence supports that mucosal immunization can be protective against *Bp* colonization, but murine studies lack the ability to evaluate one the of hallmark symptoms of pertussis, *Bp*-induced coughing.

The rat model of pertussis has been utilized to characterize *Bp* pathogenesis and evaluate coughing manifestation from *Bp* infection (36–42). Only a few studies have been performed investigating vaccine efficacy in the coughing rat model of pertussis. Hall *et al* (1998) demonstrated that the SmithKline Beecham 3-component aP vaccine (detoxified PT (PTd), FHA, and a 69kDa antigen, presumably pertactin (PRN)), the Connaught 5-component vaccine (PTd, FHA, agglutinins 2+3 (fimbriae), and PRN), and Evans whole-cell pertussis vaccine protected against cough upon intrabronchial *Bp* challenge (41). Rats administered one single human dose of DTP had a lower incidence of coughing following *Bp* challenge (39). We hypothesized that both oral and IN vaccination would protect against bacterial burden upon challenge as well as protect against *Bp* induced coughing in the rat model of pertussis by generating a protective immune response at the site of infection. To test this hypothesis, we IN and OG vaccinated and challenged Sprague-Dawley rats with 1/5th human dose of DTaP. IM-wP and IM-aP vaccinated and *Bp* challenged rats were used as positive controls to compare vaccine-mediated immunity as IM administration of DTaP is the current route of vaccine administration.

In our study, we aimed to use the rat model of pertussis to measure protection induced from mucosal vaccination of DTaP. By utilizing the coughing rat model of pertussis, protection against bacterial burden in the respiratory tract and prevention of *Bp* induced cough after immunization was critically analyzed. We also focused on evaluating the serological responses regarding vaccination followed by *Bp* challenge. Our data supports that protection can be afforded by mucosal immunization with DTaP. IN and OG immunization with DTaP not only induced systemic anti-*Bp* IgG antibodies but also

induced mucosal anti-*Bp* IgA antibodies. These data suggest that oral vaccination of DTaP can generate a humoral immune response at the respiratory mucosa in rats. IN-aP and OG-aP was also capable of protecting against *Bp*-induced cough. Furthermore, mucosal vaccination protected against bacterial burden in the respiratory tract. In conclusion, this study highlights the benefits of using the coughing rat model of pertussis to study mucosal vaccination with *Bp* vaccines.

3.3 Results

Intranasal immunization induces systemic anti-*Bp* IgG and anti-PT IgM and IgG antibody titers following booster immunization.

We hypothesized that IN-aP and OG-aP immunization would induce systemic IgM and IgG antibodies, as mucosal immunization stimulates the induction of both systemic and mucosal antibodies (27, 28, 30). To test this hypothesis, 3-week-old Sprague Dawley rats were IM-wP, IM-aP, IN-aP, and OG-aP immunized followed by a booster vaccine at 6 weeks of age with the same corresponding vaccine (**Fig. S13**). Mucosal and systemic antibodies were measured over the course of vaccination (**Fig. S13**). Minimal differences in antibody titers prior to booster vaccination was observed for all immunized groups (**Fig. 15**). Compared to all other groups, IM-wP vaccination induced a significant increase of anti-*Bp* IgM in the serum 1-week post-booster vaccination (**Fig. 15A**). IM-wP, IM-aP, and IN-aP vaccinated rats had a significant increase in anti-*Bp* IgG antibody titers following booster immunization compared to mock vaccinated challenged (MVC) rats (**Fig. 15B**). Rats vaccinated via IM-aP had a 100-fold significant increase in anti-PT IgM antibody titers one week post booster vaccine, compared to the MVC control (**Fig. 15C**). IM-aP and IN-aP vaccinated rats had a significant increase in anti-PT IgM antibodies compared

to IM-wP, and OG-aP vaccinated rats (**Fig. 15C**). This same trend was also observed in measuring anti-PT IgG antibodies (**Fig. 15D**). Here our data show that following booster immunization, IN-aP vaccinated rats developed systemic anti-*Bp* IgG and anti-PT IgG and IgM antibody titers.

Mucosal vaccination protects against cough from *Bp* infected rats.

In 2014, Warfel *et al* showed that aP vaccination was protective against pertussis disease, but failed to protect against colonization and transmission of *Bp* in the nonhuman primate model (14). We hypothesized that mucosal vaccination with DTaP would protect against *Bp* induced cough by eliciting a protective immune response at the respiratory mucosa. To test this hypothesis, vaccinated rats were subsequently intranasally challenged with *Bp* 2-weeks post-booster vaccine administration (**Fig. S13**). Every evening post-challenge, coughs were counted using whole-body plethysmography (WBP). MVC rats averaged a total of five coughs or less during monitoring for the first 5 days of infection (**Fig. 16A**). At days 6 and 7 post-challenge, average coughs per fifteen minutes increased to more than thirty coughs (**Fig. 16A**). There was a significant decrease in coughs for rats vaccinated with IM-wP, IM-aP, IN-aP, and OG-aP at days 6 and 7 post-challenge compared to MVC rats (**Fig. 16B-E**). On average, rats vaccinated with IM-wP coughed approximately 6 coughs per fifteen minutes each day (**Fig. 16B**). Rats vaccinated with IM-aP on average coughed 2 times per fifteen minutes each day (**Fig. 16C**). IN-aP immunized rats on average coughed 3 times per fifteen minutes each day, while OG-aP vaccinated rats coughed on average 4 times per fifteen minutes each day (**Fig. 16D&E**). To compare the average total number of coughs each day post-challenge, we calculated

the total number of coughs for each group per animal. We observed a significant decrease in total number of coughs in rats vaccinated with IM-aP (14 coughs) and IN-aP (27 coughs) compared to MVC rats (102 coughs) over the nine-day infection (**Fig. 16F**). OG-aP immunized rats coughed on average 35 times (**Fig. 16F**). Our data demonstrates that mucosal vaccination in rats protects against *Bp* induced cough.

Intranasal vaccination protects against pulmonary distress.

Our previous work has shown that *Bp* infected rats had a significant increase in pulmonary distress following challenge (43). Pulmonary distress can be evaluated by calculating enhanced pause (PenH). PenH functions as a representation of bronchoconstriction taking into consideration the timing between early and late expiration and the estimated maximum inspiratory and expiratory flow per breath. We hypothesized that mucosal vaccination would protect against *Bp* induced pulmonary distress, as bacterial clearance would decrease inflammation. Here, rats vaccinated with IM-wP, IM-aP, and IN-aP had a significant decrease in PenH compared to the MVC control group at days 5 and 7 post-challenge (**Fig. 17A-D**). Rats vaccinated IM-aP and IN-aP also had a significant decrease in PenH at day 6 post-challenge compared to MVC rats (**Fig. 17A&C-D**). However, there was no significant decrease in PenH in OG-aP vaccinated rats compared to MVC suggesting that the induced immune response is not sufficient enough to protect rats from *Bp* induced respiratory distress (**Fig. 17E**). Other respiratory parameters were also measured using WBP (**Fig. S14**). In brief, we observed that rats vaccinated IM-wP, IM-aP, and IN-aP had a significant decrease in pause (PAU) compared to MVC control group, which is another indicator of bronchiole restriction (**Fig. S14B**). Rats vaccinated with the

aP regardless of route also had a significant decrease in Tidal volume (TVb) compared to MVC rats, which could be crudely associated with inflammation (**Fig. S14D**). Overall, our data demonstrated that IN-aP vaccination decreases pulmonary distress of *Bp* infected rats.

Mucosal immunization induces production of *Bp* specific antibodies in the serum, while intranasal immunization also induces PT specific antibodies in the serum.

Next, we wanted to measure systemic antibody responses to *Bp* and PT following challenge. IM-wP vaccinated rats had a slight increase in anti-*Bp* IgM antibodies compared to all other vaccinated groups, albeit not significant (**Fig. 18A**). We observed a significant increase in anti-*Bp* IgG antibody titers in IM-wP, IM-aP, and IN-aP vaccinated rats compared to the MVC at day 1 post challenge (**Fig. 18B**). At day 9 post-challenge, all vaccinated rats had a significant increase of anti-*Bp* IgG antibody titers compared to the MVC control (**Fig. 18B**). Following *Bp* challenge, IM-aP and IN-aP immunized rats had a significant increase in anti-PT IgM antibodies compared to IM-wP immunized rats and MVC control. (**Fig. 18C**). Similar results were observed in measuring anti-PT IgG titers (**Fig. 18D**). IM-aP and IN-aP vaccination induced a significant increase in anti-PT IgG antibody titers compared to MVC, IM-wP, and OG-aP immunized rats after booster vaccination and at days 1 and 9 post-challenge (**Fig. 18D**). Although not significant, two of the OG-aP immunized rats had detectable anti-PT IgG antibody titers in the serum at day 9 post-challenge (**Fig. 18D**). Enzyme-linked immune absorbent spot (ELISpot) assay was used to determine the number of *Bp* specific IgG cells in the bone marrow at day 9 post-challenge. There was an increase in the number of *Bp* specific IgG cells in the bone

marrow in all vaccination groups compared to the MVC control; however, the only significant increase in *Bp* specific IgG producing cells in the bone marrow was detected in IM-wP vaccinated rats (**Fig. S15**). Our data indicate that mucosal vaccination via IN and OG immunization induced *Bp* specific IgG antibody responses.

Intranasal immunization induces production of *Bp* specific IgA antibodies in the nasal cavity

In humans, previous *Bp* infection leads to anti-*Bp* IgA antibodies in nasal secretions (18). IgA antibodies to *Bp* play a role in the inhibition of *Bp* attachment *in vitro* to epithelial cells (19). Here, we investigated if IN and OG immunization of DTaP would induce mucosal IgA antibodies in the lung and/or the nasal cavity. In the lung, three of the four IN-aP vaccinated rats had detectable anti-*Bp* IgA antibodies at day 1 post-challenge, although not significant (**Fig. 19A**). We did not detect anti-*Bp* IgA antibody titers at day 1 post-challenge in the lung of IM-wP, IM-aP, or OG-aP vaccinated rats (**Fig. 19A**). Low levels of anti-*Bp* IgA titers were measured in all vaccinated groups at day 9 post-challenge albeit not significant compared to our MVC control (**Fig. 19A**). The same trend was observed in the lung measuring anti-PT IgA titers at day 1 post-challenge (**Fig. 19B**). At day 9 post-challenge 50% of IN-aP rats and 25% of rats OG-aP vaccinated had detectable anti-PT IgA (**Fig. 19B**). In the nasal cavity, only one IN-aP vaccinated rat had detectable anti-*Bp* IgA antibody titers; however, we did measure a significant increase in anti-*Bp* IgA antibodies in IN-aP immunized rats at day 9 post-challenge compared to the MVC, IM-wP, IM-aP, and OG-aP immunized rats (**Fig. 19C**). Only one IN-aP and one OG-aP vaccinated rat had detectable amounts of anti-PT IgA in the nasal cavity at day 9 post

challenge (**Fig. 19D**). Overall, our data reveal that IN-aP immunization is capable of inducing IgA antibodies in the nasal cavity of rats.

Mucosal immunization protects against acute inflammation in the lung.

Our previous rat challenge study illustrated that intranasal *Bp* challenge gave rise to both acute and chronic inflammation in the rat lung (43). Here, we used histology to assess if mucosal immunization would protect against *Bp* induced inflammation in the lung. At day 1 post-challenge, no differences in acute inflammation were observed; however, at day 9 post-challenge, rats vaccinated with IM-aP, IN-aP, and OG-aP had a significant lower acute inflammation scoring compared to the MVC rats. (**Fig. 20A&C**). IN-aP immunized rats had a higher chronic inflammatory score at day 1 post-challenge (**Fig. 20B&D**). There were no observed differences in chronic inflammation in vaccinated rats compared to MVC rats at day 9 post-challenge (**Fig. 20D**). Total inflammation was calculated by combining both acute and chronic inflammatory scores, as rat lungs exhibited both types of inflammation. No differences in total inflammation score were observed at day 1 post-challenge; however, all vaccinated rats had a significant lower total inflammation score compared to MVC rats (**Fig. 20E**). There were no differences in lung weight, which can be used as a crude measurement for lung inflammation, following *Bp* challenge. (**Fig. S16A**). We did observe a significant increase in percent body weight change in IM-wP vaccinated rats compared to MVC control rats suggesting that wP protects against weight loss observed in non-vaccinated challenged rats (**Fig. S16B**). Our observations suggest that that mucosal vaccination protects against *Bp* induced inflammation in the lung (**Fig. 20C&E**).

Mucosal vaccination protects against *Bp* challenge.

Next, we wanted to assess if mucosal immunization could protect against *Bp* burden in the respiratory tract. Bacterial burden in the respiratory tract was determined 1hr, 1-, and 9- days post *Bp* challenge. Bacterial burden was measured at 1hr post-challenge (n=2) to assess potential bacterial loss for our original challenge dose. In the lung, trachea, and nasal lavage fluid, we measured approximately 10^6 CFUs 1hr post challenge (**Fig. 21A-C**). At day 1 post-challenge there was a significant 98.5% reduction in bacterial burden in the lung of IM-aP immunized rats compared to MVC (**Fig. 21A**). IM-wP, IM-aP, IN-aP, and OG-aP vaccinated rats all had a significant decrease in bacterial burden in the lung at day 9 post-challenge compared to MVC rats (**Fig. 21A**). There was also a significant decrease in bacterial burden in the trachea at both days 1 and 9 post-challenge in all vaccinated rats compared to MVC (**Fig. 21B**). At day 1 post-challenge, there was a significant 86-97% reduction in bacterial burden in all vaccinated rats compared to MVC rats in the nasal cavity (**Fig. 21C**). At day 9 post-challenge we did not measure any significant differences between groups as most of the bacteria were cleared from the nasal cavity (**Fig. 21C**). Overall, we observed that IN-aP and OG-aP vaccinated rats have a significant reduction in bacterial burden in the respiratory tract compared to the MVC control group at days 1 and 9 post-challenge (**Fig. 21A-C**).

wP immunization induces a proinflammatory cytokine response compared to mucosal vaccinated Sprague-Dawley rats.

Previous studies have shown that both wP immunization and *Bp* infection induces a pro-inflammatory Th1/Th17 immune response, while aP immunization promotes a more Th2 skewed response (44–50). In our current study, we measured cytokines in the lung and serum induced from vaccination and challenge. In the lung at day 1 post-challenge, we measured a significant 4-fold increase in IL-17 in MVC rats compared to IM-aP and IN-aP vaccinated rats (**Fig. 22A**). At day 9 post-challenge, IM-wP immunized rats had a significant increase in IL-17 compared to IM-aP, IN-aP, and MVC rats (**Fig. 22A**). IM-wP vaccinated rats also had a significant increase in Th1 cytokine IL-12p70 compared to MVC rats in the lung and serum at day 9 post-challenge (**Fig. 22A&B**). IM-wP vaccinated rats had a significant increase in Th2 cytokines IL-4 and IL-13 in the serum compared to IM-aP and IN-aP vaccinated rats and a significant increase in IL-4 to MVC control at day 9 post-challenge (**Fig. 22B**). IM-wP immunized rats also had a significant increase in G-CSF in the serum day 9 post-challenge compared to IM-aP, IN-aP, OG-aP, and MVC rats. Overall, we did not observe marked changes in cytokine responses between DTaP vaccinated rats compared to the MVC control group; however, rats OG-aP immunized did have a slight increase in IL-17, though not significant. The observed difference in cytokines levels could be from *Bp* challenge rather than vaccination. The increase in acute inflammatory score in the lung of IM-wP immunized rats at day 9 post-challenge could be associated to the increase in proinflammatory cytokines. Graphs showing the statistical significance between cytokines in the serum and lung are in the supplementary data (**Fig. S17&18**). Our data support that response to *B. pertussis* in IM-wP immunized animals is associated robust cytokine response compared to both naïve and aP

vaccinated rats, which is to be expected based on the work that has examined the Th17 response induced by whole cell pertussis vaccines.

Bp infection induces an increase in circulating neutrophils in the blood, as well as white blood cells and lymphocytes (51–56). In our current study, we utilized hematology and flow cytometry to evaluate these populations. Hematology analysis revealed a significant increase in blood lymphocytes in the IN aP vaccinated rats compared to MVC post-challenge; however, no other differences in white blood cell counts in the blood were observed in the other vaccinated groups. (**Fig. S19A&B**). At day 1 post-challenge, there was a significant decrease in circulating neutrophils in the blood for IN-aP immunized rats compared to MVC rats (**Fig. S19C**). Flow cytometry analysis observed minimal differences in the number of neutrophils and B cells at days 1 and 9 post-challenge in all groups (**Fig. S19E&F**). Based upon these data, subtle differences in various circulating cell populations were observed following IN-aP vaccination.

Serological responses correlate with bacterial clearance in the respiratory tract.

Currently no definitive correlates of protection (CoP) for vaccines to protect against *Bp* have been established (22). It is appreciated that Th17 responses as well as Trms correlate with strong protection in mice and baboons. Antibodies to PT/FHA/PRN do not always correlate with protection in humans. In an effort to more precisely define correlates, using the rat model, we aimed to utilize the coughing phenotype and bacterial burden to identify the nature of how each vaccine protects (OG/IN/IM; acellular or wP). Previous work in our lab performed by Wolf *et al* (2021) illustrated that serum anti-*Bp*, anti-FHA, and anti-PT IgG antibody titers in the serum following IN vaccination in mice

correlate with the decrease in bacterial burden in the lung following *Bp* challenge (28). Previous studies have shown that serum anti-*Bp* IgG antibodies induced from wP vaccination correlate with protection against bacterial burden in the lung of *Bp* challenged mice (57). Here, we hypothesized that antigen specific serum IgG and mucosal IgA antibodies correlate with decreased bacterial burden and cough, as IN-aP and OG-aP vaccination induced systemic and mucosal antibody responses. To test this hypothesis, we generated correlograms to evaluate both negative and positive correlations elicited by each vaccination route (58). Correlograms are an analysis tool that can be used to determine if the relationship observed between variables (i.e. bacterial burden and antibody titers) is random or not (59). If the relationship between the two variables is random the R^2 correlation value is or near zero (59). The relationship is considered correlative if the R^2 values approximately positive or negative one (59). Significant positive nonzero correlation values demonstrate a positive correlation between variables, while significant negative nonzero values represent a negative correlation (59). Negative correlations are observed when two variables are inversely related to one another; that is, when one variable increases, the other decreases. With these data, as bacterial burden would drop, then the correlate would increase (negative correlation; inverse). A positive correlation would mean that as bacterial burden increases so does the correlate that is being compared to.

IM-wP vaccinated rats had strong negative correlations (protective) between serum anti-*Bp* IgG antibodies to both bacterial burden in the lung ($R^2 = -0.97$) at day 1 post challenge and the nasal cavity ($R^2 = -0.84$) at day 9 post-challenge (**Fig. 23A-B**). At day 1 post-

challenge, we observed negative correlations (protective) between systemic anti-*Bp* IgM and anti-PT IgG antibodies in IM-aP vaccinated rats to bacterial burden in the nasal cavity ($R^2 = -0.64, -0.64$ respectively). Additionally, negative correlations were observed between anti-*Bp* IgM antibodies and bacterial burden in the trachea ($R^2 = -0.72$) at day 9 post-challenge (**Fig. 23C-D**). IM-aP vaccinated rats also had negative correlation between total cough counts over the course of challenge with IgG and IgM antibodies to *Bp* and PT (**Fig. 23D**). We expected that IN immunization would induce negative correlations between both serum- and mucosal-specific antibodies compared to bacterial burden in the lung, trachea, and nasal cavity at day 1 post-challenge (**Fig. 23E**). Lung anti-*Bp* IgA antibodies also negatively correlated with total cough count ($R^2 = -0.74$) and bacterial burden ($R^2 = -0.6$) in the lung at day 9 post-challenge for IN-aP vaccinated rats (**Fig. 23F**). We observed strong negative correlations between serum IgG and mucosal IgA antibody to bacterial burden in the lung ($R^2 = -0.93, -0.93$ respectively) in OG-aP vaccinated rats at day 1 post-challenge despite the overall lower serological responses (**Fig. 23G**). At day 9 post challenge, there was a negative correlation between serum anti-*Bp* IgG antibodies to bacterial burden in the lung ($R^2 = -0.81$), trachea ($R^2 = -0.49$), and nasal cavity ($R^2 = -0.52$) in OG-aP immunized rats (**Fig. 23H**). We also noticed that OG-aP vaccinated rats had negative correlations between serum IgG and mucosal IgA antibodies in the lung to total cough count ($R^2 = -0.94, -0.84$) at day 9 post-challenge (**Fig. 23H**). Positive correlations (non-protective) were observed between bacterial burden and total inflammatory score in IM-wP, IM-aP, and OG-aP immunized rats (**Fig. 23A, C, G**). Bacterial burden also positively correlated with total cough counts at day 9 post challenge (**Fig. 23B, D, F, H**). By utilizing correlograms, strong negative correlations between serum

serological responses and bacterial burden were observed in IM-wP and IM-aP immunized rats. Additionally, our data underscore the idea that both systemic and mucosal antibodies correlate with the observed *Bp* clearance in the respiratory tract and protection from *Bp* induced cough elicited from IN-aP and OG-aP vaccination highlighting the observed differences between vaccination routes.

3.4 Discussion

The immunity induced by aP vaccines is relatively short lived; thus, DTaP/Tdap vaccinated individuals are still capable of *Bp* transmission (60, 61). We have recently re-investigated the rat model of pertussis to further understand *Bp* pathogenesis from current circulating *Bp* strains (such as CDC isolate D420) (43). The coughing rat model of pertussis is a tool that can be used to evaluate bacterial burden in the respiratory tract, and also evaluate vaccine-induced immunity against cough and respiratory function (36–41, 62). In our current study, we evaluated mucosal vaccination with DTaP in the coughing rat model of pertussis. To our knowledge, this study is the first to evaluate both IN and OG administered DTaP in the coughing rat model of pertussis. The data presented here suggest that, not only does mucosal immunization protect against bacterial burden, but also against *Bp* induced cough by WBP (**Fig. 16&21**).

Vaccine mediated immunity has been studied in the coughing rat model of pertussis. Utilizing audio tape recorders, Hall *et al* (1998) illustrated that 3 and 5 component aP vaccines administered subcutaneously could protect against *Bp* induced cough (41). wP immunization administered intraperitoneally also decreased the incidence of cough in rats (39). Neither study noted protection against bacterial colonization. Here, in our study,

WBP was used to investigate *Bp* induced cough in IN-aP and OG-aP vaccinated rats, as well as measure bacterial burden in the respiratory tract. Our data supports that mucosal administration of DTaP protects against bacterial burden in the respiratory tract and reduced *Bp*-induced cough (**Fig. 16&21**). In addition, IN-aP vaccination reduced bronchial constriction in the lung that is elicited by *Bp* infection (**Fig. 18**). Previous studies evaluating wP and aP vaccines in the rat model of pertussis used one human dose per rat for immunization prior to challenge (39, 41). In an effort to best model an appropriate human to rat dose, we utilized a 1/5th human dose to prime and boost based on the relative sizes of rats compared to mice. We have reported 1/40th human dose as protective in mice and rats are roughly 10x the weight of mice (63). One caveat of this study is that we did not evaluate other human-to-rat titrations of aP. Identification of a minimal protective rat dose would allow for the investigation of vaccine efficacy of new potential antigens/adjuvants in this model (63).

Mucosal immunization has been of particular interest in the pertussis field. We and others have recently evaluated intranasal immunization of DTaP in *Bp* challenged mice (27, 28, 64–66). Intranasally DTaP vaccinated mice were protected against *Bp* challenge and also generated both systemic and mucosal antibodies (27, 28). Live attenuated strain BPZE1 administered intranasally was protective against *Bp* challenge in mice and baboons, and is currently in Phase 2 of clinical studies (33, 34, 67, 68). BPZE1 immunization induces both anti-*Bp* IgG and IgA antibodies systemically and has an increase in resident memory T cells in the lung (33, 69). Oral immunization has also been investigated as a possible vaccination strategy against pertussis. Oral immunization of heat-inactivated *Bp*

protected newborns against *Bp* challenge, as well as generated serum and saliva antibody titers (70). Recombinant technologies has led to the development of live attenuated *Salmonella* strains presenting *Bp* antigens (71, 72). Oral immunization of *Salmonella typhimurium aro* vaccine strain harboring the gene for PRN resulted in reduced bacterial colonization in the lung post *Bp* challenge (71). *Salmonella dublin aroA* mutant expressing the gene for FHA was also orally administered as a vaccine in mice (72). Vaccination with this strain induced IgG and IgA antibody titers to FHA in the serum and gut (72). Our current study shows that mucosal immunization not only induced systemic anti-*Bp* IgG but also anti-*Bp* IgA antibodies that likely play role in clearance at the mucosa (**Fig. 18-19, 21**).

CoP is defined as the immune response that is statistically accountable for the observed protection (73). While no CoP has yet to be fully agreed upon against pertussis in humans, anti-PT IgG levels >5 IU/ml are associated with protection in humans (74). In mice, IN administration of DTaP induced anti-*Bp*, anti-FHA, and anti-PT IgG antibodies while wP vaccination induced serum anti-*Bp* IgG antibodies that correlated with protection against *Bp* (28, 57). Here in our study, we generated correlograms between all vaccinated groups to identify correlations between variables in the coughing rat model of pertussis, which has yet to be established (**Fig. 23**). Our results indicate that bacterial clearance in the lung, trachea, and nasal cavity negatively correlate with systemic anti-*Bp* and -PT IgM and anti-*Bp* IgG antibodies in IM-wP and IM-aP immunized rats, while systemic and mucosal antibodies correlated with bacterial clearance in IN-aP and OG-aP vaccinated rats (**Fig. 23**). Antibodies generated following immunization also negatively correlated

with a decrease in total cough counts in vaccinated rats (**Fig. 23**). These results suggest that the increase in systemic and mucosal antibodies induced from IN and OG vaccination correlates with protection against *Bp* burden in the respiratory tract and *Bp* induced cough. It is important to note, that OG-aP immunized rats did not generate significant serum antibody titers to the whole bacterium until day 9 post-challenge (**Fig. 18B**). Also, two OG-aP immunized rats had an increase in anti-PT IgG antibody titers in the serum and anti-*Bp* IgA antibodies in the lung (**Fig. 18D, 19A**). We did however detect antigen specific B cells in the bone marrow and 3 of the 4 rats had low levels of IgA in the lung in OG-aP immunized rats (**Fig. S15**). One caveat that should be mentioned is that we did not investigate the T cell responses (Th1/Th2/Th17/T_H1/T_H2/T_H17/T_Rm/T_Em) in rats but future studies will incorporate this into the study design. We have proposed a summary for mechanism for oral vaccination of aP (**Fig. S20**). We hypothesize that this could be because limited amount of vaccine that successfully travels to the gut-associated lymphoid tissue (GALT) for the generation of an immune response. Oral vaccines have to travel through increased pH in the stomach while limited absorption and availability for antigen recognition also occur in the gastrointestinal tract (75). Increase in dose, number of doses, or delivering vaccine in an encased vehicle are potential methods to increase orally vaccinated immune responses. Targeting of vaccine to intestinal M cells for antigen presentation has also been shown to increase oral vaccine efficacy (76). Though we did not study new adjuvants here, we hypothesize adjuvants can aid in stimulating a protective mucosal immune response. These approaches could all potentially increase the efficacy observed through oral vaccination of aP. Furthermore, to deliver the vaccine to the gut, one could

envision novel deliver mechanisms such as gelatin coated chewables similar to gummy vitamins that are now popular.

In summary, mucosal vaccination not only protected against bacterial burden in the respiratory tract of challenged rats, but also protected against *Bp* induced cough and respiratory distress measured by WBP (**Fig. 16-17, 21**). It is critical that “next generation pertussis” vaccines protect against bacterial colonization in the lung, nasal cavity, and trachea, as disease manifestations are dependent on bacterial colonization of the lung and trachea, mediated by FHA and fimbriae (77, 78). Both IN and OG immunized rats generated anti-*Bp* specific IgG antibodies in the serum, while IN vaccinated also generated significant anti-*Bp* IgA antibody titers in the nasal cavity following challenge (**Fig. 18-19**). IN-aP and OG-aP immunized rats were protected against acute and total inflammation in the lung (**Fig. 20**). Our data support the potential of a mucosal vaccination against *Bp*.

Further work is needed to fully characterize the immune response generated following IN-aP and OG-aP vaccination in rats, as well as vaccine mediated immunity from vaccination in the coughing rat model of pertussis. T cell immune responses that have been shown to play a role in natural and vaccine mediated immunity against pertussis have yet to be evaluated in the coughing rat model of pertussis due to limited availability of resources to adequately measure T cell responses. Vaccine mediated memory has yet to be evaluated in the coughing rat model of pertussis. Additional research is needed to critically assess vaccine mediated memory, as it is essential that next generation of

pertussis vaccines induce longer lasting memory than current vaccines. Future work is also needed to evaluate mucosal immunization against current circulating strains of *Bp* as current strains are genetically divergent from strains of the past, with the goal of making the most efficacious vaccine against *Bp*.

3.5 Materials and methods

Vaccine composition and administration. INFANRIX (GSK Cat. 58160-810-11) acellular pertussis human vaccine (DTaP) and the National Institute for Biological Standards and Control WHO whole cell pertussis vaccine (NIBSC code 94/532) was used for this study. Vaccines were diluted with endotoxin-free Dulbecco's PBS (Thermo Fisher Scientific Cat. TMS012A) to a concentration of 1/5th human dose. Vaccines were diluted and administered no more than 1 hr. from composition. The first dose of vaccine was administered to three-week-old (50g) female Sprague-Dawley rats (Charles River Cat. 001CD). At six weeks of age, a booster vaccine of the same dose was administered, followed by *Bp* challenge at eight weeks of age. Intramuscular (IM) vaccinated rats received 100µl in the right thigh muscle of the hind limb. Intranasal (IN) immunized rats were first anesthetized with isoflurane until breathing was minimal. Rats then received 50µl of vaccine in each nostril for a 100µl dose. Oral gavage (OG) vaccinated rats received 100µl dose delivered curved 18 gauge feeding needle (Fisher Scientific Cat. NC9349775). MVC control group received 100µl of the same endotoxin free PBS used to dilute the vaccines in the right thigh muscles of the hind limb. One-week post-prime, two-week post-prime, and one-week post-boost blood was collected via saphenous blood draws for serological analysis. 5mm animal lancets (Fisher Scientific Cat. NC9891620) was used for blood draw. Blood was collected in capillary tubes (Fisher Scientific Cat.

NC9059691) for centrifugation. Blood was spun at 15,000x *g* for 3 min., serum collected and stored at -80°C until analysis.

***Bordetella pertussis* strains and growth conditions.** *Bp* strain D420 was cultured on Bordet Gengou (BG) agar (Remel™ Cat. R45232) supplemented with 15% defibrinated sheep blood (Hemostat Laboratories Cat. DSB500) (1). Bacteria cultured BG plates incubated for 48 hrs at 36°C. Using polyester swabs (Puritan Cat. 22-029-574), *Bp* was transferred into 20 ml Stainer-Scholte liquid media (SSM) in new 125 ml flasks (Thermo Fisher Scientific Cat. FB500125) (79). Bacterial cultures were allowed to grow at 36°C for 24 hrs inside a shaking incubator at 180 rpm.

Intranasal challenge. Vaccinated eight-week-old ~200g female Sprague-Dawley rats were then challenged. *Bp* was grown as illustrated above. Rats were anesthetized with ketamine and xylazine 50-100/5-10 mg/kg and challenged with 10⁸ CFUs in 100µl intranasally, 50µl in each nostril. Body weight of each rat was recorded before bacterial challenge, and body weights were taken post-euthanasia to calculate percent weight change. At days 1 and 9 post challenge, rats were then euthanized. Upon euthanasia blood was collected via cardiac puncture and transferred into ethylenediaminetetraacetic acid (EDTA) (BD Cat. 365974) and serum separation (BD Cat. 026897) tubes. Following cardiac puncture, 250µl of blood was collected into EDTA tubes for flow cytometry and ProCyte (IDEXX) analysis, while remaining blood was collected in serum separation tubes to isolate the serum via centrifugation (15,000x *g* for 3 min) and used for serological and cytokine analysis. To determine bacterial burden in the respiratory tract, the lung and

trachea was excised separately and homogenize. Lung weights were recorded following excision before homogenization. Lungs were then collected in gentleMACS C tubes (Miltenyi Biotec Cat. 130-096-334) in 2ml of PBS and homogenized using Miltenyi Biotec tissue dissociator (Cat. 130-095-927). Polytron homogenizer was used to homogenize the trachea in 1 ml PBS. Bacterial burden in the nares was determined by flushing 2mls of sterile 1x PBS through the nares and collected for serial dilution and plating. Serial dilutions of the homogenates and nasal collection were plated on BG plates supplemented with ceftibuten (Sigma-Aldrich Cat. SML0037) 10 µg/ml.

Serological analysis. Enzyme-linked immunosorbent assays (ELISA) was used to measure antibody titers of vaccinated and infected rats. *Bp* specific whole bacteria ELISA plates were coated with 50 µl of 10⁸ *Bp* grown as mentioned above for infection. Antigen specific antibody titers to PT (List Biological Laboratories #180) were measured by coating ELISA plates with 50 µl of antigen per well. Antigen coated plates incubated over night at 4°C. After incubation, plates were washed with 1x PBS-Tween 20 and blocked with 5% skimmed milk for 2 hrs at 37°C. Following blocking, ELISA plates were washed and serum from the saphenous blood draws and blood collected from cardiac puncture post-euthanasia were serially diluted down the ELISA plate and incubated for 2 hrs at 37°C. To measure respiratory IgA antibody titers in the lung and nasal lavages, lung homogenate supernatant and nasal lavage was added and incubated for 2 hrs at 37°C. After incubation, ELISA plates were washed as described above and 100µl of secondary goat anti-rat IgG (SouthernBiotech Cat. 3030-04), goat anti-rat IgM (SouthernBiotech Cat. 3020-04), or goat anti-rat IgA (MyBioSource Cat. MBS539212) was added to the plates at a dilution of 1:2,000 in PBS + 5% milk and incubated for 1 hr at 37°C. Plates were then

washed again and 100 μ l *p*-nitrophenyl phosphate substrate (Thermo Scientific Cat. 37620) was added and the plate was developed for 30 min at room temperature. After development, colorimetric signal of the ELISA plate at A_{450} was measured by a Biotek Synergy H1 microplate reader. Antibody titers were considered positive if values were higher than the baseline. Baseline value for each sample was set as double the average value of the blank, in which no serum, lung supernatant, or nasal lavage added to these well. Limit of detection was set at 50, and any samples with a titer value less than that were set to 50.

ELISpot assay. ELISpot assay (ImmunoSpot Cat. mTgG-SCE-1M/2) was used to analyze antigen specific B cells in the bone marrow. The right hind femur of the rat was removed and placed into Dulbecco's modified Eagle's medium (DMEM) and frozen at -80°C until analysis. Bones were then thawed in water bath at 37°C, and immediately transferred into spin tubes and spun at 1,000x *g* for 3 min to collect the bone marrow. Bone marrow was passed through a 70 μ m filter to create a single cell suspension. Cells were centrifuged at 350 x *g* for 5 min and the cell pellet was resuspended in CTL test B Media (ImmunoSpot). D420 was cultured as described above and coated the 96-well ELISpot plate as described by ELISA. The plate incubated overnight at 4°C. Plate was then washed with 1x PBS before cells were added. Three serial dilutions of cells (1.25×10^6 , 3.13×10^5 , and 1.56×10^5) cells added per well and incubated at 37°C overnight. Rabbit anti-Rat IgG antibody (Abcam Cat. ab6733) was used to replace the anti-murine IgG detection antibody that was with the kit. The rest of the protocol was followed as per the manufacturer's instructions. ELISpot plates imaged and analyzed using ImmunoSpot S6 Entry analyzer and CTL software.

Analysis of cough and bronchiole restriction using whole-body plethysmography.

Buxco® FinePointe™ Whole Body Plethysmography (WBP) (DSI) was used to quantify respiratory function during infection. Every day following *Bp* challenge and one day before challenge (5:00PM), rat respiratory profiles and coughs were measured. A 5 min acclimation period was used before measuring cough and other respiratory parameters. After acclimation the respiratory profile was recorded for 15 mins for each rat. Coughs were counted and represented over 15 mins. Enhanced pause (PenH) was calculated which represents bronchiole restriction during breathing. Coughs were counted based on box flow changes of the subject with classical cough-like waveforms. Patented fuzzy logic criteria was used to detect and count coughs (80). Each cough in a multi-cough event was counted individually. Frequency (F), Tidal Volume (TVb), Pause (PAU), Minute Volume (MVb), Inspiratory time (Ti), and expiratory time (Te) were also collected and analyzed during the course of infection.

Histological assessment of the lung. The left lobe of the lung was used for histological assessment. Following excision of the left lobe, the sectioned portion was fixed in 10% formalin 48 hrs at 26 °C. Following fixation, samples were embedded in paraffin and stained with H&E by the WVU Pathology Department. Stained samples were used to characterize and score inflammation of the lung. All scorings were done by a board-certified pathologist (iHisto). Individual scores were based on a standard qualitative scoring criterion: (0 – none, 1 – minimal (rare), 2 – mild (slight), 3 – moderate, 4 – marked, 5 –severe). The presence of neutrophils in the parenchyma, blood vessels, and airway was used to score acute inflammation, and chronic inflammation was characterized by

mononuclear infiltrates of the parenchyma, blood vessels, and airway. All examination and scoring were done with no knowledge of the groups.

ProCyte analysis of blood. Blood from the EDTA tubes was used to analyze white blood cell, neutrophil, and lymphocyte counts. 25-50 μ l of blood was drawn from the EDTA tubes and analyzed by the Procyte. After ProCyte analysis the rest of the blood was used for flow cytometry analysis.

Flow cytometry analysis. Blood samples were lysed with 1x Pharmlyse buffer (BD Biosciences Cat. 555899) for 20 min at room temperature. Blood samples were vortexed periodically during the 20 min incubation. After lysis, cells were resuspended in RPMI + 10% FBS to neutralize the lysis buffer and centrifuged at 1,000g x 5. Cells were then washed with the RPMI +10% FBS again. Cells were then resuspended in 1%FBS+PBS+5mM EDTA. Blood samples were then blocked with anti-CD32 (BD Pharmingen Cat. 550270) antibody for 15 min at 4°C. After blocking, the cells were labeled; CD45 Alexa flour 700 (Biolegend Cat. 202218), CD161 APC (Biolegend Cat. 205606), CD45R PE Cy7 (eBioscience Cat. 25-0460-82), His48 FITC (eBioscience Cat. 11-0570-82), CD43 PE (Biolegend Cat. 202812), and CD3 VioGreen (Miltenyi Biotec Cat. 130-119-125) (81). Samples were incubated 1 hr at 4°C in the dark. To prepare the lung samples for flow cytometry, the lung homogenate was filtered through a 70 μ m cell strainer (BioDesign Cell MicroSives Cat. N70R). The suspension was centrifuged at 1,000 x g for 5 min. The pellet was resuspended in Pharmlyse buffer and incubated at 37°C for 2 min. After incubation, the cells were centrifuged at 1,000 x g for 5 min, lysis buffer removed, and cells were blocked and labeled with antibody as described for blood samples. Both blood and lung samples were centrifuged at 1,000 x g for 5 min and the

pellets were resuspended in 0.4% paraformaldehyde and stored overnight at 4°C. Samples were washed with 1x PBS+5mM EDTA+1%FBS and resuspended in 1x PBS+5mM EDTA+1%FBS for analysis. Cell samples were analyzed on a LSR Fortessa and samples were gated and analyzed using FlowJo v10.

Cytokine analysis. Lung homogenates were centrifuged at 19,000× *g* for 4 min and the resulting supernatant was removed and stored at –80 °C until further analysis. Lung supernatant and serum cytokines were measured using a ProcartaPlex Multiplex Immunoassay kit: Th Complete 14-Plex Rat ProcartaPlex Panel (Thermo Fisher Scientific Cat. EPX140-30120-901) per the manufacturer's instructions. Cytokines with bead counts less than 35 were invalidated.

Generation of correlograms. Correlograms were created using R Studio software. Pearson correlation coefficients were calculated between each set of variables listed in the master table and then illustrated in the representative plot for each vaccine route and timepoint.

Statistical analysis. GraphPad Prizm 7 was used to analyze the data. The minimum biological replicates for the challenge studies were three for MVC control group and four rats per vaccinated groups. For statistical comparisons between vaccinated groups and the MVC control group over the entire course of the infection, two-way analysis of variance (ANOVA) was used with Dunnett's post hoc test. One-way ANOVA was used for comparison between vaccinated groups and MVC for an individual day or timepoint with Dunnett's post hoc test. Kruskal-Wallis test with Dunnett's post hoc test compared between groups for mucosal IgA comparisons. ROUT test was used to identify any

potential outliers during cytokine analysis of the lung.

Data availability. Data requests for figures provided can be addressed to the corresponding author.

Ethics statement. This challenge study was performed in accordance with our approved protocol by West Virginia University Institutional Animal Care and Use Committee (IACUC) protocol 1811019148.6.

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JMH and GJB performed vaccination and bacterial challenge. JMH and GJB monitored rats by whole body plethysmography. All authors participated in the animal experiments. JMH and TYW prepared flow cytometry samples. JMH and MAW performed cytokine analysis. JMH performed ELISA assays. MAD constructed correlograms. JMH, MB, and FHD contributed to experimental design. JMH wrote manuscript with critical revisions from all authors.

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3.7 Figures

Figure 15. IN booster vaccination induces systemic anti-*Bp* and anti-PT antibody titers. 1 and 2 weeks post prime immunization and 1 week post boost blood was collected via saphenous vein, and anti *Bp* and anti PT IgM (A-C) and IgG (B-D) specific antibodies were measured. Results are shown on a log scale and as a mean $\pm$ SEM ($n = 3-8$). Dotted line represents the limit of detection. * $P < 0.05$. ($n = 4-8$). P values were determined by two-way ANOVA with Dunnett's post hoc test compared between groups. * under each graph annotates the significance between labeled group under the y-axis and the group under the corresponding bar. Grayed out box annotates no stats calculated.

Figure 16. Intranasal and oral vaccination of acellular pertussis vaccine decreases cough of *B. pertussis* infected rats. Coughs were counted every day of the nine-day infection using whole body plethysmography. Coughs were counted for (A) mock vac. challenge rats, (B) IM-wP (C) IM-aP (D) IN-aP, and (E) OG-aP, vaccinated and challenged rats. To assess any potential differences between vaccine groups over the entire course of infection, (F) average total number of coughs for each rat per group was compared. Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test and one-way ANOVA with Dunnett post hoc test for total cough count, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ compared to the mock vac. challenged control group.

Figure 17. Intranasal vaccination decreases pulmonary restriction of *Bordetella pertussis* infected rats. Bronchiole restriction was measured over the course of infection by whole body plethysmography. Bronchiole restriction was determined by the factor Penh for (A) mock vac. challenge rats, (B) IM-wP (C) IM-aP (D) IN-aP, and (E) OG-aP vaccinated and challenged rats. Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the mock vac. challenge group.

Figure 18. Mucosal vaccination induces production of anti-*Bp* IgG, while IN immunization also induces both anti-PT IgM and IgG antibodies. ELISAs were used to determine and compare the induced serological responses from vaccinated and challenge rats in the serum. Both (A, C) IgM and (B, D) IgG serum antibody titers from immunized and challenged rats were measured post prime, boost, and challenge. Dotted line represents the limit of detection. Results are shown on a log scale and as mean $\pm$ SEM, * P < 0.05 (n = 4). P values were determined by two-way ANOVA with Dunnett's post hoc test compared between groups. * under each graph annotates the significance between labeled group under the y-axis and the group under the corresponding bar. Grayed out box annotates no stats calculated.

Figure 19. Intranasal immunization elicits the production of anti-*Bp* IgA in the respiratory tract. ELISA was used analyze antibodies in the (A, B) lung and (C, D) nasal cavity from lung homogenate supernatant and PBS flushed through the nasal cavity from vaccinated and challenge rats at days 1 and 9 post challenge. IgA titers were determined against pertussis toxin and *B. pertussis*. Dotted line represents the limit of detection. Results are shown on a log scale and as a mean $\pm$ SEM ($n = 3-4$). ** $P < 0.01$, **** $P < 0.0001$ P values were determined by Kruskal-Wallis test with Dunnett's post hoc test compared between groups.

Figure 20. Mucosal vaccination protects against acute and total inflammation in the lung of *Bp* infected Sprague-Dawley rats. Post euthanasia, left lobe of the lung was excised, sectioned, and stained with hematoxylin and eosin. Lung samples scores were based on standard qualitative toxicologic scoring criteria (0 – none, 1 – minimal (rare), 2 – mild (slight), 3 – moderate, 4 – marked, 5 –severe). (A) Representative image of acute inflammation of rat lung showing increased numbers of neutrophils and edema surrounding blood vessel (asterisk). (B) Representative image of chronic inflammation of the rat lung showing increased numbers of mononuclear cells surrounding bronchioles (asterisk). Inflammatory cells are also present in the lamina propria (arrow) and epithelium (arrowhead) of bronchioles. (C) Average acute inflammation scores of the lung are detailed by the presence of neutrophils in the parenchyma, blood vessels, and the airways. (D) Average chronic inflammation scores are distinguished by mononuclear infiltrates in the parenchyma, blood vessels, and airway of the lung. (E) Total inflammatory score calculated by the sum of the acute and chronic inflammation score of the lung. All

scoring assessments were determined with no knowledge of the groups. Results are shown as mean $\pm$ SEM ($n = 3-4$) P values were determined by two-way ANOVA followed by Dunnett's comparison test, $*P < 0.05$, $**P < 0.01$ compared to mock challenge.

Figure 21. Oral and intranasal immunization decreased *B. pertussis* bacterial burden in the respiratory tract. Bacteria were quantified by serially diluted CFUs following vaccination and intranasal challenge. CFU counts were determined from (A) lung homogenate (B) trachea and (C) nasal lavage 1.5Hr, 1-, and 9-day post *B. pertussis* challenge. Results are shown as mean $\pm$ SEM ($n=2-4$). P values were determined by one-way ANOVA with Dunnett's post hoc test, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$ compared to mock vac. challenge group.

Figure 22. Measurement of cytokines in the lung and serum at days 1 and 9 post infection. Heat map of the average percent cytokines normalized to the max cytokine measured in the (A) lung and (B) serum. MVMC (mock vaccinated mock challenge) cytokines are from rats in (Hall et al 2021). All statistical analysis comparing average cytokine values are in Fig S5-6.

Figure 23. Systemic and mucosal anti-*Bp* and anti-PT antibodies correlate with observed protection. Correlograms were generated using the observed data for IM-wP (A-B), IM-aP (C-D), IN-aP (E-F), and OG-aP (G-H). Program R was used to make correlation graphs from raw data for both day 1 and day 9 post-challenge. R^2 values were generated when generating the correlograms. Positive correlations are annotated by the blue circles, while the negative correlations are annotated by the red circles. The size of the circle annotates the strength of the correlation.

3.8 Supplemental Figures

Figure S 13. Experimental design of vaccination and challenge. Three-week old Sprague Dawley rats were vaccinated IM-wP, IM-aP, IN-aP, and OG-aP 1/5th human dose. 21 days post prime, rats were boosted with the same corresponding vaccine. At days 7, 14, and 28 post prime, blood was collected via saphenous blood draw for serological analysis. At day 35, rats vaccinated rats were intranasally challenged with 10⁸ viable *Bp*. Cough and respiratory distress was measured using whole body plethysmography for 8 days post challenge. Antibody titers in the serum, lung, and nasal cavity was measured at days 1 and 9 post challenge. Flow cytometry was used to measure neutrophil and b cell recruitment in the lung and blood at days 1 and 9 post challenge. The left lobe of the lung was sectioned and stained with H&E for analysis of inflammation at days 1 and 9 post challenge. Antigen specific IgG ELISpot assay was performed to measure antigen specific B cells in the bone marrow at days 1 and 9 post challenge. Cytokines in the lung and serum were measured post challenge. Hematology was used to measure blood cell populations post challenge. Bacterial burden in the respiratory tract was measured at days 1 and 9 post challenge. Created with Biorender.com

Figure S 14. Respiratory profile of Sprague-Dawley rats vaccinated and challenged with *B. pertussis*. Every day after 5p.m. whole body plethysmography was used to analyze the respiratory capacity of immunized and challenge rats. Measurements assessed were: (A) frequency of breathes, (B) pause (PAU), (C) minute volume (MVb), (D) tidal volume (TVb), (E) time of inspiration (Ti), and (F) time of expiration (Te). Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test,

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to mock vac. challenge group.

Figure S 15. ELISpot assay was performed to measure the number of *Bp* specific IgG cells from the bone marrow at day 9 post challenge. Results are shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by one-way ANOVA with Dunnett's post hoc test, $*P < 0.05$ compared to mock vaccinated challenge group.

Figure S 16. Measurement of lung and body weight at days 1 and 9 post challenge. (A) Lung weight as measured immediately after euthanasia before being homogenized. Body weight for each rat was measured before challenge and immediately post euthanasia. (B) Percent weight change was calculated by taking the differences between starting weight and end weight and dividing by the initial weight multiplying by 100. Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test, $*P < 0.05$ compared to the mock vaccinated challenge group.

Figure S 17. Measurement of cytokines in the lung at days 1 and 9 post infection. Cytokines in the lung supernatant were analyzed using ProcartaPlex multiplex immunoassay kit. Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test, * $P < 0.05$, ** $P < 0.01$ compared between mock vaccinated challenge and vaccinated challenge groups.

Figure S 18. Measurement of cytokines in the serum at days 1 and 9 post infection. Cytokines in the serum and lung supernatant were analyzed using ProcartaPlex multiplex immunoassay kit. Results shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA with Dunnett's post hoc test, * $P < 0.05$, ** $P < 0.01$ compared between mock vaccinated challenge and vaccinated challenge groups.

Figure S 19. Characterizing circulating cell populations in the blood and lung post infection. ProCytte hematology analyzer was used to assess (A) total white blood cells, (B) percent lymphocytes and (C) percent neutrophils in the blood post challenge. Flowcytometry analysis of circulating B cells and neutrophils in the lung and blood. (D&E) Neutrophils were gated by (CD45⁺ CD161⁻ B220⁻ CD43⁺ His48^{hi}), while (F) B cells were gate by (CD45⁺ CD45R⁺). Neutrophil and B cell quantification represented as percentage of single, CD45⁺ cells. Results are shown as mean $\pm$ SEM ($n = 3-4$). P values were determined by two-way ANOVA followed by Dunnett's comparison test, * $P < 0.05$, compared between mock vaccinated challenge group.

Figure S 20. Proposed mucosal immune response induced through oral vaccination. Upon oral vaccination of DTaP, antigens travel to the gut associated lymphoid tissue. Here, antigens are endocytosed by M cells or phagocytosed by DCs in the epithelial layer and antigens are presented to T and B cells in the lamina propria. Ensuing germinal center reaction occur in lymphoid follicles and induce *Bp* specific plasma cells capable of producing IgG and IgA antibodies. Generated plasma cells can home to secondary lymphoid tissue and travel to the bone marrow awaiting recall. Upon *Bp* challenge, plasma cells are recalled and migrate to the respiratory mucosa secreting IgG and IgA antibodies. Here antibodies can opsonize the bacteria and potentially play a role in antibody mediated phagocytosis of the bacteria with recruited neutrophils and macrophages. Other potential mechanism for the generation of antibody secreting plasma cells involves direct class switching and terminal differentiation.

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Chapter 4. Discussion

4.1 Overview

As highlighted in chapter 1, humans are the only reservoir for *Bp*, making it difficult for the generation of an ideal animal model to study pertussis. Here, we wanted to reinvestigate the coughing rat model of pertussis due to the similarities in clinical manifestations of pertussis as in humans and utilize this model to evaluate mucosal immunization of DTaP. In chapter 2, we focused on building upon the coughing rat model of pertussis utilizing current techniques and resources. We detailed the hallmark manifestations of pertussis such as paroxysmal cough, respiratory inflammation, and neutrophilia between two genetically diverging strains. To our knowledge, this is one of the first studies to utilize and establish WBP to precisely count cough and analyze respiratory distress of rats following *Bp* challenge. We also carefully detailed the serological response to *Bp* associated virulence factors such as FHA, PT, PRN, and ACT. Furthermore, we reported that IN inoculation of *Bp* more uniformly colonized the respiratory tract compared to IB inoculation that relies on high bacterial loads in the lung only. With the current circulating isolates being genetically different from strains of the past, it is critical for the evaluation of a new model to be as relevant and current as possible. As chapter 2 focused on further developing the coughing rat model of pertussis, in chapter 3, we tested the vaccine efficacy of mucosal vaccination of DTaP compared to IM administration of DTP and DTaP utilizing the coughing rat model of pertussis. To our knowledge, this is one of the first studies to investigate mucosal immunity against pertussis in rats. WBP was used to determine if both IM and mucosal vaccination was protective against *Bp* induced cough and respiratory distress. Serological responses following prime immunization, booster vaccination, and following challenge were also characterized. Our results show that

mucosal vaccination of DTP is protective against *Bp* colonization of the respiratory tract and protective against *Bp* induced cough.

4.2 Lessons learned from the coughing rat model of pertussis

As discussed in chapter 1, multiple animal models have been tested and have been fundamental in our understanding of pertussis. A total of nine publications to date have detailed using rats as a model to investigate either *Bp* pathogenesis or DTP/DTP vaccine efficacy (1–9). The last study utilizing the coughing rat model of pertussis was performed in 1999 (9). Since 1999, we have observed cyclic increases in the number of pertussis cases (**Chapter 1. Fig. 5**) (10). Pertussis resurgence combined with genetic divergent strains call for reevaluation of our current animal models of pertussis to understand *Bp* pathogenesis as it relates to humans, and the development of next generation pertussis vaccines. Rats, as a model of pertussis present with *Bp* colonization in the respiratory tract, leukocytosis, neutrophilia, pulmonary inflammation, and *Bp* induced paroxysmal cough (1–9).

One of the aims of chapter 2 was to revitalize the coughing rat model of pertussis using IN inoculation of a relatively current circulating isolate. Rats were IN challenged with *Bp*, as IN inoculation deposits bacteria in both the upper and lower respiratory tract, which is similar to bacterial placement in adolescent and adults with pertussis, rather than IB inoculation that places the bacteria specifically in the lower area of the lung (11). IN challenge places the bacteria in the nasal cavity, trachea, and lung. IN instillation was selected as the route of infection for its simplicity and control of the bacterial challenge

dose. In mice, aerosol *Bp* challenge has been investigated and studies have shown that aerosol challenge results in consistent bacterial loads in the respiratory tract following challenge, and the rate of bacterial clearance and protection observed in vaccinated and challenge mice was similar to vaccine efficacy in children (12, 13). Aerosol challenge was selected for its naturalness of infection as it relates to transmission of *Bp* in humans. Aerosol challenge has yet to be investigated in the coughing rat model of pertussis, future studies comparing routes of infection could further illuminate other pathogenic differences such as cough, respiratory distress, and bacterial burden.

We IN challenged Sprague-Dawley rats with 10^8 CFUs of *Bp* strains Tohama 1 and representative clinical isolated D420 (**Chapter 2. Fig. 5**). Strain D420 is genetically similar to 50% of isolates that have been recovered across the United States in 2000 (14). However, further challenge studies are needed to fully assess the impact of the observed genetic divergence on pathogenesis of more current circulating strains. Our data showed that IN *Bp* challenge resulted in bacterial burden in the nasal cavity, trachea, and lung (**Chapter 2. Fig. 11**). We did measure an increase in bacterial burden in D420 infected rats in the lung and nasal cavity compared to Tohama 1 infected rats (**Chapter 2. Fig. 11**). Immunofluorescence of the nasal cavity also found *Bp* attached to cilia of the nasal turbinates and NALT (**Chapter 2. Fig. 12**). Isolation and characterization of the immune populations of the NALT could be of particular interest particularly in the induction of germinal center formation.

Previous research measuring cough in the rat model of pertussis used sound-activated

tape recorders (2). Here, we accurately measured cough and bronchial constriction using WBP, for twelve days post-challenge. Isolated coughs were detected at days 1 and 2 post-challenge, with peak cough occurring at day 8 post-challenge (**Chapter 2. Fig. 6**). Overall, rats challenged with D420 had an increase in the number of coughs over the entire infection compared to mock challenge rats (**Chapter 2. Fig. 6**). The onset and peak coughing that were detected was similar to the previous studies using IB *Bp* challenge in rats (3). Peak cough measured in baboons occurred at day 4 post challenge (15). Differences in bacterial challenge doses and size of the animal could potentially play a role in the timing of cough. WBP confirmed that Sprague-Dawley rats challenged with *Bp* have an impaired respiratory capacity (**Chapter 2. Fig. 7**). Furthermore, use of WBP could further improve the baboon model of pertussis and potentially identify other potential differences in cough and respiratory signatures. Histological analysis of the lung illustrated an increase in neutrophil infiltration early during the infection followed by infiltration of monocytes and macrophages around the bronchioles at day 9 post challenge (**Chapter 2. Fig. 8**). We also measured an increase in lung weight for rats IN challenged with *Bp* (**Chapter 2. Fig. 9**). Our data suggests that cellular inflammation could play a role in the observed respiratory distress and *Bp* induced cough.

The second aim of this study was to detail serological responses following *Bp* challenge. With the development of the next generation of pertussis vaccines, characterization of serological responses has become essential in understanding vaccine mediated immunity. To date, no studies investigating the serological responses in rats through IN *Bp* challenge have been conducted. In chapter 2, antibody titers to *Bp* virulence factors

were measured following challenge. D420 infected rats had a significant increase in anti-*Bp* IgM antibody titers, while no anti-*Bp* IgM antibodies were detected in Tohama 1 infected rats (**Chapter 2. Fig. 14**). Both Tohama 1 and D420 infected rats had an increase in serum anti-*Bp* IgG antibody titers at day 12 post challenge; however, Tohama 1 infected rats had a significant increase in anti-*Bp* IgG titers compared to D420 challenged rats (**Chapter 2. Fig. 14**). D420 harbors the *ptxP3* allele and research has shown that strains harboring *ptxP3* released more PT during growth *in vitro* compared to *ptxP1* strains (16, 17). This could potentially explain the differences in IgG antibody titers, as studies have shown that PT can suppress serological responses (18, 19). Minimal antibody titers were detected to FHA following challenge and no antibody titers against PT and ACT were detected in rats challenged with Tohama 1 and D420 (**Chapter 2. Fig. 11**). Our data goes to show that minimal to no antibody titers were detected against PT, FHA, PRN, and ACT following IN *Bp* challenge; however, by day 9-12 post-challenge detectable amounts of anti-*Bp* IgM and IgG were measured.

4.3 Investigation into the mechanism of *Bp* induced cough

With the redevelopment of the coughing rat model of pertussis, mechanism behind *Bp* induced cough can be explored. Rats and nonhuman primates are the only animal models capable of reproducing paroxysmal cough following *Bp* challenge. As the rat model of pertussis capitalizes on ease of use, animal availability, and minimal husbandry cost, this model could be critical in the evaluation of mechanisms responsible for *Bp* induced cough.

Limited studies have reported mechanisms that may play a role in *Bp* elicited cough. Our

data in both chapter 2 and chapter 3 showed that Sprague-Dawley rats cough upon IN *Bp* challenge. Onset of cough occurred at day 2 post-challenge, with peak cough occurring at days 8-10 post-challenge (**Chapter 2. Fig. 6**). One potential hypothesis is that PT is responsible for *Bp* induced cough. Previous research has shown that Sprague-Dawley rats IB challenged with a PT -/- *Bp* strain did not cough (7). Furthermore, avirulent phase 4 strain of *Bp*, which does not release PT, also did not induce cough as well (7). Another potential hypothesis that needs to be investigated is that *Bp* induced cough is activated through the increased production of bradykinin (20). Bradykinin is a pro-inflammatory hormone that can cause blood vessel dilation and smooth muscle contraction (21). Several studies have shown that bradykinin mediated activation of respiratory nerves have been shown to induce cough (22–25). Aerosol inoculation of bradykinin induced cough in guinea pigs (26). Both PT and ACT could potentially play a role in GPCRs regulation of bradykinin activation and the induction of cough (27). IN inoculation of PT and ACT *Bp* mutants and the measurement of cough would begin to answer these toxins' role in cough. Also, with the emergence of genetically diverging strains, it is currently unknown how genetic divergence effects disease severity, notably *Bp* induced cough. Vaccination and challenge studies utilizing current isolates will be critical in the assessment of vaccine efficacy in the next generation of pertussis vaccines.

4.4 *Bordetella bronchiseptica* and *Bordetella parapertussis* challenge experiments in rats

Limited research has been conducted in investigating both the pathogenesis and mechanism of cough involving *Bordetella bronchiseptica* and *Bordetella parapertussis* infected rats. *Bordetella parapertussis* is a human pathogen that can also cause

whooping cough disease, while *Bordetella bronchiseptica* is the causative agent of kennel cough in animals (28). All *Bordetella* species release ACT; however, *Bp* is the only *Bordetella* species to produce PT (11). Interestingly, IB challenge with *Bordetella parapertussis* in rats did not result in cough, which was measured via sound activated video cameras (7). More recently, identification of the anti-sigma factor BspR/BtrA of *Bordetella bronchiseptica* was shown to play a role in the regulation of bacterial factors involved in cough (8). IN inoculation of a *Bordetella bronchiseptica* BspR/BtrA mutant exhibited reduction of cough (8). Research has demonstrated that *bspR* negatively regulates the expression of the type III secretion system, and deletion in *bspR* results in both the increased expression and release of type III secretion system effector molecules (29–31). However, the factors released that elicit coughing manifestation have yet to be identified (8). *Bordetella bronchiseptica* and *Bordetella parapertussis* challenge studies utilizing the coughing rat model-- detailed in chapter 2-- would provide further insight into the pathogenesis and potentially identify cough factors of these two strains. Furthermore, the rat model could serve as a preclinical model to evaluate vaccine efficacy against *Bordetella bronchiseptica* and *Bordetella parapertussis*. While vaccines are available for kennel cough, many questions regarding protection against cough, vaccine mediated immunity, and detailed systemic and mucosal serological responses induced through immunization, remained unanswered (32).

4.5 Lessons learned from mucosal immunity

Chapter 3 utilizes the coughing rat model of pertussis to evaluate vaccine efficacy from mucosal immunization of DTaP against a clinically relevant strain of *Bp*, D420. We

investigated vaccine efficacy of both intranasal and oral immunization of DTaP compared to the positive vaccine controls, IM administration of DTP and DTaP. This is the first study to investigate mucosal immunization of DTaP in the coughing rat model of pertussis. Here we evaluated vaccine mediated protection against *Bp* elicited cough, bacterial burden, bronchiole constriction, pulmonary inflammation as well as the generated serological and cytokine responses (**Table 2**).

	Time	MVC	IM-wP	IM-aP	IN-aP	OG-aP
Serological response	1-week p.p					
	2-week p.p					
	1-week p.b		<i>Bp</i> -IgM/ <i>Bp</i> -IgG	<i>Bp</i> -IgG/PT-IgM/PT-IgG	<i>Bp</i> -IgG/PT-IgM/PT-IgG	
	Day 1		<i>Bp</i> -IgG	<i>Bp</i> -IgG/PT-IgG	<i>Bp</i> -IgG/PT-IgG	
Protection against cough	Day 9		<i>Bp</i> -IgG	<i>Bp</i> -IgG/PT-IgM/PT-IgG	<i>Bp</i> -IgG/PT-IgM/PT-IgG/ <i>Bp</i> -IgA	<i>Bp</i> -IgG/ <i>Bp</i> -IgA
	Days 1-9
Protection against bronchiole constriction	Days 1-9
Cytokine response	Day 1
	Day 9
Protection against bacterial burden in the respiratory tract	Day 1
	Day 9

Table 3. Summary of vaccine mediated immune responses following *Bp* challenge in the coughing rat model of pertussis.

To date, only two studies have reported evaluating vaccine mediated protection against cough from DTaP and DTP vaccines in the rat model of pertussis (6, 7). In 1998, Hall *et al* demonstrated that both a 3 component (PT, FHA, and PRN) and 5 component (PT, FHA, PRN, and Fim 2/3) aP vaccines could decrease cough count following *Bp* challenge (6). IP immunization of DTP was also capable of protecting against *Bp* induced cough (7). Cough was counted using audio tape recorders. Protection against *Bp* colonization of the respiratory tract was not reported following vaccination and challenge. In our current study, we utilized WBP to measure coughs and bronchiole restriction from *Bp* infection. Our data demonstrated that both IN-aP and OG-aP vaccinated rats were protected against *Bp* induced cough (**Chapter 3. Fig. 16**). In addition to protection against cough, mucosal vaccinated rats were also protected against bacterial burden in the nasal cavity, trachea, and lung (**Chapter 3. Fig. 21**). In addition, IN-aP vaccinated rats protected against bronchiole constriction (**Chapter 3. Fig. 16**). In both previous studies, a total of 1 human dose of aP and DTP was administered before *Bp* challenge (6, 7). In chapter 3, we administered 1/5th a human dose of DTP or DTaP to prime and boost. 1/5th human dose was selected in efforts to try and relatively compare size differences between rats and mice. 1/40th human dose is protective against *Bp* colonization in mice, and rats are approximately 10X the size of mice (33). Other human-rat-titrations need to be explored. Finding a minimally protective dose of current DTaP would allow for proper evaluation of the addition of new vaccine antigens to DTaP or potential new adjuvants. Use of a high vaccine dose would potentially “mask” the protective capabilities of new antigen or adjuvant, as the vaccine mediated immune response would already be saturated.

4.6 Mucosal immunity

Mucosal immunization has been of interest in the pertussis field. *Bp* is strictly a mucosal pathogen, and dissemination of *Bp* is rare (34, 35). This makes generating an immune response at the site of infection critical to protection against pertussis. Intranasal vaccination route has been investigated as a potential vaccination strategy to generate a protective mucosal immune response. Studies performed in our lab and others have investigated IN DTaP vaccination in mice (36–40). IN vaccination of DTaP induced both antigen specific systemic and mucosal antibody titers to *Bp* (36, 37). IN vaccination of live attenuated strain BPZE1 has also shown to be protective in mice, baboons, and humans in phase 2 clinical trials (41–44). The observed protection is through the induction of both serum and mucosal IgG and IgA antibodies respectively, and the generation of tissue resident memory T cells (Trms) (41, 45). Oral vaccination of heat inactivated *Bp* resulted in both systemic and mucosal antibody titers in newborns (46). Mice orally vaccinated with *Salmonella* strains harboring *Bp* antigens, such as PRN and FHA, have resulted in decrease in bacterial colonization in the lung, and resulted in antibody titers both systemically and in the gut (47, 48). In rats, both IN and OG vaccination induced mucosal and systemic *Bp* specific antibody titers (**Chapter 3. Fig. 15, 18, 19**). Antigen specific B cells were also observed following mucosal vaccination of DTaP in the bone marrow following *Bp* challenge (**Chapter 3. Fig. S15**). Our data supported that mucosal vaccination for pertussis can induce both systemic and mucosal antibody titers that correlate to the observed decrease in bacterial burden. Future studies investigating T cell responses in the coughing rat model of pertussis are needed to illustrate the full protective

immune response induced from IN and OG vaccination. As discussed in chapter 1, T cell immune responses are critical in observed protection from natural immunity and mucosal vaccination.

4.7 Improvements to DTaP/Tdap

Since the introduction of DTaP, we have observed cyclic increases in the number of pertussis cases (49, 50). Since the resurgence of pertussis, improvements to the current vaccine are critical. Potential strategies include correcting antigenic sin of current DTaP vaccinated populations, use of different adjuvants and investigation of new potential vaccine antigens.

A previous report has shown that with DTaP and DTP vaccination in humans, there is a significant decrease in antibody responses to ACT compared to convalescent individuals after *Bp* infection (51). It has been hypothesized that this decrease in antibody response could be due to original antigenic sin (51). Antigenic sin describes the tendency of the immune response to utilize the immunity generated upon vaccination or previous infection upon subsequent infection. This “locks” the immune response and prevents any additional protective immunity. With the emergence of genetically divergent strains, it is important to shift from a Th2 dominant response that is generated from DTaP vaccination. One potential approach to improve the diversity of the generated vaccine immune response is through a mucosal booster vaccine. IM-aP immunization will induce systemic immunity which we hypothesize will be re-focused to the mucosa by delivery of DTaP by intranasal and/or oral vaccination (**Figure 24**).

Figure 24. Vaccine generated immune responses from intramuscular and mucosal immunization of DTaP, and booster immunization of DTaP. (A) IM vaccination of DTaP induces T cell differentiation of naïve T cells to Th2 cells. Antigen-specific B cells produce IgG4 antibodies, which protect against PT toxin that is released systemically. The generated immune responses are not recruited to the mucosa, where *Bp* colonization occurs. (B) Mucosal immunization generates both a systemic and mucosal immune response. Following mucosal immunization induces T cell differentiation of naïve T cells to Th1 and Th17 cells, resulting in the production of IFN- γ and IL-17 at the mucosa. IgG1 antibodies are produced from antigen-specific B cells as well as secretory IgA antibodies. (C) IM prime vaccination of DTaP followed by mucosal vaccination could potentially rescue against antigenic sin from repeated IM vaccination of DTaP. Adapted from (Chasaide, 2020). Created with Biorender.com

Another potential solution to combat the pertussis problem, is the addition of new vaccine antigens. As described in chapter 1, *Bp* is evolving to no longer express particular antigens in DTaP, such as PRN (52). Strains lacking PT have also been reported (53). Virulence factor ACT is a potential candidate to be included in the next generation pertussis vaccine. ACT is a virulence factor that plays a role in *Bp* persistence by inhibiting innate immune responses to *Bp* killing (54). Convalescent patients who have cleared *Bp* infection have ACT antibodies in their serum, and in murine studies, immunization of ACT has been protective (55). RTX region of ACT has also been shown to induce a serological response to ACT and be protective (33). Vaccination with *Bordetella* resistance to killing A (BrkA) has been shown to reduce bacterial colonization in mice following *Bp* challenge (56). Vaccination with autotransporters Vag8 and SphB1 have been demonstrated to decrease bacterial burden in the lung; however, did not in the upper respiratory tract (57). Protection against hallmark symptom of pertussis, paroxysmal cough has yet to be evaluated utilizing these antigens. The rat model of pertussis could provide a preclinical model to evaluate the vaccine mediated efficacy with the addition of these antigens and their protective responses against *Bp* induced cough.

The adjuvant that is currently used in DTaP is alum. Alum works by promoting strong Th2 immune responses; however, studies have shown that the optimal long-lived immune responses generated from natural infection and DTP vaccination induces Th1/Th17 immune responses (58). Adjuvants capable of generating Th1/Th17 immune responses are being explored. Vaccination with TLR4 agonists such as monophosphoryl lipid-A (MPLA) in combination with DTaP decreased bacterial burden in the lung of *Bp* challenge

mice (59). TLR9 agonist, unmethylated single-stranded CpG, increased anti-PT IgG antibody titers upon combination and vaccination with DTaP (60). CpG also stimulated a protective Th1/Th17 immune response following vaccination that protected against *Bp* colonization in the lung following challenge (60). Stimulator of interferon genes (STING) agonists have also protected against bacterial burden in the lung (61). Future studies evaluating bacterial clearance from the upper respiratory tract and protecting against cough are needed to further differentiate the ideal adjuvant for the next generation of pertussis vaccines.

4.8 Improvement of oral vaccination against pertussis

In chapter 3, we illustrated that oral vaccination of DTaP protected against *Bp* induced cough and *Bp* colonization in the nasal cavity, trachea, and lung post-challenge in the coughing rat model of pertussis (**Chapter 3. Fig. 16, 21**). The generation of antibody titers to *Bp* were not detected until day 9 post-challenge. A proposed mechanism can be seen in chapter 3 (**Fig. S20**). A possible hypothesis for this observed delay in the generation of antibody titers is that limited availability of antigen travels to the GALT. Vaccines delivered orally rely on antigens traveling through the acidic environment of the stomach before arriving at the GALT (62). Potential strategies to correct this delay in vaccine mediated immune responses include an overall increase in the antigen concentration delivered, an increase in the number of vaccine doses, or finding delivery mechanism to increase antigen presentation to the GALT. Research has shown that targeting antigens to M cells has increased vaccine efficacy (63, 64). Use of viral-like particles and vesicles containing vaccine antigens have increased antigen uptake by M cells (63, 64). Addition

of cytokines IL-12, GM-CSF, and TLR agonists have been shown to increase the overall immune response generated from oral vaccination (65–67)

4.9 Experimental oral vaccines of potential

Oral vaccination has been briefly explored as a potential route of immunization against pertussis. Newborns orally vaccinated with wP induced the production of both systemic and mucosal antibodies to *Bp* (68). Oral vaccination of killed *Bp* also resulted in systemic and mucosal antibody titers in both the serum and saliva of newborns (69). Further, vaccinated newborns had a lower incidence of pertussis in the first year of life compared to unvaccinated newborns (69). Studies in mice have also shown that oral immunization of bacterial vectors harboring *Bp* virulence factors resulted systemic and mucosal serological responses (70). In chapter 3, OG vaccination of DTaP resulted in protection against bacterial burden and *Bp* induced cough; however, OG vaccination did not protect against bronchiole constriction and the generation of systemic antibody titers were not significantly increased compared to mock vaccinated challenge rats until day 9 post-challenge (**Chapter 3. Fig. 17-18**). Several approaches could be taken to further increase vaccine efficacy of an oral vaccine, such as encapsulation of antigens or antigen targeted delivery to M cells (71). Lipid-based vehicles, such as liposomes and bilosomes, are the most commonly used vehicles for encasing and delivery of vaccine antigens (71). Hydrophilic proteins and/or peptides can be encased inside the vesicle, while hydrophobic components can be included in the outer layers (72, 73). Encapsulation of antigens in lipid based vehicles further protect the antigens from the acidic pH of the gut and other proteolytic enzymes (71). Additionally, lectin coated liposomes were capable of targeting antigens to M cells, increasing the mucosal serological responses (72, 74). Nanoparticles

and polymeric microparticles can also be used to encapsulate and deliver antigens to the gut-associated lymphoid tissue (75–77). Oral immunization of particles encapsulated with the lectins *Ulex europaeus agglutinin-1* and *Aleuria aurantia* increased mucosal IgA antibody titers and induced a IL-2 and IFN- γ Th1 mediated immune response (74, 78–80). Taken together, these data show potential approaches that could be taken to further increase vaccine mediated immunity induced from an oral vaccine against pertussis.

4.10 In summary

The coughing rat model of pertussis can be used as a model to not only study *Bp* pathogenesis and potentially the mechanisms behind paroxysmal cough, but also be used as a preclinical model for the evaluation of the “next generation” of pertussis vaccines. Utilizing the coughing rat model of pertussis, we have demonstrated that both intranasal and oral vaccination of DTaP can be protective against *Bp* induced cough and overall, we have optimized the coughing rat model to study the next generation of pertussis vaccines

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