DETERMINANTS OF SITE-SPECIFIC PROTECTIVE IMMUNITY AGAINST *BORDETELLA SPP.*

by

Amanda D. Caulfield

(Under the Direction of Eric T. Harvill)

ABSTRACT

Bordetella pertussis, the bacterial pathogen responsible for "whooping cough" causes an estimated 24 million cases of vaccine-preventable illness per year, resulting in an excess of 170,000 deaths annually. Due to these and other factors, both the CDC and NIH have listed *Bp* as a priority (re)emerging pathogen of high concern. Importantly, the incidence of whooping cough in nations with high vaccine coverage is on the rise, attributed to asymptomatic transmission that is enabled by the imperfect and waning immunity of current acellular pertussis (aP) vaccines. The inability of aP vaccines to generate sterilizing immunity in the nasopharynx has sparked a critical need to better understand the immune response to *Bp* at this initial site of infection, however, like many well-adapted pathogens, *Bp* has evolved complex mechanisms to modulate the host immune response. In an effort to better understand Bp suppression of host immunity, we have focused on a sigma factor (BtrS) that we have shown is critical to the regulation of *Bordetella spp.* immunomodulatory factors. Importantly, we observe that infection with an isogenic mutant lacking this regulator generates more robust and complete sterilizing immunity in the nasal cavity and lungs of convalescent mice. We also observed significant increases in $CD4^+$ T cells in the nasal cavity and lungs, as well as elevated IgA titers in *Bp*Δ*btrS*-

convalescent mice following secondary challenge. The observation that the Bp mutant lacking this regulator (*Bp*Δ*btrS*) induced more effective sterilizing anamnestic immunity in the nasal cavity presents the opportunity to study and understand both how *Bp* suppresses immunity *and* how enhanced protective immunity can be generated.

INDEX WORDS: *Bordetella,* pertussis, mucosal immunity, immunomodulation, otitis media

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B.S., Quinnipiac University, 2016

M.S., Quinnipiac University, 2018

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2023

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DEDICATION

This dissertation is dedicated to my fellow scientists, dreamers, and disruptors who have weathered doubt from naysayers or have ever endured the refrain “You’ll never make it.”

May we continue to prove them wrong.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my mentor, Dr. Eric Harvill, for his continuous support of my professional development and scientific journey. I am inspired by your ability to challenge each of us to overcome our self-imposed boundaries while fostering the development and rapid growth of our unique strengths. Your intentional establishment of achievable, but ambitious goals consistently push our minds and abilities *just a little farther* at every opportunity. I would not be the scientist, or person I am today without your catered mentorship.

To my dearest friends and colleagues in the Harvill lab, Dr. Kalyan Dewan, Colleen Sedney, Jillian Masters, Maiya Callender, and Yang Su: this work would not have been possible without you. Each of you has imparted profound lessons on the significance of kindness, courage, ambition, perseverance, and rigor, respectively. Thank you for all the unforgettable memories, early mornings, late nights, and "dazzling" humor you brought to the lab each day. I can only hope to carry with me a small portion of that spark to my future labs and collaborative endeavors.

I would also like to extend a special thank you to prior members of the Harvill lab Dr. Longhuan (Harry) Ma, Dr. Bodo Linz, Dr. Hamidou Illasou, and Dr. Monica Gestal for their contributions and support in this research. I was incredibly fortunate to learn from each of you early in my training.

To my committee members Dr. Frederick Quinn, Dr. Balazs Rada, Dr. Kimberly Klonowski and Dr. Vincent Starai, I would like to extend my sincerest thanks for your guidance and support, particularly in the 11th hour. Thank you for always having my best interests and development in mind. I would also like to thank past members of my committee Dr. Nancy

Manley and Dr. Pejman Rohani for their earlier support of my development and research.

And finally, to my fiancé, Philip. There are no words that can capture my gratitude for your unwavering support throughout the incredible events and extraordinary circumstances that have marked this journey. I am eternally grateful for your late-night pep talks, endless proofreading, and your enthusiasm as the sole member of my "practice audience" for every poster, talk, and presentation. I cannot begin to thank you enough for all that you have done and endured as my partner these past five years.

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CHAPTER 1

INTRODUCTION

Adapted from: Amanda D. Caulfield, Maiya Callendar, Eric T. Harvill. 2023. Generating enhanced mucosal immunity against *Bordetella* pertussis: current challenges and new directions. Frontiers *in Immunology.* Reprinted here with permission of the publisher.

INTRODUCTION

Bordetella pertussis (*Bp*) is the highly transmissible etiologic agent of pertussis, a severe respiratory disease that causes particularly high morbidity and mortality in infants and young children (1–3). Patients with pertussis disease classically exhibit bouts of intense paroxysmal coughing which may be accompanied by post-tussive vomiting, bronchopneumonia, pulmonary hypertension, hypoxia, and in severe cases, brain damage or death (4). Problematically, whooping cough remains one of the least controlled vaccine-preventable diseases worldwide with several countries experiencing recent periods of resurgence (5). Increased incidence has been correlated with a transition from whole cell pertussis (wP) vaccines to acellular (aP) vaccines composed of 1-5 detoxified antigens including pertussis toxin, pertactin, filamentous hemagglutinin, and fimbriae 2&3 (6-9). While aP vaccines are highly successful in preventing severe lower respiratory (LR) disease in most cases, the TH-2 skewed immunity they confer wanes rapidly and fails to prevent sub-clinical upper respiratory (UR) mucosal infection and transmission to new hosts. This is of particular concern as many severe cases among newborns and immunocompromised persons are transmitted *via* caregivers or family members who were not displaying clinical signs of pertussis disease (8-9). Consequently, the population-level protective effects of herd immunity are disrupted by this failure to eliminate the ongoing circulation of *Bp.*

These observations are compounded by the rapid emergence and expansion of "vaccine escape mutant" strains deficient in pertactin, the only antigen included in aP vaccines capable of generating bactericidal antibodies against *Bp* (7, 8, 11-14). It is speculated that the targeted loss of this surface-bound antigen, but not other aP components, may grant a fitness advantage as it

evades anti-PRN bactericidal antibodies to facilitate persistence within the UR tract of aP vaccinated hosts (8). This is further supported by the observation that pertactin-deficient mutant strains are emerging from a diversity of *Bp* lineages, which have now risen to dominance in many countries with broad vaccine coverage. Together these observations have prompted new research initiatives aimed at generating sterilizing mucosal immunity in the nasal cavity to prevent initial colonization and limit the ongoing circulation of *Bp* within vaccinated populations.

Of note, the issue of sub-clinical infection and transmission amongst vaccinated individuals is not limited to *Bp*. Generating sterilizing immunity in the nasopharynx is a common challenge against many respiratory pathogens including pandemic SARS-CoV-2, *Mycobacterium tuberculosis*, influenza, respiratory syncytial virus, and others (15–18). Each of these pathogens can infect, persist, and often transmit from the nasopharynx; a mucosal site that continuously encounters foreign antigen. To do this, these pathogens must bypass specialized nasopharyngeal tissue equipped with mucus layers saturated with antimicrobial peptides, beating cilia, a sea of competitive microflora, innate responders such as neutrophils & macrophages, as well as inductor and effector sites replete with dendritic cells, T cells, B cells, and secretory IgA (19–22).

In humans, these complex defensive networks are largely evaded or modulated by *Bp* long enough to facilitate growth and transmission to new hosts (19, 23–30). Increased reports of asymptomatic or sub-clinical cases of Bp infection provides clinical evidence of this ability in populations with broad vaccine coverage which warrants new research initiatives and the development of targeted mucosal vaccine strategies. To develop vaccines capable of generating

sustained protective immunity in the UR mucosa, it is critical that we first understand how these pathogens modulate or evade the host immune response in the nasopharynx and similarly determine which mucosal immune factors are involved in eventually controlling and clearing infection.

Conventional vs. Novel Animal Models of Bp Infection

Problematically, our current understanding of *Bp*-induced immunity is largely derived from research conducted using a highly pathogenic model of infection in mice, which requires unnaturally high doses of (as many as a million) bacteria delivered into the lungs to generate severe pulmonary pathology and an artificially robust immune response. While this "pneumonic" model of infection has been valuable in the development of vaccines capable of preventing severe disease, it does not allow for the study of key aspects of naturally acquired *Bp* infection, including the initial stages of nasal cavity colonization, gradual spread within the respiratory tract, bacterial persistence, shedding, and transmission to new hosts (31). Perhaps most importantly, artificially high challenge doses produce severe lung pathology and a robust immune response capable of controlling the infection in just a few weeks. The resulting disease profile bears little resemblance to that of natural infection which, in contrast, arises from low numbers of bacteria in the nasal cavity and can persist for months.

To enable the study of these critical aspects of *Bp* infection, we have recently developed an innovative mouse model that replicates the conditions of naturally occurring infection with low-dose intranasal inoculation using relatively low number of bacteria (less than 1000 are sufficient) delivered in a 5 µl droplet deposited to the tip of the nose. This "nasopharyngeal" model simulates natural infection, allowing *Bp* to initially colonize the nasal cavity in low numbers (31). The resultant infection proceeds as it does in natural human infections, allowing

us to observe, measure, and evaluate realistic host-pathogen interactions in both naïve and vaccinated hosts. Importantly, these models can be complementary to one another, with the dual aim of evaluating mucosal immune responses against sub-clinical infection as well as pulmonary or systemic responses against the most severe forms of disease. These models will be extensively employed and contrasted Chapters 3 and 4.

Intramuscular vaccine-induced serum IgG protects against severe pulmonary disease but does not control Bp in the nasal cavity

While the cause of pertussis resurgence is multifactorial, it is well-appreciated that waning immunity and flawed aP vaccine-induced immunity are involved. Problematically, vaccine-induced protection and antibody titers roughly correlate, but neither is sustained indefinitely. Prior data from several human studies have demonstrated that peripheral blood anti-pertussis immunoglobulin G (IgG) titers decay rapidly within the first-year post-vaccination and continue to decline at slower rates thereafter (32–37). However, it remains unclear at what threshold antibody-titer decay results in increased susceptibility to pertussis disease. Although it is currently believed that high anti-pertussis IgG titers contribute to prevention of severe pulmonary disease, the mechanisms of protection remain poorly understood, and appear to vary in different regions of the respiratory tract (10, 19, 22, 39–42).

In contrast to protection observed in the LR tract, vaccine-induced IgG responses are less effective in mediating UR mucosal *Bp* infection and disease. This has been repeatedly observed from conventional murine models, as high antibody titers were detected in aP vaccinated animals with no reduction in CFU in the nasal cavity, resulting in prolonged carriage (19, 21). This was similarly reported in the aP-primed baboon model, which demonstrated persistent colonization

beyond 35-days post-challenge despite high serum IgG titers against all five aP antigens. Interestingly, the lack of IgG-mediated protection is not unique to aP vaccine-induced immunity and was also reported from wP-primed baboons with modest improvement in clearance to 19/21 days post-challenge (10). Together these observations suggest aP- and wP-primed IgG responses fail to rapidly control *Bp* from within the nasopharynx, contributing to extended periods of colonization and transmissibility.

Historically, anti-pertussis IgG titers have been the most accessible indicator for immunity in both clinical and research settings. However, the dual observation that both wP and aP vaccine-induced IgG titers correlate with reduced incidence of severe pulmonary disease but fail to prevent sub-clinical infection complicates our ability to use anti-pertussis IgG as a reliable indicator for UR protection. The disconnect between high IgG titers and persistent nasopharyngeal infection may be partially attributed to pertussis toxin-specific mechanisms that block serum-mediated clearance by delaying the recruitment of neutrophils, among other immunomodulatory abilities detailed in sections below (26). These data collectively suggest that serum IgG titers alone are not a sufficient indicator for protective immunity against *Bp*, and that other, localized mechanisms of protection should be evaluated.

It is well appreciated that convalescent immunity following a course of primary infection with *Bp* generates humoral and cellular immune responses that are critical in generating more effective protection against secondary infection in both the UR and LR tracts (32). It is probable that a portion of this protective immune response in the LR is mediated by IgG, and evidence supporting its role in reducing morbidity and mortality associated with clinical disease warrants continued research into anti-pertussis IgG responses. However, when evaluating IgG responses

to new candidate antigens for improved vaccines, it is critical that we differentiate between bactericidal antibodies directed against surface-bound factors which facilitate clearance, and neutralizing antibodies which protect against toxin-induced effects (11).

The controversial role of secretory IgA against Bp

Immunoglobulin A (IgA) is produced in response to natural infection, but not from current intramuscular aP or wP vaccines. However, conflicting reports regarding the role of IgA in infection with *Bp* have confounded our ability to correlate protection with this critical component of mucosal immunity. Anti-pertussis IgA appears to reduce adherence of *Bp* to ciliated epithelium *in vitro*, which could provide some level of protection against initial colonization and/or spread through the respiratory tract (40). In addition, human anti-pertussis IgA has been shown to effectively bind FcaRI on polymorphonuclear leukocytes to stimulate phagocytosis *in vitro*, indicating that IgA can be effective in facilitating bacterial clearance, despite IgA being traditionally considered a poor complement activator relative to IgM and IgG (22, 43). However, IgA-deficient mice were previously reported to be indistinguishable from wild type mice in controlling primary or secondary *Bp* infection, suggesting IgA plays at most a modest and/or redundant role in immunity to *Bp* (42).

The strongest data to argue a protective role for IgA against *Bp* was reported from experiments investigating a recent live-attenuated vaccine candidate, BPZE1. Designed to be administered intranasally, BPZE1 is reported to generate a robust secretory IgA response which appears to correlate with protection against challenge with *Bp*. Importantly, BPZE1-vaccinated $IgA^{-/-}$ mice had significantly higher colonization on days 7 and 21 post-challenge relative to vaccinated wildtype mice (44). These data indicate that removal of IgA resulted in significantly

impaired protection generated by a candidate intranasal vaccine. Importantly, baboons vaccinated with BPZE1 also generated protective immunity with detectable *Bp*-specific serum IgA, however UR mucosal sIgA was not directly evaluated (45).

The observation of sIgA production following intranasal delivery of commercial pertussis vaccines has prompted new investigations into the merits of intranasal vs. intramuscular vaccination approaches. In a recent study, mice intranasally vaccinated with aP vaccines generated similar levels of protection and serum IgG levels relative to intramuscular vaccination and significantly higher IgA titers in the nasal cavity by day 9 post *Bp* challenge (46, 47). A small human study also observed strong mucosal and systemic immune responses following intranasal wP vaccination (over 4 doses), which is a positive indicator for applicability of this concept in human immunization (48).

These examples suggest that intranasal vaccination and/or infection are able to generate protective sIgA against *Bp via* a site-specific mechanism. It is likely that intranasal but not intramuscular delivery facilitates uptake by nasal microfold (M) cells required for translocation into nasopharynx-associate lymphoid tissue (NALT) (48). This inductive tissue is critical for the generation of mucosal immunity against other pathogens, and NALT-targeted immunization has been shown to induce both mucosal and systemic immunity. Importantly, sIgA production is specifically stimulated in mucosal effector sites, where dendritic cell-activated T-cells specifically induce clonal expansion of IgA^+ B cells (Figure 1.1). The resulting IgA^+ B cells and plasmablasts induced by nasal immunization express CCR10 and **α**4**β**1-integrin, which together facilitate migration to respiratory tissues that express the corresponding receptors CCL28 and VCAM1, respectively (49–51).

In sum, conflicting reports of IgA-mediated protection against *Bp* warrant additional investigations into sIgA and intranasal vaccination strategies that deliver antigen directly to the site of infection (50). However, linking sIgA responses with sustained protection may present an additional challenge for future vaccine applications aimed at eliciting this response given the faster decline of IgA relative to IgG titers following diagnosis of clinical (symptomatic) pertussis (43, 52).

New vaccines must generate robust Th1/Th17 responses to enable clearance of Bordetella pertussis from the nasopharynx

The inability of aP vaccines to generate robust immunity in the nasal mucosa has sparked a critical need to better understand the immune response to *Bp* at this initial site of infection, in contrast to pneumonic responses which are relatively well-characterized. To date, cellular responses specific to *Bp* in the nasal mucosa have received the most attention, however, research initiatives investigating this topic are relatively recent, and much remains unknown.

T cell-mediated immunity has long been reported to play a central role in the control and clearance of *Bp* from the respiratory tract. Specifically, proinflammatory Th1 and Th17 responses have been shown to be critical for protection against colonization with *Bp* (51, 53). Problematically, aP vaccines primarily induce a Th2- skewed response, which is not sufficient to protect the UR tract from subsequent infection (32, 51, 53). Additionally, aP vaccines fail to generate tissue-resident memory T cells (T_{RM}), which are typically maintained in respiratory tissue and respond rapidly to secondary encounter with *Bp* (54).

Natural infection and intranasal vaccination generate T_{RM} that are protective in the upper respiratory tract (Figure 4.1). Specifically, IL-17 secreting $CD4^+$ T_{RM} induce rapid neutrophilic recruitment to the nasopharynx in response to secondary challenge, which has been shown to greatly contribute to the opsonization and control of *Bp* (53–55). Following secondary infection, a cascade of proinflammatory cytokines including IL-1b, TNFa, IL-17A (from T_{RM}), IL-17C (from epithelial cells), IL-6, and IFN g recruit additional phagocytes to the site of infection including dendritic cells and macrophages which ultimately clear the infection *via* opsonization-enhanced phagocytosis (Figure 1.2). Interestingly, $IL17^+$ T_{RM} have been shown to recruit a subset of Siglec F^+ neutrophils, which have been recently described to primarily reside within the nasal mucosa and exhibit an activated phenotype with increased NETosis (56, 57). Decades of immunology research has demonstrated that the absence or removal of a single component of the immune system can result in deleterious effects to protective immunity. Notably, intramuscular aP vaccines fail to stimulate or generate multiple components of both humoral (IgA) and cellular (IL-17^+ T_{RM}, Siglec F^+ neutrophils) immunity that are critical in the mucosal response to respiratory pathogens. However, the observation that intranasal administration of aP vaccines and candidate vaccines like BPZE1 are able to generate these responses suggest we have much to learn from continued investigations in this area (32).

Disrupting immunomodulation to inform vaccine-development

An additional barrier to our understanding of the response to *Bp* in the nasopharynx is immunomodulation. Like many well-adapted pathogens, *Bp* has evolved complex mechanisms to modulate the host immune system to its advantage in active infection and can reinfect convalescent hosts after immunity wanes. These abilities are well accepted to be multifactorial,

with several known virulence factors having unique impacts on diverse aspects of the host immune response to include the suppression of neutrophilic inflammation, decreased cytokine signaling from multiple cell types, complement evasion, and suppression of serum antibody responses (23–30). Together, these abilities create a key problem in the study of pertussis immunobiology, as (modulated) convalescent immunity is historically regarded as the "gold standard" against which vaccines are designed and compared.

Fortunately, there is now a growing appreciation that *B. pertussis* is a well-adapted human-specific pathogen that is both responsive to *and* interactive with the host environment. This is foremost evidenced by the classic regulatory Bvg-AS system, which controls the expression of most virulence factors by increasing expression when the bacterium senses that it is within the host environment. In this activated state (Bvg^+), an arsenal of regulatory systems and virulence factors are expressed that can enable it to evade or modulate host immunity. A few key factors are summarized below (58-59).

To highlight a few examples: Pertussis Toxin (PT) is an AB_5 that functions to disrupt G coupled protein receptor signaling pathways, thus disrupting cAMP-dependent activities like the activation of neutrophils and macrophages (60-63). In addition to delaying phagocyte recruitment *via* PT, *B. pertussis* also evades phagocyte-mediated killing by surviving within macrophages, an ability that is shared by many other bacteria with immunomodulatory abilities (64-65).

Adenylate cyclase toxin (ACT) specifically targets $Cd11b^+$ professional phagocytes, including dendritic cells, macrophages, and neutrophils by forming cation-selective pores to permeabilize the cell membrane. This pore formation initiates a massive efflux of potassium ions, while the N-terminal AC domain functions to bind to calmodulin, catalyzing the conversion

of ATP to cAMP. Together, these functions serve to effectively kill target cells *via* osmotic-lysis and necrosis. ACT also impairs dendritic cell maturation and cytokine secretion. These effects ultimately result in IL-1b production, polarizing naïve T cells, and expanding the overall Th17 response (64-66).

Pertactin, a putative autotransporter and highly immunogenic component of acellular vaccines, appears to modulate (decrease) the secretion of pro-inflammatory cytokines (TNFa, IL6, IL8, G-CSF) and may downregulate host genes associated with cell death (30). Information on these and other immunomodulatory virulence factors can be found in Chapter 2.

Future vaccine candidates, particularly live-attenuated, may benefit from removal of these immunomodulatory factors to boost immunity. Their formulations could be supplemented with detoxified versions of these proteins if their antigens are critical in *Bp* immunity, such as pertussis toxin. It is likely that immunomodulation is particularly important in mucosal immunity to *Bp*, as the tightly controlled inflammatory responses in the nasopharynx may be further reduced to enable persistence and extend the window of transmissibility (19, 67-69).

Disrupting Whole Immunomodulatory Networks to Enhance Immunity Against Bp

An alternative approach to deleting singular immunomodulatory factors may be to disrupt whole immunomodulatory networks or systems. This approach is supported by recent work published by our lab which described deletion of the gene encoding an extra cytoplasmic sigma factor, *btrS*, to disrupt a broad range of virulence and immunomodulatory factors (67, 70-71). Using murine models of infection, we reported the immunity generated by a *Bordetella bronchiseptica (Bb)* isogenic mutant lacking this gene to fully protect against challenge with both *Bb* and *Bp*. The sterilizing immunity induced by this mutant was substantially stronger than

that generated by wild type *Bb* or *Bp* in both the upper and lower respiratory tracts, with few indications of waning protection for the life of the mouse.

BtrS, and the anti-sigma factor BtrA, are appreciated to regulate nearly 300 genes in *Bb*, including the type III secretion system (T3SS), adenylate cyclase toxin, filamentous hemagglutinin, fimbria, pertactin, SphB1, autotransporters, and many others with unconfirmed functions (71-72). However, while the conservation of this gene between *Bb* and *Bp* is high (100% identity), BtrS appears to regulate a smaller set of genes in *Bp.*

In *Bp*, BtrS is directly activated by phosphorylated BvgA (Bvg^+, virulence activated state), however, *in vitro* investigations into *Bp* BtrS, the T3SS, and other associated genes have been complicated by transcriptional inactivation common in lab-adapted strains, which can be restored upon exposure to blood/serum, however, little research has been performed in the context of active BtrS/T3SS expression in *Bp* (71-73). As a result, role(s) of BtrS, the T3SS and the effector protein, BteA, are only partially characterized in *Bp,* though, the T3SS is appreciated to subvert IL-17, IFNg, and IgG2a responses *in vivo* (74).

Given the dual observations that BtrS contributes to a broad immunomodulatory network in *Bb* and what is currently known regarding T3SS-dependent immune responses, we decided to investigate the role of BtrS in *Bp* infection. We therefore generated *Bp*Δ*btrS* mutants in a well-characterized strains (Tohama I and D420). Our preliminary experiments using these strains in murine models of *Bp* infection similarly demonstrated enhanced convalescent immunity that facilitated complete clearance of the nasal cavity within 3 days post-inoculation (pi): more effective than that observed in wP or aP-vaccinated groups, and better than WT-convalescent immunity. These results led us to hypothesize that BtrS may contribute to immunomodulatory

activity in *B. pertussis,* the disruption of which, appears to result in enhanced anamnestic immunity in convalescent animals.

Understanding the mechanisms and host factors that generate sterilizing anamnestic immunity in the nasal cavity is a high priority initiative for *Bp*, as no universally accepted correlate of protection has been established. However, it is widely appreciated that complementary roles exist for both humoral and cell-mediated immunities (summarized above), with the field rapidly shifting focus to identify bacterial and host factors required to generate both responses of sufficient strength to prevent colonization in the upper respiratory tract. Here we propose three aims that use our powerful and innovative models of murine infection combined with assays and techniques in genetics, immunology, histopathology, and microbiology to probe the host-factors stimulated by a *Bp* mutant strain suspected to have severely reduced immunomodulatory capacity. Data generated from these experiments will have important implications in our understanding of the mechanisms of protective, long-lasting immunity in the nasal mucosae that will be vital in informing the development of improved vaccines capable of preventing sub-clinical colonization and transmission of *B. pertussis.*

AIM 1: Characterize the host protective immune response to *Bp*Δ*btrS* in the lungs and nasopharynx. Our preliminary data demonstrate that Δ*b*tr*S* mutants induce more protective immunity than wild type *Bb* or *Bp*. Here we will compare the specific immunological mechanisms involved. 56 days after primary infection with *Bp* or *Bp*Δ*btrS* we will challenge with wild type Bp and assess the recruitment of lymphoid cells as well as antibody titers.

AIM 2. Identify host targets of immunomodulation *via* BtrS-dependent activity. Conversely, we can utilize these mutant strains to determine the host targets for BtrS-mediated immunomodulation.

AIM 3: Determine the individual contributions of adaptive immune factors in sterilizing immunity in the nasal mucosa. To determine whether the sterilizing immunity generated by *Bp*Δ*btrS* is primarily cell- or antibody-mediated, we will adoptively transfer immune components into B and T cell deficient mice. *Bp*Δ*btrS*- or wildtype *Bp*- induced convalescent serum or T cells will be intraperitoneally injected or intranasally transferred into target mice prior to *Bp* challenge, respectively. The numbers of *Bp* in respiratory organs thereafter will be compared to that recovered from naïve, aP/wP vaccinated, *Bp*-convalescent, and *Bp*Δ*btrS*-convalescent mice.

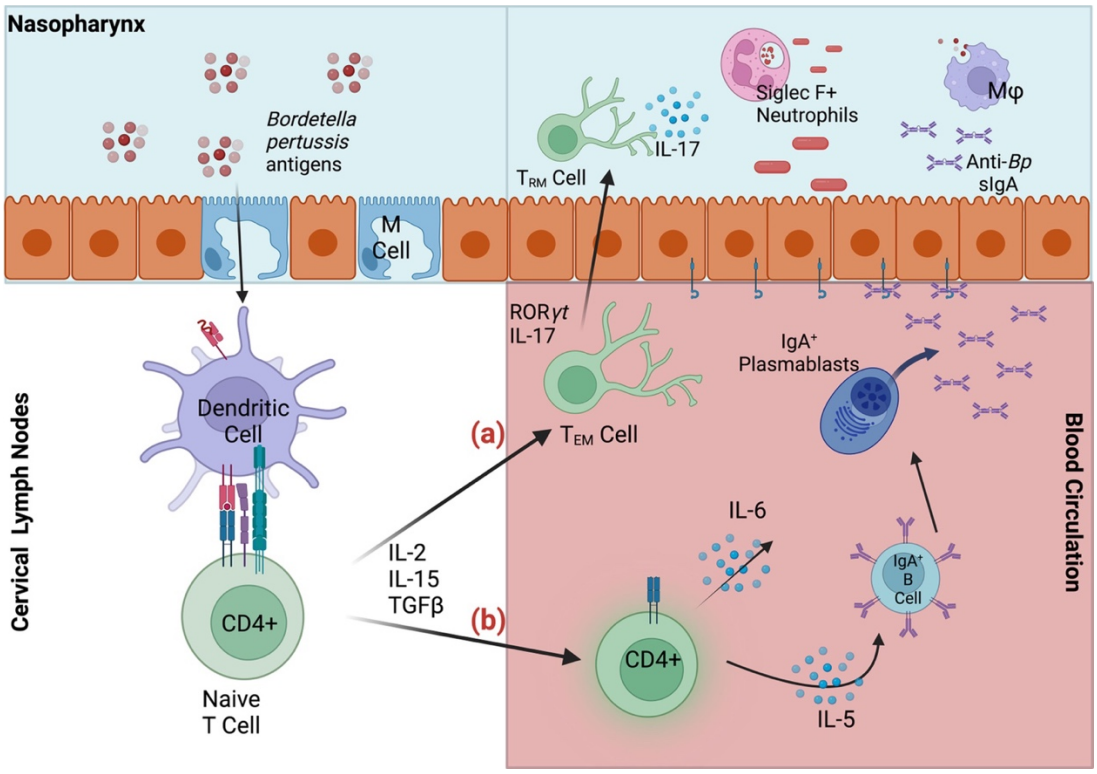


Figure 1.1: Summary of mucosal responses to *Bordetella pertussis* in nasopharynx-associated lymphoid tissue (NALT). *Bp* antigens are transported *via* microfold (M) cells into the NALT, where dendritic cells capture antigen, migrate to the lymph nodes, and stimulate the naïve $CD4^+$ T cells. (A) Activated effector $CD4^+$ T cells may then migrate to the nasal tissue, where they are maintained as T_{RM}. Upon secondary infection IL-17^+ T_{RM} expand and recruit Siglec F^+ neutrophils to the nasopharynx facilitating rapid clearance (B) Effector $CD4^+$ T cells also induce maturation of IgA-committed B cells, which migrate to the cervical lymph nodes, and to their effector site (nasal cavity). IgA^+ B cells and plasmablasts mature into plasma cells in response to cytokines IL-5 and IL-6. Dimeric IgA, secreted by differential plasma cells, bind polymeric Ig receptors, and are released into infected respiratory tissue as secretory IgA. Anti-*Bp* sIgA, along with macrophages (Mφ) and Siglec F^+ neutrophils contribute to the clearance of *Bp* in the upper respiratory tract.

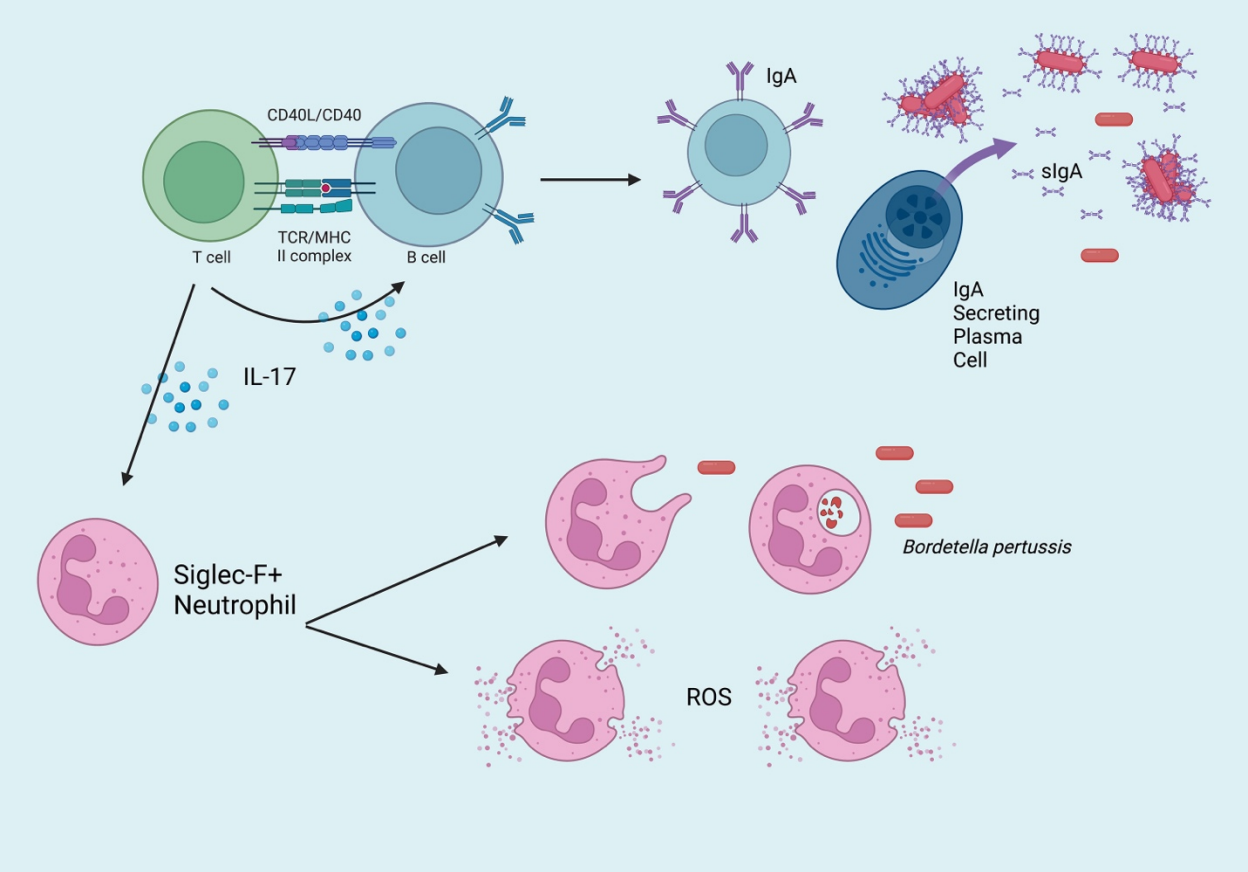


Figure 1.2: Overview of the critical mucosal-specific factors in cell-mediated immunity to *Bordetella pertussis* in the nasopharynx. Effector $CD4^+$ T cells activate IgA^+ B cells, which mature into dimeric IgA-secreting plasma cells. Once transported across the epithelium, sIgA binds *Bp*, which may bind and enable clearance by phagocytes. IL-17^+ T_{RM} recruit Siglec F^+ neutrophils to the site of infection, which eliminate *Bp via* phagocytosis or NETosis (ROS = reactive oxygen species).

REFERENCES

1. Roush SW, Murphy T. v., Basket MM, Iskander JK, Moran JS, Seward JF, et al. Historical comparisons of morbidity and mortality for vaccine-preventable diseases in the united states. J Am Med Assoc (2007). doi: 10.1001/jama.298.18.2155
2. Jackson DW, Rohani P. Perplexities of pertussis: Recent global epidemiological trends and their potential causes. Epidemiol Infect. (2014). doi: 10.1017/ S0950268812003093
3. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, et al. Global, regional and national causes of child mortality in 2008: a systematic analysis. *Lancet* (2010) 375:1969-87. doi: 10.1016/S0140-6736(10)60549-1
4. 4. Hiramatsu Y, Suzuki K, Nishida T, Onoda N, Satoh T, Akira S, et al. The mechanism of pertussis cough revealed by the mouse-coughing model. mBio (2022) 13 (2):e0319721. doi: 10.1128/mbio.03197-21
5. Frenkel LD. The global burden of vaccine-preventable infectious diseases in children less than 5 years of age: Implications for COVID-19 vaccination. how can we do better? *Allergy Asthma Proc* (2021) 42(5):378–85. doi: 10.2500/aap.2021.42.210065
6. Saso A, Kampmann B, Roetynck S. Vaccine-induced cellular immunity against *Bordetella pertussis*: Harnessing lessons from animal and human studies to improve design and testing of novel pertussis vaccines. *Vaccines* (2021) 9(8). doi: 10.3390/vaccines9080877
7. Martin SW, Pawloski L, Williams M, Weening K, Debolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: Evidence for a possible selective advantage. *Clin Infect Dis* (2015) 60(2):223–7. doi: 10.1093/cid/ciu788

8. Ma L, Caulfield A, Dewan KK, Harvill ET. Pertactin-deficient *Bordetella pertussis*, vaccine-driven evolution, and reemergence of pertussis. *Emerging Infect Dis* (2021) 27(6):1561–6. doi: 10.3201/eid2706.203850
9. Locht C. Pertussis: Where did we go wrong and what can we do about it? *J Infect.* (2016) 72. doi: 10.1016/j.jinf.2016.04.020
10. Warfel JM, Zimmerman LI, Merkel TJ. (2014). Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model, in: Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.1314688110
11. Lesne E, Cavell BE, Freire-Martin I, Persaud R, Alexander F, Taylor S, et al. Acellular pertussis vaccines induce anti-pertactin bactericidal antibodies which drives the emergence of pertactin-negative strains. *Front Microbiol* (2020) 11:2108. doi: 10.3389/fmicb.2020.02108
12. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the united states. *Clin Vaccine Immunol* (2014) 21(2):119–25. doi: 10.1128/CVI.00717-13
13. Barkoff AM, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Pertactin-deficient *Bordetella pertussis* isolates: Evidence of increased circulation in Europe, 1998 to 2015. *Eurosurveillance* (2019) 24(7):1700832. doi: 10.2807/1560-7917.ES.2019.24.7.1700832
14. Safarchi A, Octa*via* S, Luu LDW, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection

pressure in a mixed infection model. *Vaccine* (2015) 33(46):6277–81. doi: 10.1016/j.vaccine.2015.09.064

15. Petukhova G, Naikhin A, Chirkova T, Donina S, Korenkov D, Rudenko L. Comparative studies of local antibody and cellular immune responses to influenza infection and vaccination with live attenuated reassortant influenza vaccine (LAIV) utilizing a mouse nasal-associated lymphoid tissue (NALT) separation method. *Vaccine* (2009) 27(19):2580–7. doi: 10.1016/J.VACCINE.2009.02.035
16. Barnes MVC, Openshaw PJM, Thwaites RS. Mucosal immune responses to respiratory syncytial virus. *Cells* (2022) 11:1153. doi: 10.3390/cells11071153
17. Gallo O, Locatello LG, Mazzoni A, Novelli L, Annunziato F. The central role of the nasal microenvironment in the transmission, modulation, and clinical progression of SARS-CoV-2 infection. *Mucosal Immunol 2020 14:2* (2020) 14(2):305–16. doi: 10.1038/s41385-020-00359-2
18. Li W, Deng G, Li M, Liu X, Wang Y. Roles of mucosal immunity against *Mycobacterium tuberculosis* infection. *Tuberculosis Res Treat* (2012) 2012. doi: 10.1155/2012/791728
19. Soumana IH, Linz B, Dewan KK, Sarr D, Gestal MC, Howard LK, et al. Modeling immune evasion and vaccine limitations by targeted nasopharyngeal *Bordetella pertussis* inoculation in mice. *Emerging Infect Dis* (2021) 27(8):2107–16. doi: 10.3201/eid2708.203566
20. Mettelman RC, Allen EK, Thomas PG. Mucosal immune responses to infection and vaccination in the respiratory tract. *Immunity* (2022) 55(5):749–80. doi: 10.1016/j.immuni.2022.04.013

21. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* (2005) 11(Suppl 4):S45–53. doi: 10.1038/nm1213

22. Hellwig SMM, van Spriel AB, Schellekens JFP, Mooi FR, van de Winkel JGJ. Immunoglobulin a-mediated protection against *Bordetella pertussis* infection. *Infect. Immun* (2001) 69(8):4846–50. doi: 10.1128/IAI.69.8.4846-4850.2001

23. Carbonetti NH, Artamonova GV, Andreasen C, Dudley E, Mays RM, Worthington ZEV. Suppression of serum antibody responses by pertussis toxin after respiratory tract colonization by *Bordetella pertussis* and identification of an immunodominant lipoprotein. *Infect. Immun* (2004) 72(6):3350–8. doi: 10.1128/IAI.72.6.3350-3358.2004

24. Andreasen C, Carbonetti NH. Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to *Bordetella pertussis* respiratory tract infection in mice. *Infect. Immun* (2008) 76(11):5139–48. doi: 10.1128/IAI.00895-08

25. Henderson MW, Inatsuka CS, Sheets AJ, Williams CL, Benaron DJ, Donato GM, et al. Contribution of *Bordetella* filamentous hemagglutinin and adenylate cyclase toxin to suppression and evasion of interleukin-17-mediated inflammation. *Infect. Immun* (2012) 80(6):2061–75. doi: 10.1128/IAI.00148-12

26. Kirimanjeswara GS, Agosto LM, Kennett MJ, Bjornstad ON, Harvill ET. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis. J Clin Invest* (2005) 115(12):3594–6301. doi: 10.1172/JCI24609

27. Gestal MC, Whitesides LT, Harvill ET. Integrated signaling pathways mediate *Bordetella* immunomodulation, persistence, and transmission. *Trends Microbiol* (2019) 27(2):118–30. doi: 10.1016/j.tim.2018.09.010

28. Valdez HA, Oviedo JM, Gorgojo JP, Lamberti Y, Rodriguez ME. *Bordetella pertussis* modulates human macrophage defense gene expression. *Pathog Dis* (2016) 74(6). doi: 10.1093/femspd/ftw073

29. Gorgojo J, Scharrig E, Gómez RM, Harvill ET, Rodríguez ME. *Bordetella parapertussis* circumvents neutrophil extracellular bactericidal mechanisms. *PloS One* (2017) 12(1). doi: 10.1371/journal.pone.0169936

30. Hovingh ES, Mariman R, Solans L, Hijdra D, Hamstra HJ, Jongerius I, et al. *Bordetella pertussis* pertactin knock-out strains reveal immunomodulatory properties of this virulence factor article. *Emerging Microbes Infect.* (2018) 7(1):39. doi: 10.1038/s41426-018-0039-8

31. Soumana IH, Linz B, Dewan KK, Sarr D, Gestal MC, Howard LK, Caulfield AD, Rada B, Harvill ET. Modeling Immune Evasion and Vaccine Limitations by Targeted Nasopharyngeal *Bordetella pertussis* inoculation in mice. *Emerg Infect Dis*. 2021 Aug;27(8):2107-2116.

32. Solans L, Locht C. The role of mucosal immunity in pertussis. *Front Immunol* (2019) 10:3068(JAN). doi: 10.3389/fimmu.2018.03068

33. Le T, Cherry JD, Chang SJ, Knoll MD, Lee ML, Barenkamp S, et al. Immune responses and antibody decay after immunization of adolescents and adults with an acellular pertussis vaccine: The APERT study. *J Infect Dis* (2004) 190(3):535–44. doi: 10.1086/422035

34. Tomovici A, Barreto L, Zickler P, Meekison W, Noya F, Voloshen T, et al. Humoral immunity 10 years after booster immunization with an adolescent and adult formulation

combined tetanus, diphtheria, and 5-component acellular pertussis vaccine. *Vaccine* (2012) 30(16):2647–53. doi: 10.1016/j.vaccine.2012.02.013

35. Taranger J, Trollfors B, Lagergård T, Sundh V, Bryla DA, Schneerson R, et al. Correlation between pertussis toxin IgG antibodies in postvaccination sera and subsequent protection against pertussis. *J Infect Dis* (2000) 181(3):1010–3. doi: 10.1086/315318
36. van Twillert I, Han WGH, van Els CACM. Waning and aging of cellular immunity to *Bordetella pertussis. Pathog Dis* (2015) 73(8). doi: 10.1093/femspd/ftv071
37. Munoz FM, Bond NH, Maccato M, Pinell P, Hammill HA, Swamy GK, et al. Safety and immunogenicity of tetanus diphtheria and acellular pertussis (Tdap) immunization during pregnancy in mothers and infants: A randomized clinical trial. *JAMA* (2014) 311(17):1760–9. doi: 10.1001/jama.2014.3633
38. Raya BA, Srugo I, Kessel A, Peterman M, Vaknin A, Bamberger E. The decline of pertussis-specific antibodies after tetanus, diphtheria, and acellular pertussis immunization in late pregnancy. *J Infect Dis* (2015) 212(12):1869–73. doi: 10.1093/infdis/jiv324
39. Mills KHG, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell- mediated immunity in protection against *Bordetella pertussis*. *Infect. Immun* (1998) 66(2):594–602. doi: 10.1128/iai.66.2.594-602.1998
40. Tuomanen EI, Zapiain LA, Galvan P, Hewlett EL. Characterization of antibody inhibiting adherence of *Bordetella pertussis* to human respiratory epithelial cells. *J Clin Microbiol* (1984) 20(2):167–70. doi: 10.1128/jcm.20.2.167-170.1984

41. Kirimanjeswara GS, Mann PB, Harvill ET. Role of antibodies in immunity to *Bordetella* infections. *Infect. Immun* (2003) 71(4):1719–24. doi: 10.1128/IAI.71.4.1719-1724.2003

42. Wolfe DN, Kirimanjeswara GS, Goebel EM, Harvill ET. Comparative role of immunoglobulin a in protective immunity against the *Bordetella*e. *Infect. Immun* (2007) 75(9):4416–22. doi: 10.1128/IAI.00412f-07

43. van Twillert I, Marinović AAB, Kuipers B, van Gaans-Van Den Brink JAM, Sanders EAM, van Els CACM. Impact of age and vaccination history on long-term serological responses after symptomatic b. pertussis infection, a high dimensional data analysis. *Sci Rep* (2017) 7. doi: 10.1038/srep40328

44. Solans L, Debrie AS, Borkner L, Aguiló N, Thiriard A, Coutte L, et al. IL-17-dependent SIgA-mediated protection against nasal *Bordetella pertussis* infection by live attenuated BPZE1 vaccine. *Mucosal Immunol* (2018) 11(6):1753–62. doi: 10.1038/s41385-018-0073-9

45. Locht C, Papin JF, Lecher S, Debrie AS, Thalen M, Solovay K, et al. Live attenuated pertussis vaccine BPZE1 protects baboons against *Bordetella pertussis* disease and infection. *J Infect Dis* (2017) 216(1):117–24. doi: 10.1093/infdis/jix254

46. Boehm DT, Wolf MA, Hall JM, Wong TY, Sen-Kilic E, Basinger HD, et al. Intranasal acellular pertussis vaccine provides mucosal immunity and protects mice from *Bordetella pertussis. NPJ Vaccines* (2019) 4(1). doi: 10.1038/s41541-019-0136-2

47. Hall JM, Bitzer GJ, DeJong MA, Kang J, Wong TY, Wolf MA, et al. Mucosal immunization with DTaP confers protection against *Bordetella pertussis* infection and cough in sprague-dawley rats. *Infect. Immun* (2021) 89(12). doi: 10.1128/IAI.00346-21

48. Berstad AKH, Holst J, Frøholm LO, Haugen IL, Wedege E, Oftung F, et al. A nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human volunteers. *J Med Microbiol* (2000) 49(2):157–63. doi: 10.1099/0022-1317-49-2-157

49. Date Y, Ebisawa M, Fukuda S, Shima H, Obata Y, Takahashi D, et al. NALT m cells are important for immune induction for the common mucosal immune system. *Int Immunol* (2017) 29(10):471–8. doi: 10.1093/intimm/dxx064

50. Kiyono H, Fukuyama S. Nalt-versus peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* (2004) 4(9):699–710. doi: 10.1038/nri1439

51. Dubois V, Locht C. Mucosal immunization against pertussis: Lessons from the past and perspectives. *Front Immunol* (2021) 12:701285. doi: 10.3389/fimmu.2021.701285

52. Marcellini V, Mortari EP, Fedele G, Gesualdo F, Pandolfi E, Midulla F, et al. Protection against pertussis in humans correlates to elevated serum antibodies and memory b cells. *Front Immunol* (2017) 8:1158(SEP). doi: 10.3389/fimmu.2017.01158

53. Allen AC, Wilk MM, Misiak A, Borkner L, Murphy D, Mills KHG. Sustained protective immunity against *Bordetella pertussis* nasal colonization by intranasal immunization with a vaccine-adjuvant combination that induces IL-17-secreting T RM cells. *Mucosal Immunol* (2018) 11(6):1763–76. doi: 10.1038/s41385-018-0080-x

54. Wilk MM, Borkner L, Misiak A, Curham L, Allen AC, Mills KHG. Immunization with whole cell but not acellular pertussis vaccines primes CD4 TRM cells that sustain protective immunity against nasal colonization with *Bordetella pertussis*. *Emerging Microbes Infect.* (2019) 8(1):169–85. doi: 10.1080/22221751.2018.1564630

55. Wilk MM, Mills KHG. CD4 TRM cells following infection and immunization: Implications for more effective vaccine design. *Front Immunol* (2018) 9:1860(AUG). doi: 10.3389/fimmu.2018.01860

56. Borkner L, Curham LM, Wilk MM, Moran B, Mills KHG. IL-17 mediates protective immunity against nasal infection with *Bordetella pertussis* by mobilizing neutrophils, especially siglec-f+ neutrophils. *Mucosal Immunol* (2021) 14(5):1183–202. doi: 10.1038/s41385-021-00407-5

57. Matsui M, Nagakubo D, Satooka H, Hirata T. A novel siglec-f+ neutrophil subset in the mouse nasal mucosa exhibits an activated phenotype and is increased in an allergic rhinitis model. *Biochem Biophys Res Commun* (2020) 526(3):599–606. doi: 10.1016/j.bbrc.2020.03.122

58. Bone MA, Wilk AJ, Perault AI, Marlatt SA, Scheller EV, Anthouard R, et al. *Bordetella* PlrSR regulatory system controls BvgAS activity and virulence in the lower respiratory tract. Proc Natl Acad Sci USAm 2017. Available from: https://doi.org/ 10.1073/pnas.1609565114.

59. Moon K, Bonocora RP, Kim DD, Chen Q, Wade JT, Stibitz S, et al. The BvgAS regulon of *Bordetella pertussis*. MBio. 2017. Available from: https://doi.org/10.1128/mBio.01526-17.

60. Carbonetti NH. Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools. Future Microbiol 2010;5:45569.

61. Sedney CJ, Caulfield A, Dewan KK, Blas-Machado U, Callender M, Manley NR, et al. Novel murine model reveals an early role for pertussis toxin in disrupting neonatal

immunity to *Bordetella pertussis*. Front. Immunol. 2023;14:1125794. Available from: https://doi.org/10.3389/fimmu.2023.1125794.

62. Katada T, Tamura M, Ui M. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP- ribosylation of a membrane protein. Arch Biochem Biophys 1983. Available from: https://doi.org/10.1016/0003-9861(83)90212-6.

63. Rivera I, Linz B, Dewan KK, Ma L, Rice Christopher A, Kyle Dennis E, et al. Conservation of ancient genetic pathways for intracellular persistence among animal pathogenic *Bordetella*e. Front Microbiology 2019. Available from: https://doi.org/10.3389/ fmicb.2019.02839.

64. Gueirard P, Druilhe A, Pretolani M, Guiso N. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo*. Infect Immun 1998;66:171825.

65. Khelef N, Zychlinsky A, Guiso N. *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun 1993;61:406471.

66. Khelef N, Gounon P, Guiso N. Internalization of *Bordetella pertussis* adenylate cyclase-haemolysin into endocytic vesicles contri- butes to macrophage cytotoxicity. Cell Microbiol 2001;3:72130.

67. Gestal MC, Howard LK, Dewan K, Johnson HM, Barbier M, Bryant C, et al. Enhancement of immune response against *Bordetella* spp. by disrupting immunomodulation. *Sci Rep* (2019) 9(1). doi: 10.1038/s41598-019-56652-z

68. Holubova J, Stanek O, Juhasz A, Hamidou Soumana I, Makovicky P, Sebo P. The fim and FhaB adhesins play a crucial role in nasal cavity infection and *Bordetella pertussis*

transmission in a novel mouse catarrhal infection model. *PloS Pathog* (2022) 18(4):e1010402. doi: 10.1371/journal.ppat.1010402

69. Soumana IH, Dewan KK, Linz B, Rivera I, Ma L, Howard LK, et al. Modeling the catarrhal stage of *Bordetella pertussis* upper respiratory tract infections in mice. *DMM Dis Models Mech* (2022) 15(5). doi: 10.1242/dmm.049266
70. Gestal MC, Johnson HM, Harvill ET. Immunomodulation as a novel strategy for prevention and treatment of *Bordetella* spp. infections. *Front Immunol* (2019) 10:2869. doi: 10.3389/fimmu.2019.02869
71. Ahuja U, Shokeen B, Cheng N, Cho Y, Blum C, Coppola G, Miller JF. Differential regulation of type III secretion and virulence genes in *Bordetella pertussis* and *Bordetella bronchiseptica* by a secreted anti-σ factor. Proc Natl Acad Sci U S A. 2016 Mar 1;113(9):2341-8.
72. Jakub Držmíšek, Denisa Petráčková, Ana Dienstbier, Ivana Čurnová & Branislav Večere k (2023) T3SS chaperone of the CesT family is required for secretion of the anti-sigma factor BtrA in *Bordetella pertussis*, Emerging Microbes & Infections, 12:2,
73. Mattoo S, Yuk MH, Huang LL, Miller JF. Regulation of type III secretion in *Bordetella*. Mol Microbiol. 2004 May;52(4):1201-14.
74. Fennelly NK, Sisti F, Higgins SC, Ross PJ, van der Heide H, Mooi FR, Boyd A, Mills KH. *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. Infect Immun. 2008 Mar;76(3):1257-66.
75. Naninck T, Contreras V, Coutte L, Langlois S, Hébert-Ribon A, Pelletier M, et al. Intranasal inoculation with *Bordetella pertussis* confers protection without inducing

classical whooping cough in baboons. *Curr Res Microbial Sci* (2021) 2. doi: 10.1016/j.crmicr.2021.100072

76. Gestal MC, Blas-Machado U, Johnson HM, Rubin LN, Dewan KK, Bryant C, et al. Disrupting *Bordetella* immunosuppression reveals a role for eosinophils in coordinating the adaptive immune response in the respiratory tract. *Microorganisms* (2020) 8(11). doi: 10.3390/microorganisms8111808

CHAPTER 2

LITERATURE REVIEW

Bordetella pertussis

Bordetella pertussis is the highly transmissible etiologic agent of pertussis, a severe respiratory disease commonly referred to as "whooping cough" due to the characteristic inspiratory sound of gasps for air in between intense and prolonged bouts of paroxysmal coughing (1). Clinical manifestations of *B. pertussis* infections very greatly from subclinical to severe disease, which may be complicated by secondary bronchopneumonia, post-tussive vomiting, cerebral hypoxia, brain damage, and rarely, death (2,3). Despite broad vaccine coverage in many countries, the global pertussis disease burden remains high, causing an estimated 24-48 million infections and 100,000-300,000 deaths annually (4).

Historical accounts of whooping cough date back to Guillaume de Baiullou's 1578 report of an outbreak among children in Paris. However, additional historical descriptions of pertussis-like disease and modern genomic analysis suggest *B. pertussis*—and its close relatives—to have been infecting humans for thousands of years (5-8).

B. pertussis is quite fastidious *in vitro* and was not isolated and characterized until 1906 when Bordet and Gengou developed their namesake medium consisting of potato extract and blood (9). The difficulty in culturing and working with *B. pertussis in vitro* is considered a reflection of its fragility and sensitivity to the external environment as a human-restricted pathogen.

Despite the availability and broad use of effective vaccines, *B. pertussis* remains a pathogen of high concern on a global scale. This is largely due to higher pertussis- related child

mortality rates in low-income countries, combined with an observed resurgence of cases in several high-income countries despite broad vaccine coverage. While the cause(s) of pertussis resurgence are widely debated, several explanations have supporting data. These include the reduced efficacy of newer acellular vaccines, rapid waning of acellular vaccine-induced immunity, and increased reporting due to heightened awareness and sensitivity of new diagnostic tests (2-3,10-13). It is important to consider that these explanations are not mutually exclusive, and some combinations of these, and potentially other contributors, may be at work.

The *Bordetella genus*

The *Bordetella* genus was established by Moreno-Lopez in 1952, named in tribute to Jules Bordet (14). The genus belongs to the family *Alcaligenaceae,* and currently consists of 16 known species, most of which are respiratory pathogens of mammals (15). The *Bordetella* genus has historically been subdivided into two groups: classical and non-classical *Bordetella*e. Classical *Bordetella*e include *Bordetella bronchiseptica, Bordetella pertussis,* and *Bordetella parapertussis,* which are closely related species that were the first to be cultured and studied. The "non-classical" *Bordetella*e consist of 13 additional species that have been isolated from humans, other mammals, and environmental sources (5, 16).

- *Bordetella pertussis* is a human-restricted pathogen known for causing the most severe forms of pertussis, or whooping cough (1).
- *Bordetella parapertussis* causes whooping cough-like disease in humans but is believed to cause less-severe leukocytosis and illness than *B. pertussis*. A different lineage of *B. parapertussis* has been isolated from healthy and pneumonic sheep reservoirs, revealing distinct human and ovine clades of this species (1, 17-19).

- *Bordetella bronchiseptica* causes pathogenic respiratory infections in a broad range of mammalian hosts. It is the etiologic agent of kennel cough in dogs, atrophic rhinitis in pigs, snuffles in rabbits, as well as chronic and asymptomatic infections in other mammals. Despite frequent exposure to animal reservoirs, human clinical cases of *B. bronchiseptica* are rare, mostly seen in immunocompromised patients (20, 21). Genomic analyses indicate that both *B. pertussis* and *B. parapertussis* evolved from a *B. bronchiseptica*-like ancestor (5-7).
- *Bordetella holmesii* is a rising agent of interest, as it has been isolated with increasing frequency from patients with whooping cough-like illness. It can often be co-isolated from clinical infections with *B. pertussis*, and is associated with invasive infections resulting in bacteremia, meningitis, endocarditis, pneumonia, etc. Its reservoir is unknown (22-25).
- *Bordetella hinzii* is a commensal bacterium in birds with the ability to cause pathogenic infections. Human respiratory and digestive infections are opportunistic but are increasing in frequency (26,27).
- *Bordetella pseudohinzii* is a recently described species, originally isolated from laboratory mice. This pathogen readily infects both the respiratory tract and middle ears of host mice, eliciting chronic and progressive damage and hearing loss. It has recently been used as a model organism for the study of middle ear infections, as disease progression mimics that of chronic otitis media in humans (16,28,29).

- *Bordetella trematum* has been isolated from both human wounds and chronic ear infections. It is often associated with polymicrobial infections in diabetic and immunocompromised patients. Its reservoir is unknown (30-31).
- *Bordetella avium* is a common respiratory pathogen of poultry (bordetellosis) and it is known to result in high (80%-100%) mortality in young turkeys. Human infections are rare and opportunistic (32-34).
- *Bordetella petrii* is an environmental species initially isolated from an anaerobic bioreactor, making it the first known *Bordetella* species to be capable of growing in anaerobic conditions. It has more recently been isolated from the respiratory tract of immunocompromised human patients (1,35).

The evolution of *Bordetella pertussis*

Prior to the development of advanced molecular technologies, *B. pertussis, B. parapertussis,* and *B. bronchiseptica* were regarded as subspecies based on comparative 16S rDNA analysis. However, more recent genetic analyses including whole genome sequencing, genome content, and insertion sequence (IS) characterization, etc., have supported the view of these as three distinct species, with *B. pertussis* and *B. parapertussis* evolving independently from a *B. bronchiseptica*-like ancestor (7,36,37).

Additional phylogenetic and metagenomic analyses have recently suggested the entire *Bordetella* genus to have more ancient origins as soil and water microbes. This is not only evidenced by metagenomic analysis of environmental soil and water samples, but also by the conservation of genes associated with growth in soil and with complex interactions with common environmental amoeba (5, 38). Specifically, *B. bronchiseptica* has demonstrated a

remarkable ability to evade predation from *Dictyostelium discoidium* by avoiding digestion following phagocytosis by feeding stage trophozoites. The bacteria later "hijack" the complex amoebic replication/dissemination cycle by migrating with amoebae to the sori (fruiting bodies) where the bacteria grow in numbers and are highly efficiently disseminated along with amoebic spores (8,39).

Importantly, recent animal models confirm this interaction to permit transmission of *B. bronchiseptica* from environmental amoeba to mice, raising questions about whether the ability to survive in feeding trophozoites facilitated the evolution of environmental *Bordetella*e into mammalian pathogens (8,39,40). This evolutionary relationship is evidenced by the conservation of genes generally associated with intracellular survival. While *B. pertussis* appears to have lost the ability to use amoeba as an amplifying vector, it still uses a similar set of genes that mediate resistance to oxidative stress and acidic pH to survive within host phagocytes (40,41).

On the genomic level the evolution and speciation of *B. pertussis* from *B. bronchiseptica* is marked by the expansion of IS element copy numbers, specifically IS481, which facilitates frequent recombination events resulting in inversions, transversions, deletions, and inactivation of gene segments (42-43). Specifically, the *B. pertussis* genome contains over 250 IS elements; far more than any other *Bordetella* species (Table 2.1). The large copy number of IS elements has resulted in the construction of a unique genome, characterized by a mosaic-like arrangement of genes, numerous pseudogenes, and an overall smaller size (3.9Mbp, 3,576 genes) compared to that of *B. bronchiseptica* (5.338 Mbp, 4,579 genes) (8).

The apparent loss of over 1,000 genes that are retained by *B. bronchiseptica* is believed to be related to the closed life cycle of *B. pertussis* in humans, a pattern which is also mirrored in

the evolution of *B. parapertussis* and *B. holmesii* (7,44). Each of these species possesses a smaller genome and narrower host range when compared to their ancestral lineages (Figure 2.1).

Analysis of a large number of recent *B. pertussis* isolates suggests additional ongoing speciation, likely the result of population bottlenecks in which lineages with higher fitness in differing and changing human populations expanded from several "founding" clones (44). However, the evolution and adaptation of *B. pertussis* to human hosts and their changing dynamics of contact and vaccine-induced immunity continues. Of particular interest is the recent variation in the expression of bacterial factors included in current vaccines. The most notable example is the recent expansion of numerous pertactin-deficient strains, which is believed to contribute to the bacterium's ability to evade vaccine-induced host immunity (10). This topic will be covered in a later section.

Epidemiology & resurgence of pertussis disease

As a human-restricted pathogen, *B. pertussis* has no known reservoirs, but transmits with high efficiency between human hosts *via* respiratory droplets. Importantly, the CDC identifies the initial catarrhal phase of infection to be the most contagious, permitting transmission to vulnerable hosts before the onset of classical symptoms and diagnosis of symptomatic whooping cough (45). Circulation of *B. pertussis* among human populations is also aided by undiagnosed infections that may be asymptomatic or result in indistinct mild illness. Waning immunity is believed to play a role in enabling these sub-clinical infections, as they occur in individuals with both convalescent and vaccine-induced immunity (3,46-49).

In the pre-vaccine era, pertussis was recognized as one of the deadliest infectious illnesses, and one of the leading causes of disease-related infant mortality worldwide (3,50,51). Data from the United States in the years spanning 1940-1948 revealed pertussis infections to

result in the death of nearly three times the number of infants (under 12 months of age) than measles, mumps, varicella, rubella, scarlet fever, diphtheria, polio, and meningitis combined (46). Due to the severity of illness seen in neonates, and the majority of reported cases of pertussis occurring in children younger than 6 years old, whooping cough was primarily classified as a child-specific disease. However, it was later recognized that older children and adults may be more likely to present only mild symptoms of infection if they were previously infected or (later) immunized (50). This may have led to reduced estimates of pertussis disease among adults; however, pertussis remained a top priority for vaccine development initiatives primarily aimed at reducing infant mortality, as well as adolescent and adult morbidity.

Vaccine development initiatives led to fruition beginning in the 1920's with variations of whole bacteria, or bacterial suspensions with different formulae. Widespread vaccination campaigns using killed whole cell *B. pertussis* (wP) vaccines began in the 1950's and were later included in the WHO Expanded Programme on Immunization in 1974. Shortly after, pertussis incidence and mortality rates dropped to record lows in the United States and many other participating countries (52-53). Problematically, pertussis numbers in some countries resurged beginning in the 1980's and 1990's, despite wide wP vaccine coverage, and continued at higher levels than those observed in the 1970's (54).

The varying secondary effects of wP vaccines prompted some countries to abandon or reduce the frequency of wP vaccination. The incidence of pertussis in countries that ceased vaccination increased substantially, leading many to quickly resume previously established immunization programs and initiatives (55). In response to concerns regarding wP vaccine reactogenicity, acellular pertussis (aP) vaccines were developed and later distributed in the mid 1990's. Rather than containing whole bacteria (with a myriad of antigens), aP vaccines were

designed to contain only a selection of 3-5 inactivated and/or detoxified antigens. This led to a diverse array of global vaccine use, with many countries remaining on wP vaccines, while others adopted differing aP formulations with varying schedules (7).

Changes in transmission dynamics have been reported following the introduction of effective aP and aP pertussis vaccines, exchanging child-to-child transmission for adolescent/adult-to-infant transmission (56). Recent reports suggest this shift to be a result of waning immunity, as neither convalescent nor vaccine induced immunity are sustained permanently (57).

Following the implementation of aP vaccines, several high-income countries reported an alarming resurgence in the 2000's and 2010's. In many of these countries, incidence rates of adult and infant pertussis had reached levels not seen since the pre-vaccine era. An investigation by the WHO in 2014 confirmed higher infant mortality and pertussis-related hospitalizations in Australia, Portugal, the United Kingdom, Israel, and the United States, all of which had switched to aP vaccines approximately 10 years prior. However, this trend was not seen in all countries that used acellular vaccines, leading to a high degree of speculation as to the cause of resurgence. Despite continued efforts in prevention and control, *B. pertussis* persists as a pathogen of high concern on a global scale, causing an estimated 24-48 million infections and 100,000-300,000 deaths annually (4,58-60).

Clinical Disease Manifestations & Treatment of *Bordetella pertussis* Infection

Diagnosis & Disease Progression

The diagnosis of clinical pertussis is complicated by the wide spectrum of disease presentations which can range from asymptomatic infection to life-threatening respiratory illness

(60). Given the rarity of severe pertussis disease in countries with wide vaccine coverage, clinical suspicion of whooping cough is typically preceded by known patient exposure, presentation of characteristic (severe) symptoms, or knowledge of a localized outbreak. These factors often result in under-reporting of pertussis infection in cases where symptoms are mild or generally indistinguishable from other infectious respiratory illnesses (61-62).

"Classical pertussis" cases are typically characterized by:

- An incubation period of 7-10 days.
- An initial catarrhal phase lasting 1-2 weeks, which resembles a non-specific respiratory infection and possible mild cough without fever.
- A mild to severe paroxysmal phase lasting 3-8 weeks with paroxysmal cough, inspiratory "whoops," and possible post-tussive vomiting.
    - In young infants, classical pertussis may cause apnea and cyanosis without cough
- A convalescent phase where symptoms subside over several (2-12) weeks (60) (Figure 2.2).

Complications of severe pertussis are more common in neonates, and may include pneumonia, seizures, encephalopathy, cerebral hypoxia, pulmonary hypertension, secondary viral or bacterial infections, among others. Severe pertussis infections in infants may cause permanent brain damage from hypoxia. Death can occur in the most extreme cases, typically in infants under 6 months of age (4,63).

Adult (and adolescent) *B. pertussis* infections are less predictable and can range from asymptomatic/sub-clinical to severe "classical pertussis." Atypical pertussis disease presentation may also occur in regions where children are effectively vaccinated, resulting in milder disease

manifestations. “Mild” pertussis may present as a non-specific respiratory infection that begins in the nasal cavity, and progresses with limited standard cough (without inspiratory whoops) (3,47). Those with mild or atypical infections are still capable of transmitting *B. pertussis* to at-risk persons or groups.

Pertussis is a notifiable disease in most countries, and diagnosis can be made by assessment of disease presentation alone or in combination with laboratory tests including culture, polymerase chain reaction (PCR), and serology at appropriate time points in infection.

- Culture should be used to confirm infections during the catarrhal phase, and into the first 2 or 3 weeks of the paroxysmal phase of infection. It is currently the gold standard diagnostic method and is the most effective. Additionally, cultures may be used to test the susceptibility of specific isolates to antibiotic treatments. Unfortunately, culture requires at least four to five days of incubation once a sample has been received, and a trained microbiologist. While cultures can be used to diagnose infant infection shortly after the onset of cough, adults are more likely to delay consultations until culture is no longer sensitive.

- PCR is the most widely used diagnostic as it is more rapid and sensitive, especially at later stages in infection. Unfortunately, PCR generally detects IS481, a repeated signature which is not specific to *B. pertussis*, and may indicate infection with other *Bordetella* species. This limitation can be problematic when identifying the cause of an outbreak (58).

- Three weeks after the onset of paroxysmal coughing, serology may be used to diagnose infection. This method uses enzyme-linked immunosorbent assay (ELISA) to measure *B. pertussis*-specific anti-pertussis toxin (anti-PT) antibody titers. Infections may also be

diagnosed through the confirmation of secondary cases with culture or PCR due to the high transmissibility of *B. pertussis* (59-61).

Treatment

Appropriate antibiotic treatment can be effective in reducing transmissibility in patients with confirmed *B. pertussis* infection but may not reduce the time course of symptoms. This is often the case, as *B. pertussis* infections are not diagnosed until the disease has progressed with more severe and distinct symptoms.

Infants under 1-2 years of age may be hospitalized under supportive care, particularly if complications arise. Effective antibiotics include erythromycin, azithromycin, and clarithromycin as first-line therapeutics. Antibiotic resistance against these drugs is uncommon, though trimethoprim-sulfamethoxazole may be used as an alternative if patient allergy or bacterial resistance to other first-line drugs emerge (64).

Bacterial Characteristics & Microbiology of *Bordetella pertussis*

B. pertussis is a strictly aerobic gram-negative coccobacillus with a size range of 0.2-0.3μm X 0.5-0.8μm (9). It was previously regarded as non-motile, however, recent research has confirmed *B. pertussis* to both encode and express a functional flagellar apparatus under specific environmental conditions (see Bvg below) (65). It grows at an optimal temperature between 35-37°C, near human body temperature. *B. pertussis* primarily obtains energy by oxidizing amino acids and is incapable of fermenting carbohydrates (66).

B. pertussis is relatively difficult to culture but can produce colonies on Bordet Gengou blood agar plates when incubated at optimum temperatures over 4-5 days. Colonies will appear small, circular, grey/white in color, and are hemolytic. Blood and/or starch components are often

added to solid growth media to overcome sensitivity to detergents and unsaturated fatty acids (66). *B. pertussis* can also be cultured in liquid Stainer Sholte (SS) medium containing necessary amino acids (glutamic acid, proline, and cysteine), and growth factors (nicotinamide & glutathione). SS media is often supplemented with heptakis (2,6-O-dimethyl-β-cyclodextrin) to complex problematic fatty acids (66). *B. pertussis* can be difficult to identify as it exhibits only a few distinctive biochemical characteristics. It is hemolytic, catalase positive, oxidase positive, and demonstrates nitrate reductase activity (68). For these reasons, identification is typically confirmed by molecular methods.

Virulence Factors & Host-Pathogen Interactions

B. pertussis pathogenesis is mediated by several different toxins and virulence factors. Many of these factors are under the control of a "master" virulence regulon (discussed below) and may be displayed on the surface of the bacterium or are secreted into the host environment though the use of varied secretion systems. Each of these virulence factors differentially contributes to pathogenesis and the unique disease profile of *B. pertussis.*

Regulation of Virulence Expression: the Two-Component BvgAS System

The majority of virulence factors discussed in this section are expressed under the control of the two-component *Bordetella* virulence gene (BvgAS) system. The sensor, BvgS, is a 135-kDa sensor histidine kinase composed of a periplasmic domain, linker region, and phosphotransfer domain (69). BvgS mediates phosphorylation of the regulator, BvgA, and the concentration of phosphorylated BvgA affects the transcription of multiple factors involved in infection. The varying levels of phosphorylated BvgA correspond to two distinct phases, namely, Bvg^+ and Bvg^- (69-70).

When the bacterium detects that it is within the host environment (37ºC), the membrane-embedded sensor, BvgS, is active and phosphorylates cytoplasmic BvgA. The increase in phosphorylated BvgA directly activates expression of numerus virulence-associated genes (*vags*), including pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), filamentous hemagglutinin (FHA), pertactin (PRN), fimbriae (FIM), BrkA, and others. Under these conditions, the Bvg^+ phase is active, and the bacterium is considered virulent (69-71).

BvgR, a 32-kDa repressor is also expressed in the Bvg^+ phase, and serves to inhibit the expression of virulence-repressed genes (*vrg*). Expectedly, *vrg* genes are active in the absence of BvgR in the Bvg- phase, which is characterized by inactive BvgS in response to external signals including low temperature (below 25ºC) and the presence of certain salts. In this phase, BvgA is not phosphorylated, thus, *vag* genes are not actively transcribed (69).

The transcription and metabolic changes when *Bordetella spp.* are in the Bvg^- phase are typically associated with survival outside mammalian hosts and is not known to have a direct function in *B. pertussis* infection. However, infection with fixed Bvg^- phase bacterial mutants in rhesus macaques was shown to evade host clearance and persist in the nasal cavity (69). Additional *in vitro* studies have suggested the Bvg^- phase to play a role in transmission of *B. pertussis,* however, this has not yet been demonstrated in animal models (69).

It should be noted that the two-component BvgAS system is not the only one used by *B. pertussis.* Additional regulators include PlrSR (associated with infection and BvgAS response in the lower respiratory tract) and RisAS (associated with resistance to oxidative stress and persistence) (71-72).

Cytotoxicity-Associated Virulence Factors

Lipopolysaccharide

Lipopolysaccharide (LPS) is a large molecule produced by most gram-negative bacteria. As a main component of the outer membrane, LPS can serve many functions, and is typically comprised of 3 domains: (1) a hydrophobic moiety called lipid A, (2) an oligosaccharide core containing 2-keto-deoxyoctulosonic acid (Kdo), and (3) a sero-specific polysaccharide composed of repeating units termed the "O-antigen." Unlike *B. parapertussis* and *B. bronchiseptica*, *B. pertussis* LPS lacks the O-antigen domain due to absence of the encoding *wbm* locus. Due to the lack of O-antigen, *B. pertussis*-specific "LPS" is referred to as lipooligosaccharaide (LOS) (73-75).

O-antigen has been shown to play a critical role in serum complement resistance by preventing C3b deposition onto the bacterial surface in closely related species including *B. bronchiseptica* and *B. parapertussis.* Thus, the loss of O-antigen in *B. pertussis* renders the bacterium highly susceptible to host serum complement. To compensate for this, *B. pertussis* employs other machanisms that aid in complement evasion including those mediated by BrkA, Vag8, and FHA, among others (discussed below) (74, 76-77).

Pertussis Toxin

PT is a 105 kDa ADP-ribosylating toxin and is expressed in the Bvg+ phase (78-79). It is classified as an AB_5 toxin, with one catalytic subunit (A or S1), and a binding domain (B) comprised of subunits S2-5. The binding of PT to host cells is mediated *via* sialic acid-containing glycoproteins in the B domain (80). Entry into host cells is then facilitated by endocytosis, followed by retrograde transport from the Golgi complex to the endoplasmic reticulum. At this site, the A subunit is released into the cytosol following a conformational change in the B subunit (81).

Once in the cytosol, the A unit catalyzes ADP-ribosylation of membranous G proteins, locking these proteins in an inactive state (80-82). Specifically, PT hydrolyses cellular NAD and transfers the resultant ADP-ribose to a specific cysteine residue within the C terminal end of the target G protein (80). This activity inhibits G-protein coupled signaling pathways, affecting various cAMP-dependent activities in host cells including neutrophils and macrophages (83-86).

Recent data generated using a novel neonatal model of infection indicate PT to be critical in mediating neonatal- specific immune components to prevent host control and rapid clearance of *B. pertussis* from the lungs of mouse pups (87). Additional *in vivo* studies using adult animal models have found PT to reduce the secretion of pro-inflammatory cytokines and chemokines in the lung that aid in the recruitment of neutrophils and macrophages. In later stages, leukocytosis manifests as a characteristic effect of *B. pertussis* infection, along with PT-mediated suppression of antibody responses. Together, these effects allow *B. pertussis* to evade opsonization and phagocytosis (80,83-86).

In addition to G-protein-based immunomodulation, PT is also known to interact with pancreatic beta cells to increase insulin production. This contributes to a positive feedback loop wherein more insulin is produced continuously, leading the host into a hypoglycemic state. The resultant low blood sugar deprives host cells, including immune cells, of required glucose. But this has no direct effect on *B. pertussis* which is unable to metabolize glucose (88-89).

It should be noted that PT is expressed only by *B. pertussis* and is not known to be expressed by *B. parapertussis* or *B. bronchiseptica.* Therefore, the effects of PT on pathogenesis result in a disease that is unique to *B. pertussis* infection. While not responsible for the characteristic cough during disease, PT appears to work in concert with other virulence factors to effectively mediate colonization, proliferation, and transmission between hosts. Intriguingly, PT

had even more impressive effects on infection in immune hosts, blocking antibody-mediated clearance of *B. pertussis* (89). Serologic diagnostic techniques often exclusively measure antibody titers against PT, and due to its exclusivity to *B. pertussis*, it is an essential component of all aP vaccine formulations (90).

Adenylate Cyclase Toxin

Adenylate cyclase toxin (ACT), also known as adenylate cyclase hemolysin (AC-Hly) is a 177-kDa RTX toxin. It consists of 5 main regions: (1) an N terminal adenylate cyclase domain (catalytically active), (2) a central hydrophobic domain, (3) a hydrophobic joining region, (4) a C-terminal repeat toxin, and (5) a C-terminal signal sequence. This toxin is produced by *B. pertussis, B. bronchiseptica,* and *B. parapertussis* in the Bvg+ phase, its export being mediated by a type I secretion system composed of CyaB, D, and E (91-92).

ACT oligomers function to form small pores in target cells, which is the basis for its hemolytic activity. It specifically targets Cd11b-positive professional phagocytes, including dendritic cells, macrophages, and neutrophils by forming cation-selective pores to permeabilize the cell membrane (91). This pore formation initiates a massive influx of calcium and efflux of potassium ions.

ACT monomers mediate AC domain translocation into host cells, where it is internalized *via* CR3-mediated vesicular transport. Interestingly, ACT was the first bacterial adenylate cyclase to demonstrate the ability to enter eukaryotic cells (93). Once internalized, the N-terminal AC domain functions to bind to calmodulin, catalyzing the conversion of ATP to cAMP. Interestingly, its enzymatic activity can be increased by a factor of 1000X in the presence of host calmodulin (94). Together, these functions serve to effectively kill target cells, particularly macrophages, *via* osmotic-lysis and necrosis (84-85,95-96). ACT also impairs

dendritic cell maturation and cytokine secretion. These effects ultimately result in IL-1β production, polarization of naïve T cells, and overall Th17 response expansion (97-99).

Tracheal Cytotoxin

Tracheal Cytotoxin (TCT) is a 921 dalton muramyl peptide and is a product of the normal breakdown of peptidoglycan in gram negative bacteria as they grow. Secretion of this toxin is not under control of the BvgAS system. It is believed to function by working in synergy with LPS to increase the production of type II inducible nitric oxide (iNOS), which kills ciliated cells in the trachea and other areas of the respiratory tract (100).

Dermonecrotic Toxin

Dermonecrotic toxin (DNT) was the first toxin identified in the *Bordetella* genus, and is produced by all classical *Bordetella*e. It is known to cause necrotic lesions and may be responsible for tissue damage in various *Bordetella* infections, including those with *B. pertussis* (101). DNT is a 160-kDa AB toxin composed on an N-terminal binding domain and a C-terminal active domain. Once endocytosed in a dynamin-dependent manner, DNT primarily targets RhoGTPases in host cells. It catalyzes polyamination and deamidation of target GTPases resulting in a loss of hydrolyzing ability. This causes unregulated transduction of positive signals that cause a variety of effects in host cells (101-104).

DNT lacks a signal sequence and is not known to be actively secreted into the host environment. Rather, higher concentrations of active DNT can be found in cultures with killed bacteria, suggesting it may play a role in the cytoplasm during infection, and is largely released into the host environment following bacterial cell death (103).

Adhesion-Associated Virulence Factors

B. pertussis expresses numerous proteins to facilitate adhesion to host cells and tissues, for example, the mucosal epithelial cells within the human respiratory tract. The best studied adhesins are the fimbrial proteins (Fim2 and Fim3), filamentous hemagglutinin (FHA), and pertactin (PRN). Each of these proteins has additional functions that facilitate infection including immunomodulation.

Fimbriae

B. pertussis expresses fimbriae composed of long repeating chains of either Fim2 (22kDa) or Fim3 (22.5kDa) subunits, respectively, with an additional FimD located at the tip. The FimD subunit has been shown to exhibit adhesion abilities (71). In the periplasm, assembly is mediated by two proteins; the usher FimB and the chaperone FimC. Once complexed with the chaperone, release is mediated by the attachment of the final subunit, FimD (72). The expression of both Fim2 and Fim3 are regulated by the BvgAS regulon (105).

An additional level of regulation comes in the form of serotype variation. Both promoters of the Fim2 and Fim3 genes have a homopolymeric stretch of approximately 15 cytosines, which regulates both high and low expression depending on the number of cytosines present in the stretch. As a result, *B. pertussis* may express (and produce) varying levels of one, both, or neither fimbriae (105).

Once assembled and secreted to the bacterial surface, Fim2 functions to bind sulphated sugar-containing receptors (71). Both Fim2 and Fim3 have similar heparin binding sites for fibronectin (106). FimD, with its adhesin properties, mediates the binding of *B. pertussis* to monocytes. Together these effects work to amplify attachment of the bacterium *via* another

virulence factor, filamentous hemagglutinin (FHA) (107). Due to their immunogenicity and induction of agglutinin antibodies in human infection, Fim2 and Fim3 are often incorporated as antigens in 5-component acellular pertussis vaccines.

Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA) is a major adhesin expressed by *B. pertussis* and other *Bordetella*e (108). Its production involves cleavage from a 367-kDa precursor into a 220-kDa functional protein, which may be present at the surface or secreted into its host environment (109-110). FHA expression is controlled by the BvgAS regulon, and it is secreted by a two-partner secretion system (TPSS), with FHA as one component and FhaC as the other (111). Anchored in the bacterial membrane, the N-terminus of FhaC is believed to interact with FHA to facilitate secretion through its C terminal end. There, the FHA-precursor protein is processed by SphB1, and mature FHA is secreted into the host environment (112).

Mature FHA is composed of two regions of tandem 19-residue repeats (R1 and R2), followed by four binding domains: an Arg-Gly-Asp (RGD domain), a carbohydrate recognition domain (CRD), a sulphated carbohydrate/heparin binding domain (HBD), and a putative integrin binding domain (113). With these, FHA demonstrates numerous binding activities including lactose and galactose-containing glycolipids on ciliated epithelium, and galactose-containing glycol-conjugates on macrophages (114-116). Interestingly, these binding capabilities are synergistically dependent on the activity of ACT (117).

FHA is proposed to have immunomodulatory functions due to its ability to interact with both epithelial and immune cells. It can also bind to LRI/IAP on macrophages, which increases CR3-binding activities in the complement cascade and inhibits the synthesis of pro-inflammatory IL-12 (118-119). These effects are suspected to aid in the delay of adaptive immunity, thus

prolonging bacterial persistence in the respiratory tract. Due to its ability to induce a strong immune response, FHA is also included in most formulations of aP vaccines.

Pertactin

Pertactin, or PRN, is a 69-kDa autotransporter and putative adhesin that is highly conserved and expressed by *B. pertussis* and other classical *Bordetella*e. Of note, it was the first identified autotransporter in a *Bordetella* species, and its expression is controlled by the BvgAS regulon (120).

Unprocessed pertactin consists of four main regions: (1) an N-terminal signal sequence, (2) a passenger domain, (3) an RGD adhesin motif, and (4) a C terminal translocator domain (121-123). Pertactin is post-translationally modified *via* cleavage of the signal peptide following its transport into the periplasm. The translocator domain is cleaved following its passage through the outer membrane, where it facilitates the formation of a hydrophobic pore (122-123).

In respiratory infections, pertactin has been reported to facilitate attachment to eukaryotic cells and may be important for colonization of the respiratory tract (124). In the swine model, pertactin was also reported to play a role in resisting neutrophil mediated clearance of *B. bronchiseptica* (124). However, additional reports using varied animal models describe different or lesser effects of pertactin-deletion in experimental infection ranging from no change in respiratory colonization to increased fitness in vaccinated hosts (125-126). This is further complicated by *in vitro* descriptions of pertactin playing a role in the ability of select *Bordetella* species to invade and survive within mammalian cells (123,127).

Investigations into the immunomodulatory abilities of pertactin report an effect on the secretion of pro-inflammatory cytokines (TNFα, IL-6, IL-8, G-CSF), and regulation of genes

associated with host cell apoptosis. In doing so, pertactin may aid in the survival from certain facets of both innate and adaptive immunity (128-129).

Pertactin is highly immunogenic and is a component in most aP vaccines. Interestingly, anti-pertactin IgG titers have been found to decay less rapidly than those against other *Bordetella* antigens and may provide longer lasting protection (130). However, a recent surge in circulating pertactin-deficient *B. pertussis* strains has raised concern that vaccine-conferred protection against pertactin (and other antigens) may be driving the selection of bacterial clones lacking pertactin, and thereby evading this specific immunity. Pertactin-deficiency is by far the most common variant, rising to 85% of US isolates by 2018 (131). This topic will be discussed in more depth in Chapter 5 (Figure 2.3).

Synergy between *B. pertussis* virulence factors

The unique disease that manifests from infection with *B. pertussis* is partially a result of synergistic interplay between various virulence factors. Adhesins like FHA, PRN, and Fim2/3 allow the bacterium to attach to a variety of host cells and tissue types within the respiratory tract (107,114-117,121). Here it is important to note that these adhesins do not function alone, but in combination with each other and additional virulence factors including PT, ACT, TCT, and LPS. Tissue integrity and cell structure are disrupted (without regeneration) from the effects of TCT and LPS, which hinders cilia-mediated removal of bacteria from the respiratory tract. These once-ciliated tissues become coated in mucus, which increasingly accumulates as the disease progresses to the paroxysmal coughing phase (1,75,91).

Through virulence interactions and diverse binding to receptors within the host, several bacterial proteins facilitate the immunomodulation of both innate and adaptive host responses. Many members of the *Bordetella* genus, including *B. pertussis*, have demonstrated the ability to

survive and persist within macrophages, a key cell type involved in eliminating invading pathogens (39). However, these interactions extend beyond survival, with some virulence factors like PT, ACT, and PRN demonstrating the ability to affect the production of certain cytokines, limit immune cell recruitment, and directly kill immune cells (ACT) (80,83-86,97,128).

While the full extent of these interactions has yet to be elucidated, it remains clear that *B. pertussis* and its close relatives are highly evolved pathogens that are both responsive and interactive with their host environment in an effort to maximize replication, persistence, and transmission. These concepts are summarized in Figure 2.4.

B. pertussis Evolution & Variation in the Vaccine Era

The reemergence of pertussis disease in countries with high vaccine coverage has led to speculation and investigations into the factors that may be involved. Waning immunity as a feature in both convalescent and vaccine-conferred protection has been a long-standing concern, and much consideration has been given to selecting the most effective (and safest) vaccine formulations and schedules. However, despite these careful considerations, cases of pertussis in many countries are still on the rise (12).

Globally, *B. pertussis* has demonstrated evolutionary changes and adaptation to the modern vaccine era. Isolates from both vaccinated and unvaccinated patients have been analyzed to reveal new variations in virulence factor genes and expression. For example, polymorphisms in the promoter of pertussis toxin (*ptxP*) have resulted in the selection and expansion of a specific allele, *ptxP3*, which is associated with an increase in disease severity as it results in increased production of PT (approximately 1.6X the normal range) (132). The expansion of *B. pertussis* strains with the *ptxP3* allele is a fairly recent development and is believed to be driven by the selective pressure to survive in populations with broad vaccine-induced immunity. Today,

ptxP3 is one of the most dominant of at least nine alleles, lending support to this hypothesis (57, 132).

Perhaps more compelling is the recent emergence of pertactin-deficient strains, which now comprise up to 85% of circulating isolates in the United States (131). Interestingly, many of these pertactin-deficient strains possess different disruptions in the corresponding gene, indicating that the loss of pertactin may have been selected for multiple times in facilitating the expansion of several clones (133). While it has been postulated that this too is the result of vaccine-driven selective pressure, pertactin-deficiency has been reported in many, but not all countries that have adopted aP vaccines (133-137). There have also been rare reports of *B. pertussis* lacking PT, however, PT deletion and variation has been demonstrated to result in impaired fitness, offering an explanation as to why these mutations have not expanded (126). Pertactin, however, is more likely to share some redundant functions with the several other autotransporter and adhesins contained within the *B. pertussis* genome, lessening the severity of fitness cost associated with its disruption.

As the prevalence of circulating *B. pertussis* strains with variations in aP vaccine factors continues to rise, so too does the incidence of clinical pertussis in affected populations. This has led researchers to postulate that antigenic variation in *B. pertussis* may be contributing to the rise in the incidence of whooping cough, by enabling the pathogen to evade the immunity directed against the smaller number of antigens used in aP vaccines. However, most of the data presented to support this inference is largely correlative.

Current & Future Prevention of *B. pertussis* Infection

In recent years, much attention has been given to the resurgence of pertussis in select vaccinated populations, however, it should be noted that both wP and aP vaccines have been

largely effective in preventing severe disease. Without these vaccines, the burden of pertussis would have continued to be staggering on a global scale. However, in recognition of resurgence events, many researchers now seek to improve the efficacy of current vaccine formulations and schedules. To accomplish this, additional work is being performed to develop enhanced animal models of *B. pertussis* infection (138).

Conventional animal models were initially designed to replicate the most severe instances of disease in order to study pathogenesis and robust immune responses capable of clearing infection. Consequently, these models generally involve extremely high doses of *B. pertussis* deposited deep in the lungs of mice. This results in a severe disease profile that is unlike that of natural infections, which arises from small numbers in the nasal cavity and persists for long periods of time (138). Recent work describes the development of a new mouse model of natural and persistent *B. pertussis* infection (139). Importantly, this model has already proven to be valuable in the study of natural disease progression, including colonization, spread within the respiratory tract, and persistence. This promising model may be applied to future studies seeking to assay improved vaccine candidates capable of preventing not only severe disease, but transmissible sub-clinical and mild *B. pertussis* infections as well.

Experimental models investigating transmission of *B. pertussis* have historically been restricted to non-human primates, which are costly and require use of specialized facilities (11). Therefore, the development of more cost effective and accessible animal models of pertussis shedding and transmission have been a priority among specialized research groups. Use of these animal models is expected to elucidate which bacterial factors are involved in transmission, but may also aid in the development of vaccines capable of preventing or decreasing transmission between hosts.

Live (attenuated) and glyco-conjugate vaccine formulations are being explored as alternatives to current formulations, however, much focus has been directed at improving the efficacy of aP vaccines themselves (13, 138,140-141). This has the potential to include additional antigens and adjuvants capable of enhancing the immune response to *B. pertussis* or extending the duration of vaccine-induced immunity (142).

Recommended prevention methods include adherence to vaccine schedules, maternal immunization, "cocooning," and continued distribution of effective vaccines. In populations where vaccine coverage is high and readily accessible, the "cocooning" strategy, whereby all family members that may come into contact with a susceptible newborn infant are vaccinated, is largely aimed at reducing pertussis transmission, morbidity, and mortality in neonates (143-144). However, this method has not yet been proven to be highly effective in an experimental model and is difficult to assess in current practice.

The inaccessibility of healthcare for both diagnosis and treatment of pertussis in low-income countries is a significant barrier to coordinated efforts aimed at preventing pertussis outbreaks and rampant transmission. Additional challenges to prevention initiatives are rising in select high-income countries, where anti-vaccine movements are escalating, despite continued prevention-based education and vaccine distribution initiatives. For these reasons, and others mentioned above, pertussis disease remains a challenging obstacle for both research and public health initiatives.

TABLE 71.1 Genome size, insertion sequence element copy number, and host range for select *Bordetella* species.

Species	Genome size (Mbp)	No. genes	No. IS elements	Host range
B. bronchiseptica	5.338	4759	5	Mammals: dogs, cats, pigs, mice, rabbits, horses, sheep, humans, *etc*.
B. parapertussis	4.773	4169	31	Humans & sheep
B. pertussis	4.806	3576	252–272	Humans
B. hinzii	4.885	4456	0	Turkeys, immunocompromised humans
B. holmesii	3.699	3139	198–212	Humans
B. petrii	5.287	4718	53	Environment, humans

Table 2.1. Genome size, insertion sequence, element copy number, and host range for select *Bordetella* species.

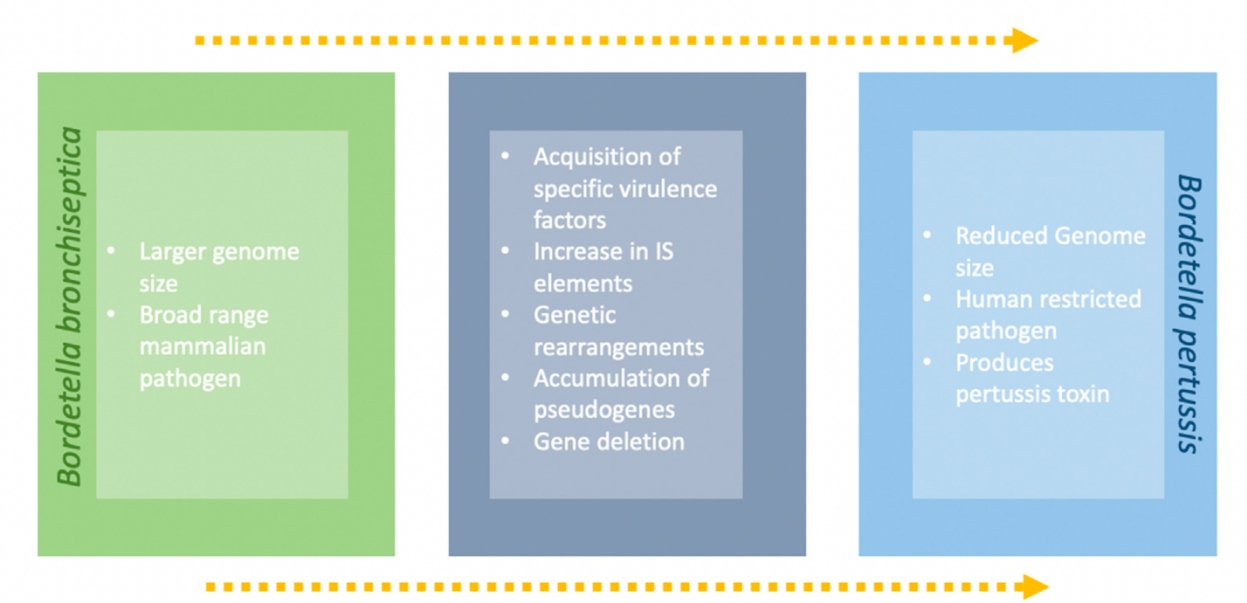


Figure 2.1 Summary of the evolution of *B. pertussis* from *B. bronchiseptica.*

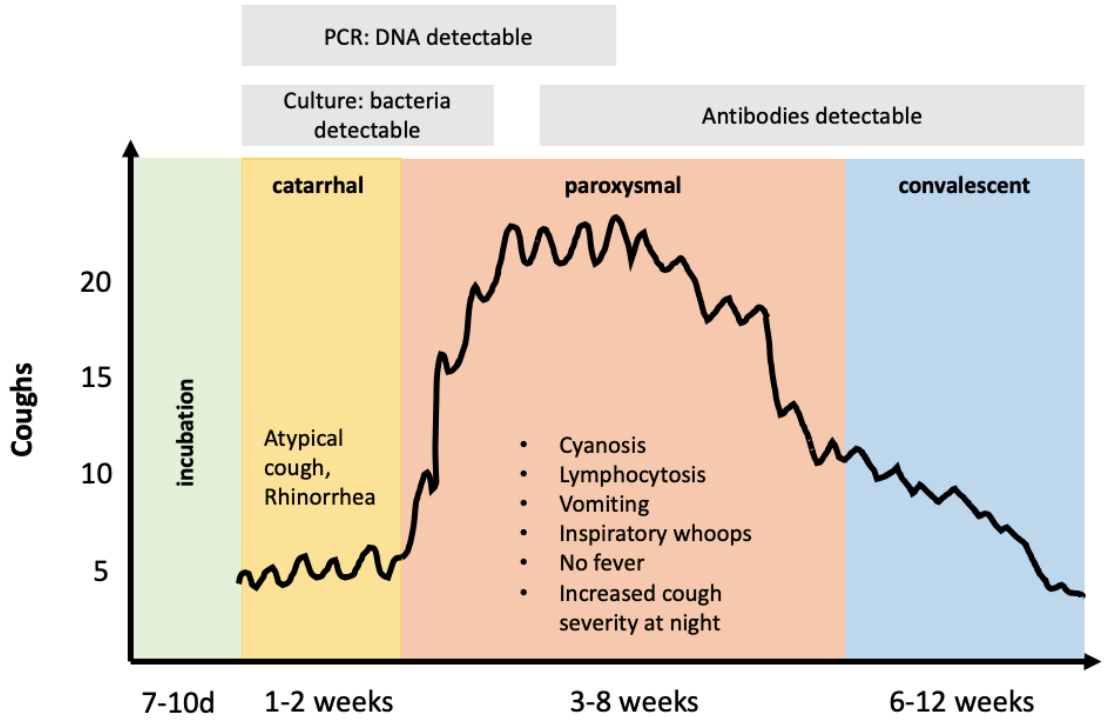


Figure 2.2: The different phases of whooping cough disease, cough severity, and appropriate diagnostic techniques.

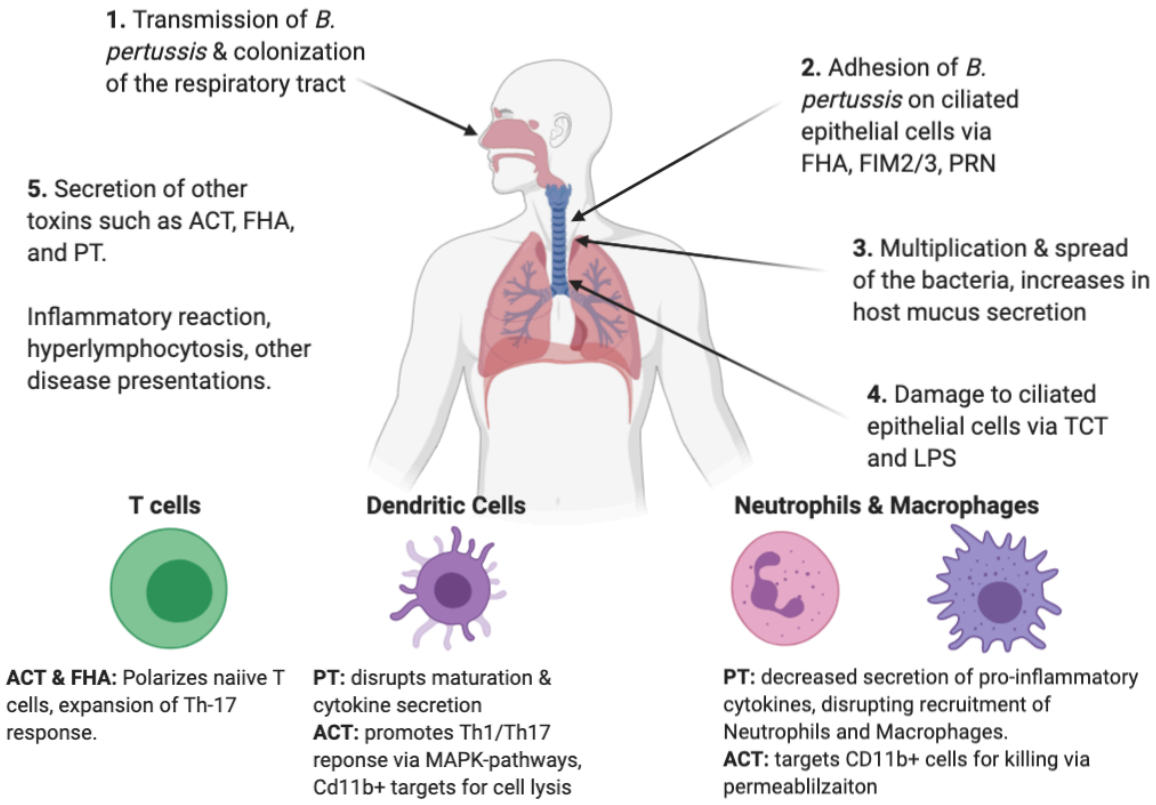


Figure 2.3: Summary of pertussis disease progression and toxin effects on different cell types.

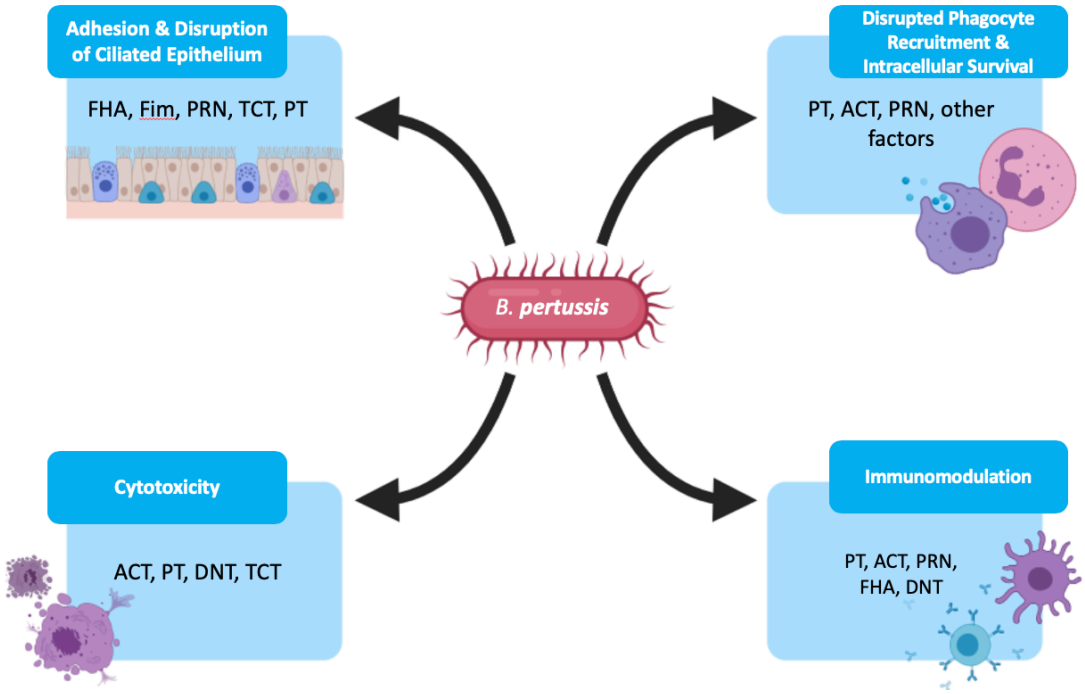


Figure 2.4: Brief overview of the synergistic and overlapping effects of virulence factors in pertussis disease.

REFERENCES

1. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev 2005. Available from: https://doi.org/10.1128/CMR.18.2.326- 382.2005.

2. Crowcroft N, Miller E, Crowcroft N, Miller E. Pertussis epidemiol- ogy. Pertussis 2018. Available from: https://doi.org/10.1093/oso/ 9780198811879.003.0004.

3. Kilgore PE, Salim AM, Zervos MJ, Schmitt HJ. Pertussis: microbi- ology, disease, treatment, and prevention. Clin Microbiol Rev 2016. Available from: https://doi.org/10.1128/CMR.00083-15.

4. Crowcroft NS, Pebody RG. Recent developments in pertussis. Lancet. 2006. Available from: https://doi.org/10.1016/S0140-6736 (06)68848-X.

5. Soumana IH, Linz B, Harvill ET. Environmental origin of the genus *Bordetella*. Front Microbiol 2017. Available from: https:// doi.org/10.3389/fmicb.2017.00028.

6. Mooi FR. *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. Infection, Genet Evolution 2010. Available from: https://doi.org/10.1016/j.meegid.2009.10.007.

7. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis and Bordetella bronchiseptica.* Nat Genet 2003;35:3240. Available from: https://doi.org/ 10.1038/ng1227.

7. Taylor-Mulneix DL, Soumana IH, Linz B, Harvill ET. Evolution of *Bordetella*e from environmental microbes to human respiratory pathogens: amoebae as a missing link. Front Cell Infect Microbiol 2017. Available from: https://doi.org/10.3389/fcimb.2017.00510.

8. Bordet J, Gengou O. Le Microbe de la Coqueluche. Les Annales de l'Institut Pasteur 1906.

9. Martin SW, Pawloski L, Williams M, Weening K, Debolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. Clin Infect Dis 2015. Available from: https://doi.org/10.1093/cid/ciu788.

10. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci USA 2014. Available from: https://doi.org/10.1073/pnas. 1314688110.

11. Jackson DW, Rohani P. Perplexities of pertussis: recent global epidemiological trends and their potential causes. Epidemiol Infect 2014. Available from: https://doi.org/10.1017/ S0950268812003093.

12. Bouchez V, Hegerle N, Strati F, Njamkepo E, Guiso N. New data on vaccine antigen deficient *Bordetella pertussis* isolates. Vaccines. 2015. Available from: https://doi.org/10.3390/ vaccines3030751.

13. Moreno-Lopez M. El genero *Bordetella*. Microbiol Esp 1952;5:17781.

14. Part C Brenner DJ, Krieg NR, Staley JT, editors. Family III. Alcaligenaceae. Bergey's Manual of Systematic Bacteriology, Vol. 2. Berlin: Springer; 2005. p. 64771.

15. Ivanov YV, Linz B, Register KB, Newman JD, Taylor DL, Boschert KR, et al. Identification and taxonomic characterization of *Bordetella* pseudohinzii sp. nov. isolated from laboratory-raised mice. Int J Syst Evolut Microbiol 2016. Available from: https:// doi.org/10.1099/ijsem.0.001540.

16. Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, Tatti KM, et al. Pathology and pathogenesis of fatal *Bordetella pertussis* infection in infants. Clin Infect Dis 2008. Available from: https://doi.org/10.1086/589753.

17. Eldering G, Kendrick P. Bacillus parapertussis: a species resem- bling both Bacillus pertussis and Bacillus bronchisepticus but identical with neither. J Bacteriol 1938;35:56172.

18. Porter JF, Connor K, Donachie W. Isolation and characterization of *Bordetella parapertussis*-like bacteria from ovine lungs. Microbiology. 1994. Available from: https://doi.org/10.1099/ 13500872-140-2-255.

19. Woolfrey BF, Moody JA. Human infections associated with *Bordetella bronchiseptica.* Clin Microbiol Rev 1991;4:24355.

20. Gueirard P, Weber C, Le Coustumier A, Guiso N. Human *Bordetella bronchiseptica* infection related to contact with infected animals: persistence of bacteria in host. J Clin Microbiol 1995;33:20026.

21. ittet LF, Emonet S, Schrenzel J, Siegrist CA, Posfay-Barbe KM. *Bordetella* holmesii: an under-recognised *Bordetella* species. Lancet Infect Dis 2014. Available from: https://doi.org/10.1016/ S1473-3099(14)70021-0.

22. Mir-Cros A, Codina G, Mart´ın-Go´ mez MT, Fa` brega A, Mart´ınez X, Jane´ M, et al. Emergence of *Bordetella* holmesii as a causative agent of whooping cough, Barcelona, Spain. Emerg Infect Dis 2017. Available from:https://doi.org/10.3201/eid2311.170960.

23. Rodgers L, Martin SW, Cohn A, Budd J, Marcon M, Terranella A, et al. Epidemiologic and laboratory features of a large outbreak of pertussis-like illnesses associated with cocirculating *Bordetella* holmesii and *Bordetella* pertussis Ohio, 2010-2011. Clin Infect Dis 2013;56:32231.

24. Weyant RS, Hollis DG, Weaver RE, Amin MFM, Steigerwalt AG, O'Connor SP, et al. *Bordetella* holmesii sp. nov., a new gram-negative species associated with septicemia. J Clin Microbiol 1995;33:17.

25. Fabre A, Dupin C, Be´ne´zit F, Goret J, Piau C, Jouneau S, et al. Opportunistic pulmonary *Bordetella* hinzii infection after a*via*n exposure. Emerg Infect Dis 2015. Available from: https://doi.org/ 10.3201/eid2112.150400.

26. Cookson BT, Vandamme P, Carlson LC, Larson AM, Sheffield JV, Kersters K, et al. Bacteremia caused by a novel *Bordetella* species, "B. hinzii.". J Clin Microbiol 1994;32:256971.

27. Perniss A, Schmidt N, Gurtner C, et al. *Bordetella* pseudohinzii targets cilia and impairs tracheal cilia-driven transport in naturally acquired infection in mice. Sci Rep 2018. Available from: https:// doi.org/10.1038/s41598-018-23830-4.

28. Dewan KK, Taylor-Mulneix DL, Campos LL, et al. A model of chronic, transmissible Otitis Media in mice. PLoS Pathog 2019. Available from: https://doi.org/10.1371/journal.ppat.1007696.

29. Vandamme P, Heyndricks M, Vancanneyt M, Hoste B, De Vos P, Falsen E, et al. *Bordetella* trematum sp. nov., isolated from wounds and ear infections in humans, and reassessment of Alcaligenes denitrificans Ru¨ger and Tan 1983. Int J Syst Bact 1996;46:84958.

30. Castro TRY, Martins RCR, Dal Forno NLF, Santana L, Rossi F, Schwarzbold AV, et al. *Bordetella* trematum infection: case report and review of previous cases. BMC Infect Dis 2019. Available from: https://doi.org/10.1186/s12879-019-4046-8.

31. Kersters K, Hinz K-H, Hertle A, Segers P, Lievens A, Siegmann O, et al. *Bordetella* avium sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int J Syst Bact 1984;34:5670.

32. Temple LM, Weiss AA, Walker KE, Barnes HJ, Christensen VL, Miyamoto DM, et al. *Bordetella* avium virulence measured *in vivo* and in vitro. Infect Immun 1998.

33. Beach NM, Thompson S, Mutnick R, Brown L, Kettig G, Puffenbarger R, et al. *Bordetella* avium antibiotic resistance, novel enrichment culture, and antigenic characterization. Veter Microbiol 2012. Available from: https://doi.org/10.1016/j. vetmic.2012.05.026.

34. Weigand MR, Peng Y, Batra D, Burroughs M, Davis JK, Knipe K, et al. Conserved patterns of symmetric inversion in the genome evolution of *Bordetella* respiratory pathogens. mSystems 2019;4: e0070219. Available from: https://doi.org/10.1128/mSystems. 00702-19.

35. Mooi FR, He Q, Guiso N. Phylogeny, evolution, and epidemiology of *Bordetella*e. In: Locht C, editor. *Bordetella*: Molecular Microbiology. Wymondham, UK: Horizon Bioscience; 2007. p. 1745.

36. Diavatopoulos DA, Cummings CA, Schouls LM, Brinig MM, Relman DA, Mooi FR. *Bordetella* pertussis, the causative agent of whooping cough, evolved from a distinct, human-associated line- age of *B. bronchiseptica*. PLoS Pathog 2005. Available from: https://doi.org/10.1371/journal.ppat.0010045.

37. Linz B, Ma L, Rivera I, Harvill ET. Genotypic and phenotypic adaptation of pathogens: Lesson from the genus *Bordetella*. Curr OpInfect Dis 2019. Available from: https://doi.org/10.1097/ QCO.0000000000000549.

38. Taylor-Mulneix DL, Bendor L, Linz B, Rivera I, Ryman VE, Dewan KK, et al. *Bordetella bronchiseptica* exploits the complex life cycle of Dictyostelium discoideum as an amplifying transmission vector. PLoS Biol 2017. Available from: https://doi.org/ 10.1371/journal.pbio.2000420.

39. Strassmann JE, Shu L. Ancient bacteriaamoeba relationships and pathogenic animal bacteria. PLoS Biol 2017. Available from: https://doi.org/10.1371/journal.pbio.2002460.

40. Rivera I, Linz B, Dewan KK, Ma L, Rice Christopher A, Kyle Dennis E, et al. Conservation of ancient genetic pathways for intracellular persistence among animal pathogenic *Bordetella*e. Front Microbiology 2019. Available from: https://doi.org/10.3389/ fmicb.2019.02839.

41. Moran NA, Plague GR. Genomic changes following host restric- tion in bacteria. Curr OpGenet Dev 2004. Available from: https:// doi.org/10.1016/j.gde.2004.09.003.

42. Weigand MR, Peng Y, Loparev V, Batra D, Bowden KE, Burroughs M, et al. The history of *Bordetella pertussis* genome evolution includes structural rearrangement. J Bacteriol 2017. Available from: https://doi.org/10.1128/JB.00806-16.

43. Linz B, Ivanov YV, Preston A, Brinkac L, Parkhill J, Kim M, et al. Acquisition and loss of virulence-associated factors during genome evolution and speciation in three clades of *Bordetella* spe- cies. BMC Genomics 2016. Available from: https://doi.org/ 10.1186/s12864-016-3112-5.

44. Liang JL, Tiwari T, Moro P, Messonnier NE, Reingold A, Sawyer M, et al. Prevention of pertussis, tetanus, and diphtheria with vac- cines in the United States: Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2018. Available from: https://doi.org/10.15585/mmwr.rr6702a1.

45. Kendrick PL. Can whooping cough be eradicated? J Investig Dermatol 2012. Available from: https://doi.org/10.1093/infdis/ 132.6.707.

46. Senzilet LD, Halperin SA, Spika JS, Alagaratnam M, Morris A, Smith B. Pertussis is a frequent cause of prolonged cough illness in adults and adolescents. Clin Infect Dis 2001. Available from: https://doi.org/10.1086/320754.

47. Philipson K, Goodyear-Smith F, Grant CC, Chong A, Turner N, Stewart J. When is acute persistent cough in school-age children and adults whooping cough? A prospective case series study. Br J Gen Pract 2013. Available from: https://doi.org/10.3399/ bjgp13X670705.

48. Yesmin K, Shamsuzzaman SM, Chowdhury A, Khatun K, Alam J. Isolation of potential pathogenic bacteria from nasopharynx from patients having cough for more than two

weeks. Bangladesh J Med Microbiol 2012. Available from: https://doi.org/10.3329/bjmm.v4i2.10826.

49. Cherry JD. Pertussis in the preantibiotic and prevaccine era, with emphasis on adult pertussis. Clin Infect Dis 1999. Available from: https://doi.org/10.1086/515057.

50. Gordon JE, Hood RI. Whooping cough and its epidemiological anomalies. Am J Med Sci 1951. Available from: https://doi.org/ 10.1097/00000441-195109000-00011.

51. Roush SW, Murphy TV, Basket MM, Iskander JK, Moran JS, Seward JF, et al. Historical comparisons of morbidity and mortal- ity for vaccine-preventable diseases in the United States. J Am Med Assoc 2007. Available from: https://doi.org/10.1001/jama.298.18.2155.

52. Cherry JD. Historical review of pertussis and the classical vaccine. J Infect Dis 1996. Available from: https://doi.org/10.1093/infdis/174.supplement_3.s259.

53. Domenech de Celle`s M, Magpantay FMG, King AA, Rohani P. The pertussis enigma: reconciling epidemiology, immunology and evolution. Proc Biol Sci 2016. Available from: https://doi.org/ 10.1098/rspb.2015.2309.

54. Grimprel E, Baron S, Levy-Bruhl D, Garnier JM, Njamkepo E, Guiso N, et al. Influence of vaccination coverage on pertussis transmission in France. Lancet 1999;354:1699700.

55. Zepp F, Heininger U, Mertsola J, Bernatowska E, Guiso N, Roord J, et al. Rationale for pertussis booster vaccination throughout life in Europe. Lancet Infect Dis 2011;11:55770.

56. Heininger U, Cherry JD, Stehr K. Serologic response and anti- body-titer decay in adults with pertussis. Clin Infect Dis 2004. Available from: https://doi.org/10.1086/381439.

57. Loeffelholz M. Towards improved accuracy of *Bordetella pertussis* nucleic acid amplification tests. J Clin Microbiol 2012; 50:218690.

58. Guiso N. What to do and what not to do in serological diagnosis of pertussis: recommendations from EU reference laboratories. Eur J Clin Microbiol Infect Dis 2011;30:30712.

59. Cherry JD, Tan T, Wirsing Von Ko¨nig CH, Forsyth KD, Thisyakorn U, Greenberg D, et al. (2012). Clinical definitions of pertussis: Summary of a global pertussis initiative roundtable meeting, Clinical Infectious Diseases; 2011. Available from: https://doi.org/10.1093/cid/cis302.

60. Solano R, Crespo I, Ferna´ndez MI, Valero C, A´ lvarez MI, Godoy P, et al. Underdetection and underreporting of pertussis in children attended in primary health care centers: do surveillance systems require improvement? Am J Infect Control 2016. Available from: https://doi.org/10.1016/j.ajic.2016.03.033.

61. Raguckas SE, VandenBussche HL, Jacobs C, Klepser ME. Pertussis resurgence: diagnosis, treatment, prevention, and beyond. Pharmacotherapy. 2007. Available from: https://doi.org/ 10.1592/phco.27.1.41.

62. Wood N, McIntyre P. Pertussis: review of epidemiology, diagno- sis, management and prevention. Paediat Respir Rev 2008. Available from: https://doi.org/10.1016/j.prrv.2008.05.010.

63. Tiwari T, Murphy TV, Moran J. Recommended antimicrobial agents for the treatment and postexposure prophylaxis of pertussis: 2005 CDC Guidelines. MMWR.

Recommendations and Reports: Morbidity and Mortality Weekly Report. Recommendations and Reports / Centers for Disease Control; 2005.

64. Hoffman CL, Gonyar LA, Zacca F, Sisti F, Fernandez J, Wong T, et al. *Bordetella pertussis* can be motile and express flagellum- like structures. MBio. 2019. Available from: https://doi.org/ 10.1128/mBio.00787-19.

65. MacArthur I, Belcher T, King JD, Ramasamy V, Alhammadi M, Preston A. The evolution of *Bordetella pertussis* has selected for mutations of acr that lead to sensitivity to hydrophobic molecules and fatty acids. Emerg Microbes Infect 2019. Available from: https://doi.org/10.1080/22221751.2019.1601502.

66. Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J Gen Microbiol 1970;63:21120.

67. Loeffelholz MJ. *Bordetella*. In: Murray PR, editor. Manual of Clinical Microbiology. America. American Society for Microbiology; 1999. p. 7806.

68. Moon K, Bonocora RP, Kim DD, Chen Q, Wade JT, Stibitz S, et al. The BvgAS regulon of *Bordetella pertussis*. MBio. 2017. Available from: https://doi.org/10.1128/mBio.01526-17.

69. Karataev GI, Sinyashina LN, Medkova AY, Semin EG, Shevtsova ZV, Matua AZ, et al. Insertional Inactivation of Virulence Operon in a Population of Persistent *Bordetella pertussis* bacteria. Genetika. 2016;.

70. Bone MA, Wilk AJ, Perault AI, Marlatt SA, Scheller EV, Anthouard R, et al. *Bordetella* PlrSR regulatory system controls BvgAS activity and virulence in the lower respiratory

tract. Proc Natl Acad Sci USAm 2017. Available from: https://doi.org/ 10.1073/pnas.1609565114.

71. Jungnitz H, West NP, Walker MJ, Chhatwal GS, Guzma´n CA. A second two-component regulatory system of *Bordetella* bronch- iseptica required for bacterial resistance to oxidative stress, production of acid phosphatase, and *in vivo* persistence. Infect Immun 1998.

72. Buboltz AM, Nicholson TL, Karanikas AT, Preston A, Harvill ET. Evidence for horizontal gene transfer of two antigenically dis- tinct O antigens in *Bordetella bronchiseptica*. Infect Immun 2009. Available from: https://doi.org/10.1128/IAI.01448-08.

73. Harvill ET, Preston A, Cotter PA, Allen AG, Maskell DJ, Miller JF. Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. Infect Immun 2000. Available from: https://doi.org/10.1128/IAI.68.12.6720-6728.2000.

74. Geuijen CA, Willems RJ, Hoogerhout P, Puijk WC, Meloen RH, Mooi FR. Identification and characterization of heparin binding regions of the Fim2 subunit of *Bordetella pertussis*. Infect Immun 1998;66:225663.

75. Ishigaki K, Shinzawa N, Nishikawa S, Suzuki K, Fukui-Miyazaki A, Horiguchi Y. Ectopic expression of O antigen in *Bordetella pertussis* by a novel genomic integration system. MSphere 2018. Available from: https://doi.org/10.1128/msphere.00417-17.

76. Jongerius I, Schuijt TJ, Mooi FR, et al. Complement evasion by *Bordetella pertussis*: implications for improving current vaccines. J Mol Med 2015;93:395402. Available from: https://doi.org/ 10.1007/s00109-015-1259-1.

77. Hazenbos WL, Geuijen CA, van den Berg BM, Mooi FR, van Furth R. *Bordetella pertussis* fimbriae bind to human monocytes *via* the minor fimbrial subunit FimD. J Infect Dis 1995;171:9249.

78. Caroff M, Karibian D. Structure of bacterial lipopolysaccharides. Carbohydr Res 2003;338:243147.

79. Caroff M, Brisson J-R, Martin A, Karibian D. Structure of the *Bordetella pertussis* 1414 endotoxin. FEBS Lett 2000;477:814.

80. Brummelman J, Veerman RE, Hamstra HJ, Deuss AJM, Schuijt TJ, Sloots A, et al. *Bordetella pertussis* naturally occurring iso- lates with altered lipooligosaccharide structure fail to fully mature human dendritic cells. Infect Immun 2015. Available from: https://doi.org/10.1128/IAI.02197-14.

81. Beddoe T, Paton AW, Le Nours J, Rossjohn J, Paton JC. Structure, biological functions and applications of the AB5 toxins. Trends Biochem Sci 2010. Available from: https://doi.org/ 10.1016/j.tibs.2010.02.003.

82. Locht C, Barstad PA, Coligan JE, Mayer L, Munoz JJ, Smith SG, et al. Molecular cloning of pertussis toxin genes. Nucleic Acids Res 1986;14:325161.

83. Carbonetti NH. Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools. Future Microbiol 2010;5:45569.

84. Hewlett E, Wolff J. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J Bacteriol 1976;127:8908.

85. Hazes B, Boodhoo A, Cockle SA, Read RJ. Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. J Mol Biol 1996. Available from: https://doi.org/10.1006/jmbi.1996. 0277.

86. Sedney CJ, Caulfield A, Dewan KK, Blas-Machado U, Callender M, Manley NR, et al. Novel murine model reveals an early role for pertussis toxin in disrupting neonatal immunity to *Bordetella pertussis*. Front. Immunol. 2023;14:1125794. Available from: https://doi.org/10.3389/fimmu.2023.1125794.

87. Katada T, Tamura M, Ui M. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP- ribosylation of a membrane protein. Arch Biochem Biophys 1983. Available from: https://doi.org/10.1016/0003-9861(83)90212-6.

88. Kirimanjeswara GS, Agosto LM, Kennett MJ, Bjornstad ON, Harvill ET. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. J Clin Investig 2005. Available from: https://doi.org/10.1172/JCI24609.

89. Meade BD, Kind PD, Manclark CR. Altered mononuclear phago- cyte function in mice treated with the lymphocytosis promoting factor of *Bordetella pertussis*. Dev Biol Standard 1985.

90. Weingart CL, Weiss AA. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. Infect Immun 2000. Available from: https://doi.org/10.1128/IAI.68.3.1735-1739.2000.

91. Schaeffer LM, Weiss AA. Pertussis toxin and lipopolysaccharide influence phagocytosis of *Bordetella pertussis* by human mono- cytes. Infect Immun 2001. Available from: https://doi.org/ 10.1128/IAI.69.12.7635-7641.2001.

92. Yajima M, Hosoda K, Kanbayashi Y, Nakamura T, Nogimori K, Mizushima Y, et al. Islets-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats: Purification and characterization. J Biochem 1978. Available from: https://doi.org/10.1093/oxfordjournals.jbchem.a131904.

93. Furman BL, Sidey FM, Wardlaw AC. Role of insulin in the hypo- glycaemic effect of sublethal *Bordetella pertussis* infection in mice. Br J Exp Pathol 1986;.

94. Arico B, Rappuoli R. *Bordetella* parapertussis and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J Bacteriol 1987. Available from: https://doi.org/10.1128/ jb.169.6.2847-2853.1987.

95. Confer DL, Eaton JW. Phagocyte impotence caused by an inva- sive bacterial adenylate cyclase. Science 1982;217:94850.

96. Glaser P, Ladant D, Sezer O, Pichot F, Ullmann A, Danchin A. The calmodulin-sensitive adenylate cyclase of *Bordetella* pertus- sis: cloning and expression in Escherichia coli. Molec Microbiol 1988;2:1930.

97. Betsou F, Sebo P, Guiso N. CyaC-mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase-hemolysin. Infect Immun 1993;61:35839.

98. Gueirard P, Druilhe A, Pretolani M, Guiso N. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo*. Infect Immun 1998;66:171825.

99. Khelef N, Zychlinsky A, Guiso N. *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun 1993;61:406471.

100. Khelef N, Gounon P, Guiso N. Internalization of *Bordetella* per- tussis adenylate cyclase-haemolysin into endocytic vesicles contri- butes to macrophage cytotoxicity. Cell Microbiol 2001;3:72130.

101. Fedele G, Spensieri F, Palazzo R, Nasso M, Cheung GY, Coote JG, et al. *Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PLoS One 2010;5:e8734.

102. Guiso N, Rocancourt M, Szatanik M, Alonso JM. *Bordetella* ade- nylate cyclase is a virulence associated factor and an immu- nopro- tective antigen. Microb Pathog 1989;7:37380.

103. Boehm DT, Hall JM, Wong TY, Divenere AM, Sen-Kilic E, Bevere JR, et al. Evaluation of adenylate cyclase toxoid antigen in acellular pertussis vaccines by using a *Bordetella pertussis* challenge model in mice. Infect Immun 2018. Available from: https://doi.org/10.1128/IAI.00857-17.

104. Mooi FR, van der Heide HG, ter Avest AR, Welinder KG, Livey I, van der Zeijst BA, et al. Characterization of fimbrial subunits from *Bordetella* species. Microb Pathog 1987;2:47384.

105. Geuijen CAW, Willems RJL, Mooi FR. The major fimbrial sub- unit of *Bordetella pertussis* binds to sulfated sugars. Infect Immun 1996;64:265765.

106. Mooi FR, Jansen WH, Brunings H, Gielen H, van der Heide HG, Walvoort HC, et al. Construction and analysis of *Bordetella* per- tussis mutants defective in the production of fimbriae. Microb Pathog 1992;12:12735.

107. Flak TA, Heiss LN, Engle JT, Goldman WE. Synergistic epithe- lial responses to endotoxin and a naturally occurring muramyl peptide. Infect Immun 2000;68:123542.

108. Matsuzawa T, Fukui A, Kashimoto T, Nagao K, Oka K, Miyake M, et al. *Bordetella* dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. J Biol Chem 2004;279:286672.

109. Cowell JL, Hewlett EL, Manclark CR. Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*. Infect Immun 1979;25:896901.

110. Brockmeier SL, et al. Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory dis- ease in swine. Infect Immun 2002;70:48190.

111. Schmidt G, Goehring UM, Schirmer J, Lerm M, Aktories K. Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for rho GTPases. J Biol Chem 1999;274:3187581.

112. Arai H, Sato Y. Separation and characterization of two distinct hemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochim Biophys Acta 1976;444:76582.

113. Delisse-Gathoye AM, Locht C, Jacob F, Raaschou-Nielsen M, Heron I, Ruelle JL, et al. Cloning, partial sequence, expression, and antigenic analysis of the filamentous hemagglutinin gene of *Bordetella pertussis*. Infect Immun 1990;58:2895905.

114. Domenighini M, Relman D, Capiau C, Falkow S, Prugnola A, Scarlato V, et al. Genetic characterization of *Bordetella pertussis* filamentous haemagglutinin: a protein processed from an unusu- ally large precursor. Mol Microbiol 1990;4:787800.

115. Delattre AS, Saint N, Clantin B, Willery E, Lippens G, Locht C, et al. Substrate recognition by the POTRA domains of TpsB trans- porter FhaC. Mol Microbiol 2011;81:99112.

116. Coutte L, Willery E, Antoine R, Drobecq H, Locht C, Jacob- Dubuisson F. Surface anchoring of bacterial subtilisin important for maturation function. Mol Microbiol 2003;49:52939.

117. Kajava AV, Cheng N, Cleaver R, Kessel M, Simon MN, Willery E, et al. Beta-helix model for the filamentous haemagglutinin adhesin of *Bordetella pertussis* and related bacterial secretory pro- teins. Mol Microbiol 2001;42:27992.

118. Tuomanen EI, Hendley JO. Adherence of *Bordetella pertussis* to human respiratory epithelial cells. J Infect Dis 1983;148:12530.

119. Prasad SM, Yin Y, Rodzinski E, Tuomanen EI, Masure HR. Identification of a carbohydrate recognition domain in filamentous hemagglutinin from *Bordetella pertussis*. Infect Immun 1993;61:27805.

120. Relman DA, Domenighini M, Tuomanen E, Rappuoli R, Falkow S. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc Natl Acad Sci USA 1989;86:263741.

121. Zaretzky FR, Gray MC, Hewlett EL. Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella* pertus- sis: a role for toxin-filamentous haemagglutinin interaction. Mol Microbiol 2002;45:158998.

122. Ishibashi Y, Nishikawa A. *Bordetella pertussis* infection of human respiratory epithelial cells up-regulates intercellular adhe- sion molecule-1 expression: role of filamentous hemagglutinin and per- tussis toxin. Microb Pathog 2002;33:11525.

123. Kinnear SM, Boucher PE, Stibitz S, Carbonetti NH. Analysis of BvgA activation of the pertactin gene promoter in *Bordetella pertussis*. J Bacteriol 1999.

124. Makoff AJ, Oxer MD, Ballantine SP, Fairweather NF, Charles IG. Protective surface antigen P69 of *Bordetella pertussis*: Its characterization and very high level expression in Escherichia coli. Nat Biotechnol 1990. Available from: https://doi.org/10.1038/nbt1190-1030.

125. Emsley P, McDermott G, Charles IG, Fairweather NF, Isaacs NW. Crystallographic characterization of pertactin, a membrane- associated protein from *Bordetella pertussis*. J Mol Biol 1994. Available from: https://doi.org/10.1006/jmbi.1994.1029.

126. Hannah JH, Menozzi FD, Renauld G, Locht C, Brennan MJ. Sulfated glycoconjugate receptors for the *Bordetella pertussis* adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. Infect Immun 1994;62:50109.

127. Leininger E, Roberts M, Kenimer JG, Charles IG, Fairweather N, Novotny P, et al. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that

promotes adherence of mammalian cells. Proc Natl Acad Sci USAm 1991. Available from: https://doi.org/10.1073/pnas.88.2.345.

128. Hovingh ES, Mariman R, Solans L, Hijdra D, Hamstra HJ, Jongerius I, et al. *Bordetella pertussis* pertactin knock-out strains reveal immunomodulatory properties of this virulence factor arti- cle. Emerg Microbes Infect 2018. Available from: https://doi.org/ 10.1038/s41426-018-0039-8.

129. Edwards JA, Groathouse NA, Boitano S. *Bordetella bronchiseptica* adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A. Infect Immun 2005. Available from: https://doi.org/10.1128/IAI.73.6.3618-3626.2005.

130. Inatsuka CS, Xu Q, Vujkovic-Cvijin I, Wong S, Stibitz S, Miller JF, et al. Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. Infect Immun 2010. Available from: https://doi.org/10.1128/IAI.00188-10.

131. Safarchi A, Octa*via* S, Luu LDW, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infec- tion model. Vaccine. 2015. Available from: https://doi.org/ 10.1016/j.vaccine.2015.09.064.

132. Mooi FR, Van Loo IHM, Van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. *Bordetella pertussis* strains with increased toxin produc- tion associated with pertussis resurgence. Emerg Infect Dis 2009. Available from: https://doi.org/10.3201/eid1508.081511.

133. Cherry JD. Pertussis: challenges today and for the future. PLoS Pathog 2013. Available from: https://doi.org/10.1371/journal. ppat.1003418.

134. De Gouw D, Hermans PWM, Bootsma HJ, Zomer A, Heuvelman K, Diavatopoulos DA, et al. Differentially expressed genes in *Bordetella pertussis* strains belonging to a lineage which recently spread globally. PLoS ONE 2014. Available from: https://doi.org/10.1371/journal.pone.0084523.

135. King AJ, van der Lee S, Mohangoo A, van Gent M, van der Ark A, van de Waterbeemd B. Genome-wide gene expression analy- sis of *Bordetella pertussis* isolates associated with a resurgence in pertussis: elucidation of factors involved in the increased fit- ness of epidemic strains. PLoS ONE 2013. Available from: https://doi.org/10.1371/journal.pone.0066150.

136. Barkoff AM, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Pertactin-deficient *Bordetella pertussis* isolates: Evidence of increased circulation in Europe, 1998 to 2015. Eurosurveillance 2019. Available from: https://doi.org/10.2807/1560-7917.ES.2019.24.7.1700832.

137. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the United States. Clin Vaccine Immunol 2014. Available from: https://doi.org/10.1128/CVI.00717-13.

138. Ma L, Caulfield A, Dewan KK, Harvill ET. Pertactin-Deficient *Bordetella pertussis*, Vaccine-Driven Evolution, and Reemergence of Pertussis. Emerg Infect Dis. 2021 Jun;27(6):15611566. Available from: https://doi.org/10.3201/eid2706.203850. PMID: 34014152; PMCID: PMC8153889.

139. Lam C, Octa*via* S, Ricafort L, Sintchenko V, Gilbert GL, Wood N, et al. Rapid increase in pertactin-deficient *Bordetella pertussis* isolates, Australia. Emerg Infect Dis 2014. Available from: https://doi.org/10.3201/eid2004.131478.

140. Zomer A, Otsuka N, Hiramatsu Y, Kamachi K, Nishimura N, Ozaki T, et al. *Bordetella pertussis* population dynamics and phylogeny in Japan after adoption of acellular pertussis vaccines. Microb Genomics 2018. Available from: https://doi.org/10.1099/ mgen.0.000180.

141. Melvin JA, Scheller EV, Miller JF, Cotter PA. *Bordetella pertussis* pathogenesis: current and future challenges. Nat Rev Microbiol 2014. Available from: https://doi.org/10.1038/ nrmicro3235.

142. Hiramatsu Y, Miyaji Y, Otsuka N, Arakawa Y, Shibayama K, Kamachi K. Significant decrease in pertactin-deficient *Bordetella pertussis* isolates, Japan. Emerg Infect Dis 2017. Available from: https://doi.org/10.3201/eid2304.161575.

143. Argondizo-Correia C, Rodrigues AKS, De Brito CA. Neonatal immunity to *Bordetella pertussis* infection and current prevention strategies. J Immunology Res 2019. Available from: https://doi. org/10.1155/2019/7134168.

144. Feunou PF, Kammoun H, Debrie AS, Mielcarek N, Locht C. Long-term immunity against pertussis induced by a single nasal administration of live attenuated B. pertussis BPZE1. Vaccine 2010. Available from: https://doi.org/10.1016/j.vaccine.2010. 08.017.

145. Skerry CM, Mahon BP. A live, attenuated *Bordetella pertussis* vaccine provides long-term protection against virulent challenge in a murine model. Clin Vaccine Immunol 2011. Available from: https://doi.org/10.1128/CVI.00371-10.

146. Mielcarek N, Debrie AS, Raze D, Bertout J, Rouanet C, Younes AB, et al. Live attenuated B. pertussis as a single-dose nasal vac- cine against whooping cough. PLoS Pathog 2006. Available from: https://doi.org/10.1371/journal.ppat.0020065.

CHAPTER 3:

DISRUPTING BTRS-MEDIATED IMMUNOMODULATION ENHANCES MUCOSAL & PULMONARY IMMUNITY AGAINST *BORDETELLA PERTUSSIS*

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ABSTRACT

Bordetella pertussis (Bp), the bacterial pathogen responsible for "whooping cough", causes an estimated 24 million cases of vaccine-preventable illness per year, resulting in an excess of 170,000 deaths annually. Importantly, the incidence of whooping cough in nations with high vaccine coverage is on the rise, attributed to asymptomatic transmission that is enabled by inadequate Th2-skewed immunity generated by current acellular pertussis (aP) vaccines. The inability of aP vaccines to generate sterilizing immunity in the nasopharynx has sparked a critical need to better understand the immune response to *Bp* at this initial site of infection, however, like many well-adapted pathogens, *Bp* has evolved complex mechanisms to modulate the host immune response. In an effort to better understand *Bp* suppression of host immunity, we have focused on a sigma factor (BtrS) that we have previously shown to be critical to the regulation of *Bordetella spp.* immunomodulatory factors. Importantly, we observe that infection with an isogenic mutant lacking this regulator generates more robust and highly effective immunity in the nasal cavity and lungs of mice, enabling rapid clearance that surpassed wildtype and vaccine-induced immunities. Investigations into the host response to this mutant revealed significant increases in $CD4^+$ T cells in the nasal cavity and lungs, as well as elevated IgA titers in *Bp*Δ*btrS*-convalescent mice following secondary challenge. The observation that a *Bp* mutant lacking this regulator induced more effective anamnestic immunity in the upper and lower respiratory tracts presents the opportunity to study and understand both how *Bp* suppresses immunity *and* how enhanced protective immunity can be generated; addressing the critical need to understand the host immune response to *Bp* in the nasal mucosa, and how that response may be enhanced to eliminate or reduce ongoing sub-clinical transmission within vaccinated populations.

INTRODUCTION

Bordetella pertussis (Bp) is a highly transmissible, human-adapted bacterium that causes an estimated 24-48 million cases of vaccine-preventable infection each year[1-3]. Importantly, the incidence of whooping cough in nations with high vaccine coverage is on the rise and has been recognized by both the CDC and NIAID as a priority (re)emerging pathogen of high concern. In several countries with broad vaccine coverage, recent periods of resurgence have been linked to a transition from more reactogenic whole cell pertussis (wP) vaccines to less reactogenic, but also less efficacious acellular pertussis (aP) vaccines composed of 3-5 detoxified antigens[4-5]. While capable of preventing severe disease in most cases, the Th2-skewed immunity generated by aP vaccines wanes rapidly and fails to confer sterilizing immunity against *Bp*, enabling sub-clinical infections of the host nasopharynx, bacterial shedding, and transmission between vaccinated individuals[4-7]. Consequently, the population-level protective effects of herd immunity are disrupted by the resultant circulation of *Bp.*

Understanding the immunological mechanisms that generate sterilizing anamnestic immunity in the nasal cavity is a high priority initiative for *Bp*, as no universally accepted correlate of protection has been established[8-9]. It is widely appreciated that complementary roles exist for both humoral and cell-mediated immunity, with increasing evidence that site-specific mucosal immunity is critical in protection against colonization with *Bp.* This evidence is consistent with use of convalescent immunity from natural *Bp* infection as the "gold standard" for protective immunity[10-13]. Problematically, like many well-adapted pathogens, *Bp* has evolved complex mechanisms to modulate the host immune response to its own advantage[14], suggesting it may be erroneous to consider natural, but *modulated* immunity to be the highest standard we can achieve.

Importantly, recent initiatives in characterizing and generating enhanced immunity against *Bp* have begun to *disrupt* its virulence capacity by deleting or detoxifying specific virulence factors (BPZE1 live-attenuated vaccine, for example[15-16]). We have taken the alternative approach by disrupting whole immunomodulatory networks to generate a live-attenuated vaccine candidate "Bbvac" against *Bordetella bronchiseptica*, a close ancestral relative of *B. pertussis* and the causative agent of Kennel Cough in dogs, as well as other respiratory infections in various other mammalian hosts[17]. Importantly, this candidate vaccine has been experimentally shown to generate robust, sterilizing immunity against *B. bronchiseptica, B. pertussis,* and *B. parapertussis via* deletion of an extracytoplasmic sigma factor, BtrS, involved in regulation of multiple immunomodulatory factors [17-19].

Our lab and others have reported that BtrS regulates expression of a diverse network of genes, which together suppress or modulate the host immune response, resulting in suppressed anamnestic immunity. Disruption of the BtrS network was found to elicit an enhanced immune response characterized by increases in $CD4^+$ and $CD8^+$ T cells, T resident memory cells (T_{RM}), the induction of bronchus-associated lymphoid tissues (BALT), as well as increased *Bb*-specific IgG titers[17-22]. Together these effects resulted in more robust protective immunity than was induced by WT *B. bronchiseptica*, confirming our suspicion that convalescent immunity to WT infection is NOT the best immune response possible.

Due to the high conservation of *btrS* (100% identity) but differential expression of BtrS-regulated factors reported between *Bb* and *Bp,* we queried whether similarly enhanced immune responses could be generated using an isogenic mutant lacking *btrS* in *Bp.* Here, we generate and evaluate *Bp*Δ*btrS* mutants in two *Bp* strains, Tohama I and D420, with the aim of utilizing complementary murine models to probe the role of BtrS-regulated networks in upper respiratory

(mucosal) and pulmonary immunity against *Bp.* Our data indicate that BtrS-regulated factors contribute to active immunomodulation in *B. pertussis* infection. However, infection with an isogenic mutant lacking *btrS* generates enhanced protective immunity (relative to WT-convalescence and vaccine-induced immunities) in both the upper and lower respiratory tracts of mice that correlates with increased recruitment/expansion of CD4+ T cells and mucosal IgA titers.

MATERIALS & METHODS

Bacterial Strains & Culture

Gentamicin-resistant derivatives of *B. pertussis* strains (Tohama I & D420) and a streptomycin derivative of *B. bronchiseptica* (RB50) were maintained in frozen stock at -80°C and struck out on Bordet-Gengou agar (Difco, BD. cat 248200) supplemented with 10% defibrinated sheep's blood (Hema Laboratories). Plates were incubated for 3-5 days at 37°C until mature colonies formed. Liquid cultures were inoculated from plates and grown over night in Stainer-Scholte broth supplemented with 0.5% Heptakis (2,6-di-O-methyl-β-cyclodextrin) (Sigma H0513) in a 37°C shaker at 200 rpm. Liquid cultures were grown to an OD of 0.1 for murine experimental inoculum.

Generation of Isogenic Mutant Strains

Gentamicin-resistant *Bp* strains Tohama I (WT*Bp*) and D420 were used to generate isogenic mutants lacking *btrS* (*Bp*Δ*btrS* and D420Δ*btrS*, respectively) using derivatives of the unmarked allelic exchange vector pSS4245. Briefly, gDNA from each respective strain was used to generate flanking fragments corresponding to the ends of the target *btrS* sequence. The fragments were joined and inserted into pss4245 *via* PIPE cloning following linearization of the plasmid (all primers are listed in Supplementary Figure 2). The constructs were transformed into

E. coli strains DH5α and SM10λpir. In preparation for mating, *Bp* strains were grown for 5 days under Bvg^- phase conditions (BG agar containing 50 mM $MgSO_4$), then co-incubated with SM10λpir carrying the construct on BG agar containing 50 mM $MgSO_4$ for 4 hours. Cointegrates were selected by plating on BG agar plates containing streptomycin, gentamicin, and 50 mM $MgSO_4$. Following growth at 37°C for 4-5 days, cointegrates were identified *via* PCR and struck out onto plates without $MgSO_4$ (Bvg^+-phase conditions) and incubated for 5 days at 37°C. Emerging colonies were screened and determined to have undergone a second recombination event *via* PCR to identify mutant bacteria[21].

Murine Respiratory Challenges & CFU Enumeration

Wild-type (C57BL6/J), B6.129S2-*Tcra*^{tm1Mom}/J, and *Rag-1*$^{-/-}$ (*Rag1*^{tm1Mom}*/J*) mice were obtained from Jackson Laboratories and housed in a pathogen-free vivarium at the University of Georgia. Mice were lightly sedated *via* inhalation of 5% isoflurane and inoculated by gently pipetting inoculum though the external nares (500-1,000 CFU in 5µL PBS or $5X10^5$ CFU in 50µL PBS.) For experiments using ~1,000 CFU, mice were pre-treated with 45µg of gentamicin suspended in 15mL PBS 3 times at 12-hour intervals to perturb the microbiota, facilitating infection at lower dose as previously described[22].

To collect serum and enumerate CFU in the respiratory organs, mice were euthanized *via* CO_2 asphyxiation (1.5L/min) followed by cervical dislocation. Blood was collected immediately following euthanasia *via* cardiocentesis. Mice were necropsied to collect nasal cavities, NALT, trachea, lungs, spleen, and middle ears as previously described[22-23]. Harvested tissues were collected in 1mL of sterile PBS containing 5-7 ceramic beads (2.8mm, Omni International), and homogenized using a bead homogenizer (Fischerbrand Model: Bead Mill 24). Serial dilutions

were plated on BG-Gent agar and incubated for 4-5 days at 37°C, after which colonies were counted and bacterial load was calculated.

Immunization & Secondary Challenge

For vaccination experiments, we used intraperitoneal or intranasal delivery as indicated. Heat-killed whole cell (wP) vaccine was generated using $5X10^7$ CFU/200µL PBS of WT*Bp* or *Bp*Δ*btrS,* and acellular vaccines were prepared to $1/20^{th}$ of the human dose in 200µL PBS (Adacel TdaP; Soofi Pasteur). Two weeks following aP vaccination, mice received an equal booster dose. Fifty-six days following initial vaccination, mice were inoculated with (500-1,000 CFU in 5µL or $5X10^5$ CFU in 50µL as described above). CFU were enumerated as described previously.

Tissue preparation, Staining & Flow Cytometry

Five mice per group were euthanized *via* CO_2 inhalation. Nasal cavities (including NALT) and lungs were harvested, placed in 1mL of RPM1 1640 medium and homogenized *via* syringe plunger or glass pestle against a 40µm cell strainer. Cell suspensions were centrifuged at 1,500rpm for 10 minutes, the supernatant was collected, and remaining erythrocytes were lysed with ACK lysing buffer. After washing with DPBS, the cells were incubated with 0.35µL (of 1:100 dilution) of Zombie Aqua *via*bility dye (Biolegend) for 20 minutes, washed with DPBS, and incubated with 1µL Fc Block (Biolegend) for 30 minutes. Surface marker staining using 0.35µL antibody/sample was conducted using a mastermix of antibodies for 30 minutes – 1 hour. Cells were fixed with BD Stabilizing fixative (Ref 338036), washed, and resuspended in 250uL DPBS. Acquisition of data was performed using the Acea Novocyte Quanteon. Data were analyzed with FlowJo v.10.0 or NovoExpress software. Gating strategies and panel information are provided in Supplementary Figures S3.4 and S3.6.

ELISA

Anti-*Bp* Serum IgG and nasopharyngeal IgA antibodies were quantified as previously described[22]. To summarize, WT*Bp* was grown to an OD of 0.6 as above. Bacteria were heat killed at 65°C for 30 minutes, carefully added to wells in equal 100uL volumes, and incubated at 37°C for 4 hours. Plates were washed and blocked overnight at 4°C with blocking buffer (PBS with 0.1% Tween 20 and 1% BSA). Four microliters of serum or 100uL of nasopharyngeal supernatant were used to measure total antibodies through two-fold serial dilutions. Goat-anti-mouse-HRP-conjugated secondary antibody (Invitrogen) was applied and incubated for 1 hour at 37°C. SureBlue (SeraCare cat. 5120-0076) was used to develop the reaction for 3-5 minutes and was terminated with 1M HCl. The plates were read at an OD of 450nm. The titer was determined to be the reciprocal of the lowest dilution in which an OD of 0.1 was obtained.

Splenic, Pulmonary T cell & Serum Adoptive Transfer

Immune serum (peripheral blood) and T cells (spleen and lungs) for adoptive transfer were isolated from convalescent mice following primary infection with $5X10^5$CFU of either D420 or D420Δ*btrS* on day 56 post-inoculation. Serum was collected as described above. Whole convalescent spleens and lungs were isolated from convalescent animals, placed in 1mL of RPM1 1640 medium, and homogenized *via* syringe plunger against a 40μm cell strainer. Cell suspensions were centrifuged at 1,500rpm for 10 minutes and remaining erythrocytes were lysed with ACK lysing buffer.

Splenic cells were resuspended to approximately 10^6 cells/200μL PBS, and intraperitoneally injected into $Rag1^{-/-}$ mice. Pulmonary T cells were isolated *via* the magnetic StemCell EasySep Mouse T cell isolation kit, pooling where necessary. Approximately 10^5

pulmonary T cells were isolated and transferred into Rag $1^{-/-}$ mice *via* intranasal delivery in a 50uL volume. An additional group received 50µL PBS *via* intraperitoneal injection as a control. All mice were intranasally challenged with $5X10^5$CFU/50µL of D420 two hours post-transfer. Mice were euthanized on D14 post-inoculation (pi), and CFU were enumerated as above.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (v9.0 or 10.1). Two-tailed unpaired Student's t-tests, One-way ANOVA, and Two-way ANOVA were performed as appropriate, and are indicated in the corresponding figure legends. The mean ± standard error (error bars in figures) was determined for all appropriate data.

Ethics Statement

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Georgia in Athens, GA. (A2022 04-001-Y1-A3 *Bordetella*-Host Interactions, A2022 04-025-Y3-A3 Breeding Protocol, and A2021 02-009-Y3-A3 Studies on the virulence of circulating strains of *Bordetella species.*) All animals were monitored to ensure health and minimize distress. Animals that had reached terminal endpoints were removed from the study and euthanized *via* carbon dioxide asphyxiation to prevent unnecessary suffering.

RESULTS

BtrS contributes to effective colonization of the respiratory tract

To investigate the potential role of BtrS in colonization using the conventional pneumonic model of infection, groups (n=5) of C57BL/6 mice were lightly sedated *via* isoflurane inhalation and intranasally inoculated with 50µL of PBS containing $5X10^5$ CFU of

WT*Bp* or *Bp*Δ*btrS.* On days 3, 7, 14, 28, and 56pi mice were euthanized *via* CO_2 inhalation. Respiratory tissues, spleen and middle ears were collected from all mice, homogenized, and plated on BG (Bordet Gengou) blood agar plates. Bacteria were enumerated following 5 days of incubation at 37°C (Figure 3.1a-e, respectively). WT*Bp* infection profiles were consistent with previous reports, however, *Bp*Δ*btrS* was generally recovered at lower levels throughout the time course, with statistically significant differences in the nasal cavity on D14pi (Fig 3.1a), and in the lungs on days 3, 14, and 28pi (Fig 3.1c). All respiratory organs were cleared of infection regardless of strain by D56pi. Colonization of the spleen, used as an indicator for systemic dissemination in severe infections, also indicated differential colonization, with *Bp*Δ*btrS* initially reaching higher levels before rapid clearance by D14pi, while WT*Bp* was still recovered from infected mice, albeit at relatively low levels (Fig 1d). Bacterial burden was also assessed in the middle ears of mice as previously described[24], with the mutant being maintained at lower levels relative to WT*Bp* (Figure 3.1e). These results collectively suggest that BtrS, or more likely its dependent bacterial factors, may be involved in colonization and/or resisting immune-mediated control of *Bp* in the respiratory tract.

However, interestingly, mice challenged with a nasopharyngeal dose (500-1,000 CFU in 5µL) demonstrated a reverse phenotype, whereby the numbers of *Bp*Δ*btrS* bacteria surpassed that of WT*Bp*, with significantly higher bacterial load of the mutant strain recovered from the nasal cavities on days 14 and 28pi (Fig. 3.1f). While bacteria were not recovered from the lungs at any point using our nasopharyngeal infection model, sporadic numbers were recovered from the trachea and spleen, as indicated in Fig. S3.1a-b). While unexpected, these results may suggest a differential role for BtrS-regulated factors in natural infection within the nasopharynx as opposed to severe pneumonic infection.

***Bp*Δ*btrS*-convalescent immunity enables complete clearance of pneumonic challenge in the lungs by D3pi**

Informed by our prior reports of enhanced anamnestic immune responses using a *B. bronchiseptica* isogenic mutant lacking *btrS*[19], we postulated that the differential colonization profiles reported in Figure 3.1 may be due to elevated immune responses against our mutant strain in the absence of BtrS as an immunomodulatory factor. Thus, we aimed to investigate the strength of *Bp*Δ*btrS*-mediated anamnestic/convalescent immunity relative to WT*Bp* convalescence, aP and wP vaccine-induced immunities, with a naïve PBS control.

Groups (n=5) of C57BL/6 mice were lightly sedated *via* isoflurane inhalation and inoculated with 50µL of $5X10^5$ CFU of wildtype *Bp,* or *Bp*Δ*btrS*, or were intraperitoneally vaccinated with $1/20^{th}$ of the human dose of either aP or wP vaccine in 100µL PBS. Twenty-eight days following initial aP vaccination, mice were boosted with an additional dose. On day 56 post-inoculation/vaccination, mice were again lightly sedated with isoflurane, and challenged with $5X10^5$ CFU in 50µL PBS (pneumonic model, Fig. 3.2) of wildtype *Bp* delivered through the nares. On days 3, 7, 14, 28, and 56pi bacterial load was determined as above.

The conventional high dose pneumonic challenge resulted in expected colonization curves for naïve, aP, and wP vaccinated mice. Naïve mice demonstrated the highest bacterial load in the lower respiratory tract, followed by aP, and then wP, each of them establishing clearance in the lungs by days 56, 28, and 14pc, respectively. WT*Bp*-convalescent mice demonstrated fairly robust protection, with low levels of bacteria recovered from the lungs through D14pc. Excitingly, *Bp*Δ*btrS*-convalescent mice demonstrated complete clearance from the lungs on or before D3pc, significantly better than protection generated from natural infection with WT*Bp* (Fig 3.2c,e-f). However, no group was able to clear bacteria from the nasopharynx,

with *all* groups remaining colonized past D56pc. (All statistical comparisons are provided in Fig. S3.2.)

Bp*Δ*btrS*-convalescent immunity enables complete clearance of nasopharyngeal challenge by *D3pi

In order to evaluate convalescent immunity against a natural (nasopharyngeal) challenge dose, groups (n=5) of C57BL/6 mice were vaccinated, boosted, or infected as above. On day 56pi, mice were lightly sedated *via* isoflurane inhalation and intranasally treated 3 times with 45µg of gentamicin in 15µL PBS every 12 hours to perturb prohibitive resident microbiota[22]. Two hours following the last dose, all mice were challenged with 500-1,000 CFU of WT*Bp* (nasopharyngeal model, Figure 3.3). On days 3, 7, 14, 28, and 56pi bacterial load was determined as above.

In the nasopharyngeal infection model naïve mice followed a time course that roughly approximates that of normal human infections, with bacterial numbers growing rapidly, peaking above 10^4 CFU in the first few weeks and slowly declining thereafter, but persisting for at least 56 days (Figure 3.3a). The aP vaccinated group had a blunted peak but infection persisted for at least as long as naïve animals. Convalescent and wP vaccinated animals had better protection, with lower bacterial numbers at most time points, but most animals remained infected for over a month. Interestingly, wP-vaccinated mice demonstrated an improved ability to control infection relative to WT-convalescence, which supports our observation that natural infection-induced immunity is imperfect and can be improved upon. However, only the animals convalescent from prior *Bp*Δ*btrS* infection were completely protected against colonization, which strongly supports our previous observation that the immunity generated by this mutant is stronger than that

generated by wildtype *Bp* (Figure 3.3a-c). (All statistical comparisons and lower respiratory tract colonization data are provided in Figure S3.3).

BtrS suppresses T cell-mediated immunity

To evaluate the cellular response to *Bp*Δ*btrS* in the pneumonic experimental system as above (ensuring maximum immune response), we replicated all treatment groups from Fig. 2 (n=5 per group), boosted where indicated, and challenged all mice with $5X10^5$ CFU of WT*Bp.* Immune responses were evaluated at a single timepoint (D3pi) to capture early responses to challenge given the rapid clearance enabled by *Bp*Δ*btrS*. Mice were euthanized as above, and tissue samples were processed, stained, and analyzed as described in the methods. Using the gating strategy depicted in Figure S3.4, we first identified $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells, as well as $CD4^+$ T_{RM} ($CD3^+$, $CD4^+$, $CD62L^-$, $CD44^+$, and $CD69^+$). Flow cytometric analysis of T cell populations indicated significantly higher recruitment of $CD4^+$ T cells to the nasal cavities and lungs of *Bp*Δ*btrS*-convalescent mice relative to WT*Bp*-convalescent mice (Figure 3.4a). No significant differences or trends were found regarding $CD8^+$ populations (Figure 3.4b). As expected, there was a general trend toward higher $CD4^+$ T_{RM} cells recovered from the nasal cavities and lungs of both convalescent groups due to the localized infection and exposure to *Bp* antigens, in contrast to intraperitoneally vaccinated mice. *Bp*Δ*btrS*-convalescent mice had slightly higher numbers of $CD4^+$ T_{RM} recovered from both respiratory tissues that was not statistically significant. The data overall indicate that bacterial factors reliant on BtrS block $CD4^+$ T cell recruitment to infected lungs.

To further investigate the role(s) of T cells in the improved protection conferred by *Bp*Δ*btrS*, we challenged C57BL6/J or $TCRa^{-/-}$ mice (lacking functional alpha beta T cells) with $5X10^5$ CFU of WT*Bp* or *Bp*Δ*btrS.* Mice were euthanized on D28pi when we previously noted a

difference in colonization during primary infection. In C57BL6/J mice, we again observed significantly lower CFU recovered from the lungs of *Bp*Δ*btrS*-infected mice, however, *higher* numbers of *Bp*Δ*btrS* were recovered from the lungs of TCRa$^{-/-}$ mice relative to WT*Bp*. These data suggest that T cell-mediated immunity is modulated by BtrS and is required for effective control of *Bp*Δ*btrS* (Figure 3.5).

***Btr*S-dependent factors contribute to active immunomodulation**

For both *B. bronchiseptica* as well as *B. pertussis,* it remained unknown whether enhanced Δ*btrS*-convalescent immunity resulted from increased expression of immunogens/antigens or decreased active immunomodulation. To distinguish between these, we generated groups of *Bb* (strain RB50) or RB50Δ*btrS*-convalescent mice (n=5) using the same dose and method as above. Two additional groups of mice were intranasally immunized with heat-killed whole cell RB50 or RB50Δ*btrS* (5x10^7 CFU/mL in 50µL of PBS), which should contain potential immunogens/antigens but not express active immunomodulation. On D56 post-vaccination/infection, mice were challenged with 5X10^5 CFU of a gentamicin-resistant RB50 (to differentiate between any remaining bacteria from primary infection). Mice were euthanized on D3pi, and respiratory organs were harvested. The nasal cavities and lungs were processed for flow cytometry to evaluate recovered B cells and CD4$^+$/CD8$^+$ T cell populations. (Gating strategy is available from Figure S3.6).

Flow cytometric analysis and resultant data appear to indicate robust B and T cell responses in live RB50-convalescent mice, as expected and previously reported[17,19]. However, intranasal vaccination with HK RB50Δ*btrS* resulted in significantly lower recovery of B cells, as well as CD4$^+$ and CD8$^+$ T cells when compared to WT RB50 and RB50Δ*btrS* (Figure S3.7). The failure of a HK RB50Δ*btrS* to generate robust immune responses suggests that BtrS-dependent

immunomodulation in the context of the normal infection process is likely the result of active immunomodulation, for example *via* secreted bacterial factors that inhibit cell recruitment and/or activation.

We conducted a parallel experiment using a *btrS* mutant generated in D420, a virulent and recent clinical isolate of a relevant and currently circulating strain of *Bp.* Groups of 5 mice were initially intranasally treated with either 50µL PBS, live D420Δ*btrS*, or HK D420Δ*btrS*. On day 56pi all groups were challenged with $5X10^5$ CFU of WT D420. Three days following secondary challenge, mice were euthanized and tissues were processed as above. PBS control mice were highly colonized. CFU recovered from both the nasal cavities and lungs of challenged mice suggest the D420Δ*btrS* convalescent immunity to be quite protective, enabling full clearance of the lower respiratory tract by D3pi. Intranasal vaccination with HK D420Δ*btrS* resulted in immunity that was sufficient to largely reduce infection, however, both tissues remained colonized.

Flow cytometric analysis of the nasal cavities and lungs similarly indicated that D420Δ*btrS*-convalescent mice had the strongest response in the nasopharynx and lungs, with significantly higher populations of $CD4^+$ T cells, as well as $CD4^+$ T_{RM} relative to both PBS and HK D420Δ*btrS*-vaccinated groups (Fig. 3.6 a- b). Interestingly, HK D420Δ*btrS*-treated mice demonstrated similar $CD8^+$ T cell responses to the convalescent group, which were both elevated relative to the PBS control. These data collectively suggest that modulation of $CD4^+$ responses is dependent on factors secreted in the course of *Bp* infection. (Gating strategy is available from Figure S3.6)

BtrS contributes to suppression of IgA responses in the nasopharynx

We assessed the levels of anti-pertussis nasopharyngeal IgA and serum IgG in primary (D28pi) vs. secondary (D1 post-challenge) infection with WT D420 vs. D420Δ*btrS*. Interestingly, anti-pertussis IgA responses were significantly higher in D420Δ*btrS*-convalescent mice than WT D420-convalescent mice following secondary challenge, which was not observed in the primary infection groups (Figure 3.7 a-b). Serum anti-pertussis IgG titers, however, showed no difference between D420 groups, regardless of convalescence.

Site specific T cell-mediated immunity

To assess the relative contributions of serum, general (peripheral) T cell mediated immunity, and tissue specific immunities generated by WT D420 and D420Δ*btrS*, groups (n=5) of C57BL6/J mice were inoculated with $5X10^5$ CFU of either the WT or the mutant strain. After 56 days, convalescent serum, splenic cells, and pulmonary T cells were isolated from C57BL6/J mice and prepared for adoptive transfer into $Rag1^{-/-}$ mice. Convalescent serum and splenic transfer were administered *via* intraperitoneal route, and isolated pulmonary T cells were delivered intranasally. An additional group received 50µL PBS *via* intraperitoneal injection as a control. Two hours post-transfer all treatment groups were challenged with $5X10^5$CFU/50µL of WT D420. Mice were euthanized on D14 post-inoculation (pi), and CFU were enumerated to determine the relative level of protection enabled by each transfer group.

Briefly, WT D420-convalescent serum enabled significant reductions in bacterial load relative to the PBS control in the trachea and lungs. Interestingly, transfer of D420Δ*btrS*-convalescent serum granted less protection than the WT equivalent in the lower respiratory tract, with significant differences recorded in the trachea. Serum transfer did not affect nasal cavity colonization following high dose challenge.

Splenic and T cell adoptive transfers strongly indicated increased protection against respiratory challenge with pulmonary T cells, with D420Δ*btrS*-convalescent T cells demonstrating significant reductions in CFU relative to other groups. Similarly, to the serum transferred mice, neither splenic nor pulmonary T cells were successful in reducing CFU in the nasal cavity. The protection afforded to D420Δ*btrS* pulmonary T cell recipients support our previous observation that Δ*btrS*-convalescent immunity appears to be T cell mediated. Additionally, the highest degree of protection was generated using tissue-specific T cells from D420Δ*btrS*-convalescent mice, suggesting a possible role for BtrS in preventing localized T cell mediated immunity, possibly *via* $T_{RM.}$

DISCUSSION

Our lab and others have reported that BtrS regulates a diverse network of genes involved in host interactions and/or modulating host responses, including adenylate cyclase toxin, filamentous hemagglutinin, fimbria, pertactin, SphB1, autotransporters, the type III secretion system (T3SS), T3SS effectors, and many others with unknown functions[24]. It has been reported that disruption of BtrS-mediated regulation in *B. bronchiseptica* results in enhanced immunity measurable by multiple metrics, including specific T and B cell responses, elevated IgG & IgA, as well as the involvement of eosinophils in protection against bacterial infection. Use of Δ*btrS* mutants as a research tool to elucidate specific immunomodulatory mechanisms has also proven valuable. However, to date, nearly all experiments *in vitro* and *in vivo* have been performed using *B. bronchiseptica* mutants and lower respiratory (pulmonary) models of infection.

Here we report the first experiments in *B. pertussis* aimed at characterizing the immune responses to BtrS-regulated networks in the upper and lower respiratory tracts of mice. Excitingly, our data supports the observation of enhanced convalescent immunity is induced by

*Bp*Δ*btrS* mutant strains that facilitate complete clearance of the nasal cavity and lungs within 3 days post-secondary challenge: more effective protective immunity than that induced by wildtype *Bp* infection, wP, or aP vaccination. Of note, these observations were not accompanied by increased, but rather decreased CFU recovery from the respiratory tract during primary infection, indicating that these effects did not result from increased immune stimulation due to a higher bacterial load in the respiratory tract, but from the effects of *btrS* deletion.

We report that infection with either of two mutant strains (TohamaIΔ*btrS* or D420Δ*btrS*) results in enhanced convalescent immunity, characterized by significantly increased recovery of $CD4^+$ T cells with indications of increased $CD4^+$ T_{RM} cells and elevated IgA titers relative to wildtype-convalescence. Additionally, our data support that BtrS modulates host immunity *via* active expression and/or secretion of bacterial factors that impede the development of $CD4^+$ responses to infection. However, much remains to be elucidated. It has been reported that BtrS regulates very different genes in *B. bronchiseptica* and *B. pertussis*, with little known about the gene targets in the latter. Identification of regulated genes and systematic evaluation of their effects on host immunity may be beneficial in further determining the role(s) of these gene in *Bp* biology and pathogenesis. Together these data provide dual insights into how *Bp* modulates host immunity, but also how we can disrupt immunomodulatory networks, regulators, and virulence factors to observe unimpeded immunity against *Bp* as a tool for immunological discovery and vaccine development.

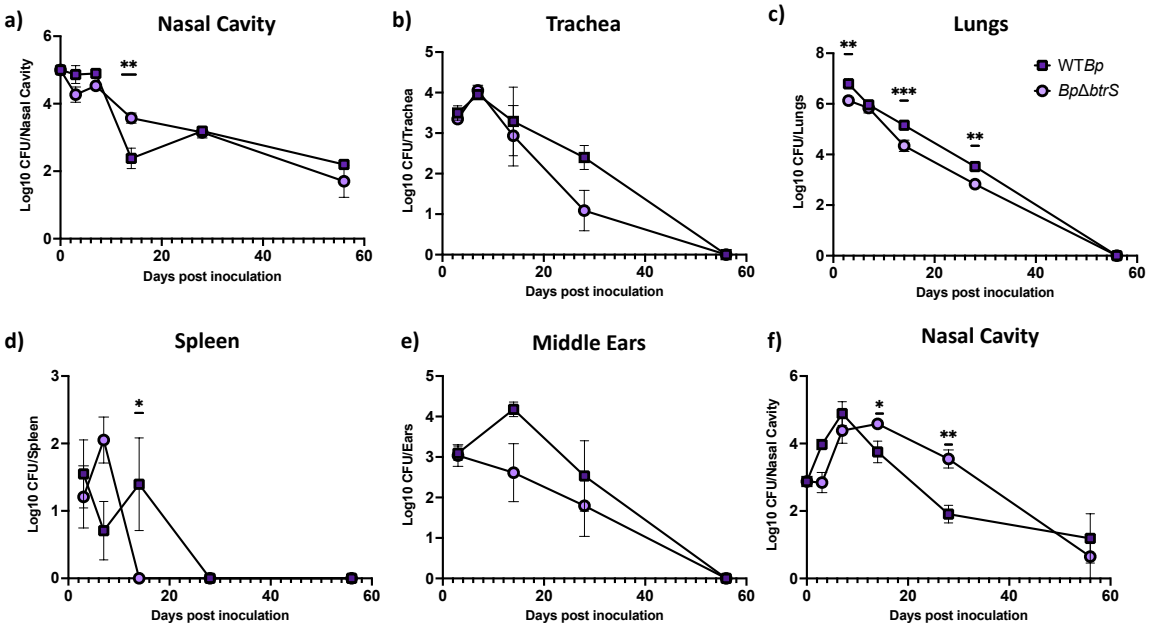


Figure 3.1 Recovery of wild type *B. pertussis* (Bp) or an isogenic mutant lacking *btrS* (BpΔ*btrS*) from the respiratory tract of C57BL/6J mice following primary pneumonic (**a-e**) or nasopharyngeal (**f**) challenge. (n=5, experiment conducted three times). Statistical significance was calculated using Two-Way ANOVA. Error bars show the standard error of the mean. n=5 per group. [ns p> 0.0332, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

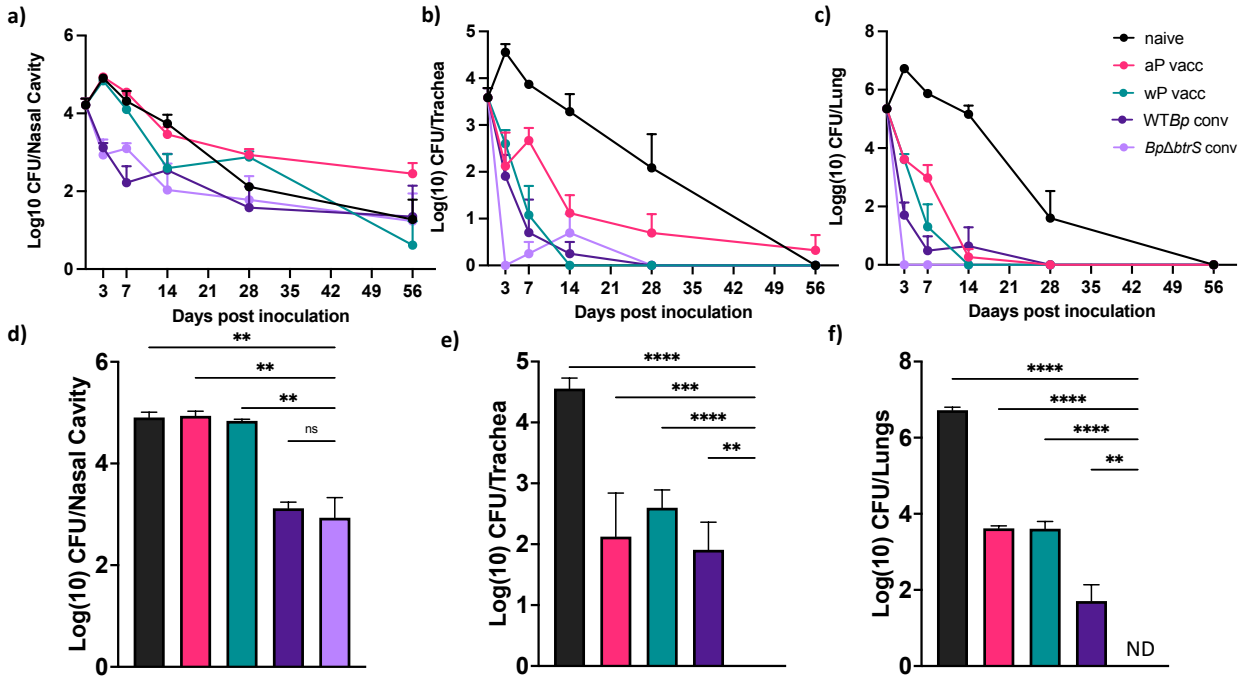


Figure 3.2: *Bp*Δ*btrS*-convalescent immunity enables complete clearance of pneumonic challenge in the lungs by D3pi. Number of CFU recovered from the (a) nasal cavity, (b) trachea, and (c) lungs of mice on days 3, 7, 14, 28, and 56 post-secondary challenge. Isolated comparison and statistical analysis of D3pc CFU recovered from the (d) nasal cavities, (e) trachea, and (f) lungs. Statistical significance was calculated *via* Two-way ANOVA. Error bars show the standard error of the mean. ND= not detected. n=5 per group. [ns p> 0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

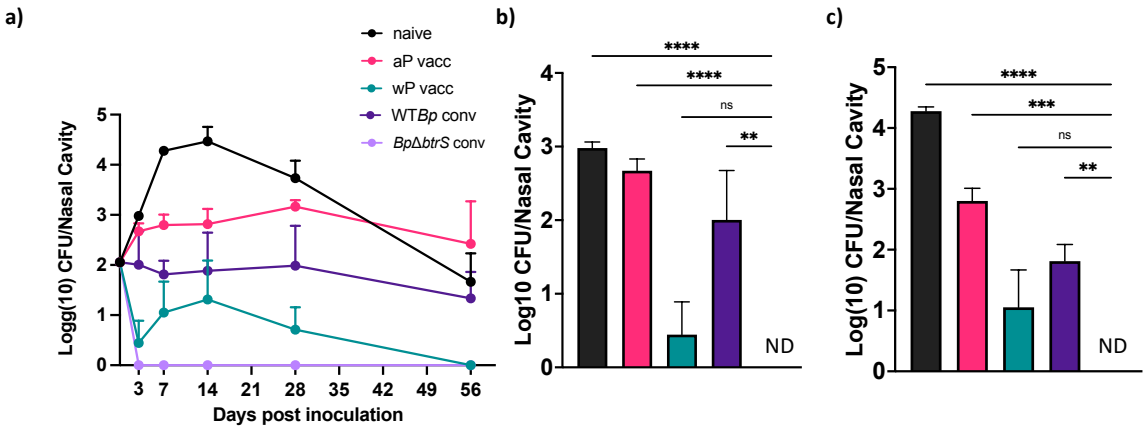


Figure 3.3: *BpΔbtrS*- convalescent immunity enables complete clearance of nasopharyngeal challenge by D3pi. Number of CFU recovered from the (a) nasal cavities of mice on days 3, 7, 14, 28, and 56 post-secondary challenge. Isolated comparison and statistics of CFU recovered on (b) D7pc and (c) D14pc from the nasal cavities. Statistical significance was calculated *via* Two-way ANOVA. Error bars show the standard error of the mean. ND= not detected. n=5 per group. [ns p> 0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

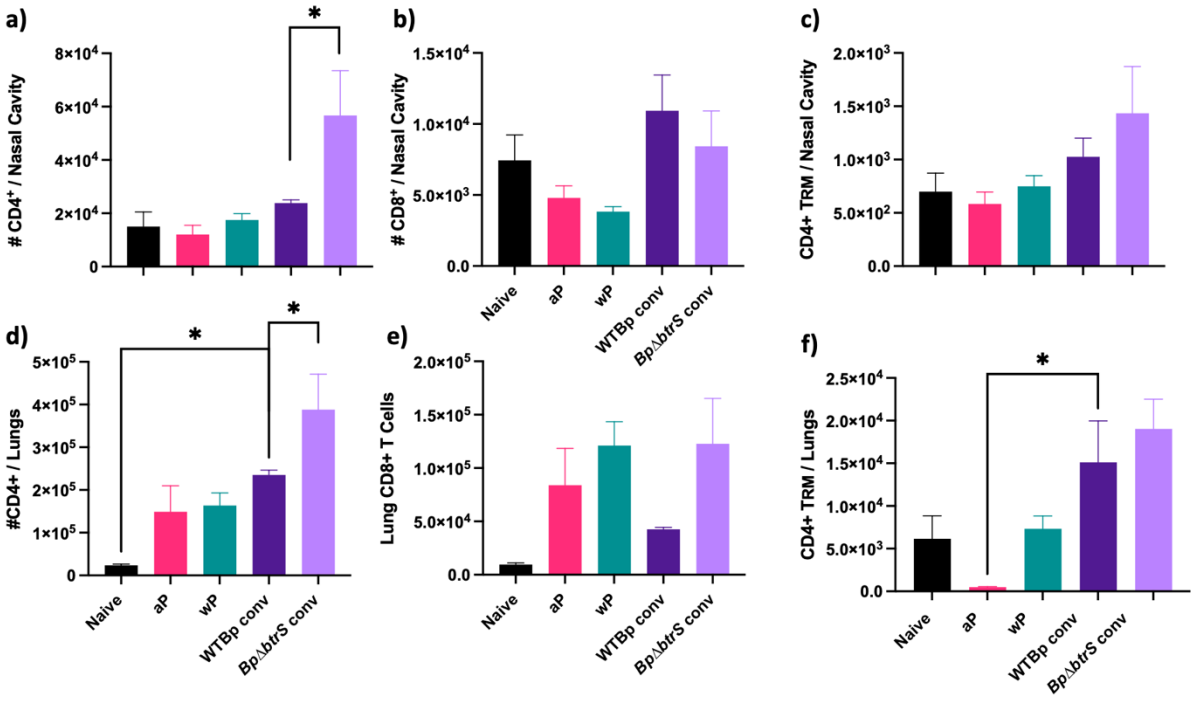


Figure 3.4 Flow cytometric analysis of lymphoid cells recruited to the nasal cavities of vaccinated or WT/*Bp*Δ*btrS*-convalescent mice on D3pi. a) nasal cavity $CD4^+$ T cells, b) nasal cavity $CD8^+$ T cells, (c) nasal cavity $CD4^+$ T_{RM}, (d) pulmonary $CD4^+$ T cells, (e) pulmonary $CD8^+$ T cells, (e) pulmonary $CD4^+$ T_{RM}. Statistical significance was calculated *via* One-way ANOVA. Error bars show the standard error of the mean. n=5 per group. [ns $p > 0.0332$, *$p < 0.0332$].

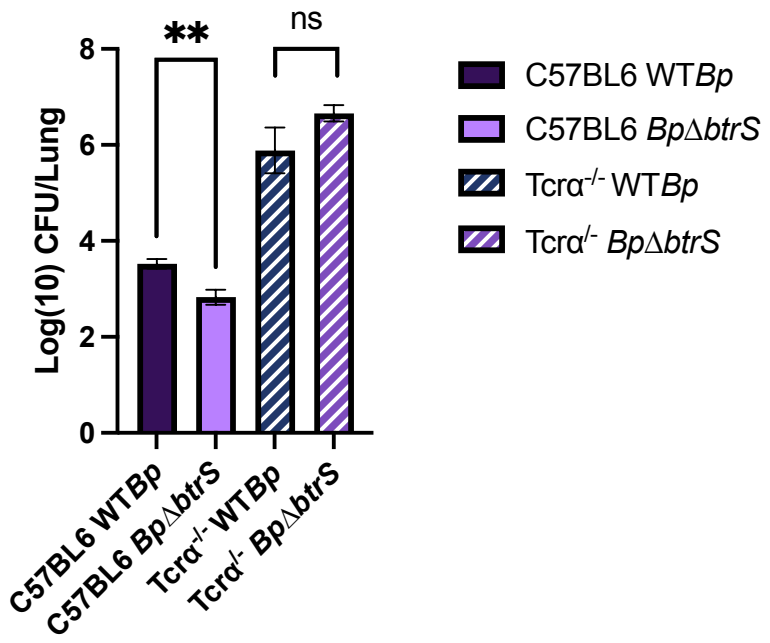


Figure 3.5 BtrS is required for Bp to modulate T cell responses. C57BL6/J or Tcra-/- mice were inoculated with $5X10^5$ CFU of either WT*Bp* or *Bp*Δ*btrS*. On d28pi lungs were harvested and bacterial CFU were enumerated. Statistical significance was calculated using Student's t test. Error bars show the standard error of the mean. n=5 per group. [ns $p > 0.0332$, **$p < 0.0021$.]

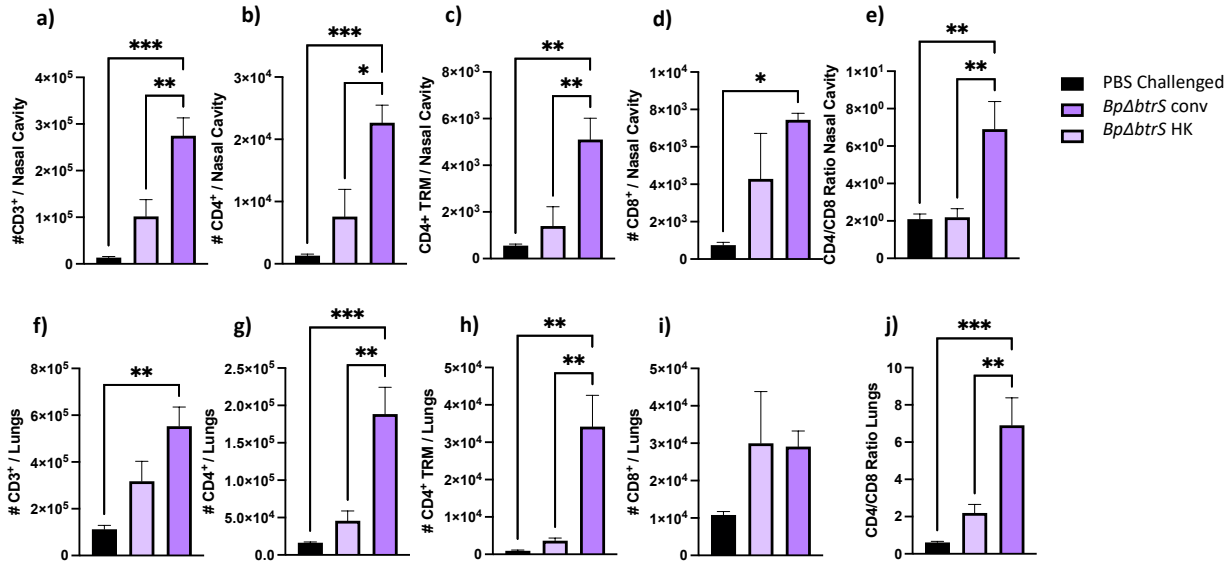


Figure 3.6 BtrS contributes to active immunomodulation.

Total $CD4^+$, $CD8^+$, and $CD4^+$ T_{RM} cells recovered from naïve, D420Δ*btrS*-convalescent, and heat killed D420Δ*btrS*-immunuzed mice following high dose challenge ($5X10^5$ CFU in 50µl PBS). Statistical analysis was calcualted *via* One-way ANOVA. Error bars show standard error of the mean. n= 5 [ns p> 0.0332, *p<0.0332 **p<0.0021, ***p<0.0002].

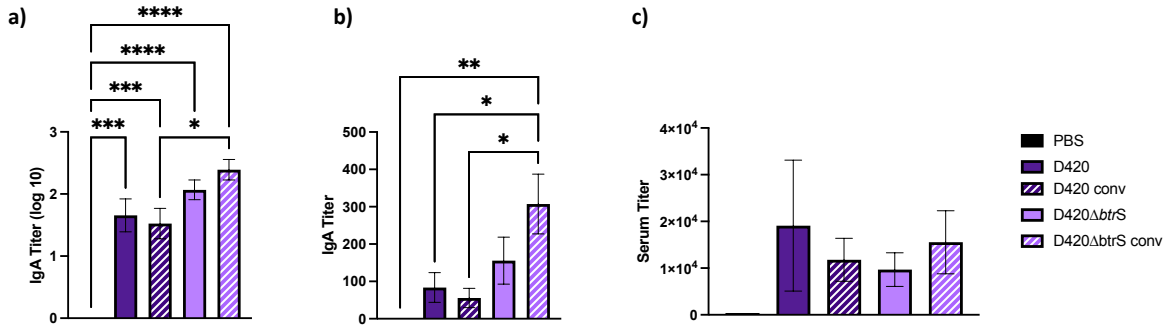


Figure 3.7 BtrS contributes to suppression of IgA responses in the nasopharynx.

Nasopharyngeal anti-*pertussis* IgA antibody titers (a) Log10, (b) linear scale, (c) serum anti-pertussis IgG titers recovered from D420 vs. D420Δ*btrS* during primary (D28pi) vs. secondary (D1pc) infection. Statistical analysis was calcualted *via* One-way ANOVA. Error bars show standard error of the mean. n= 5 [ns p> 0.0332, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

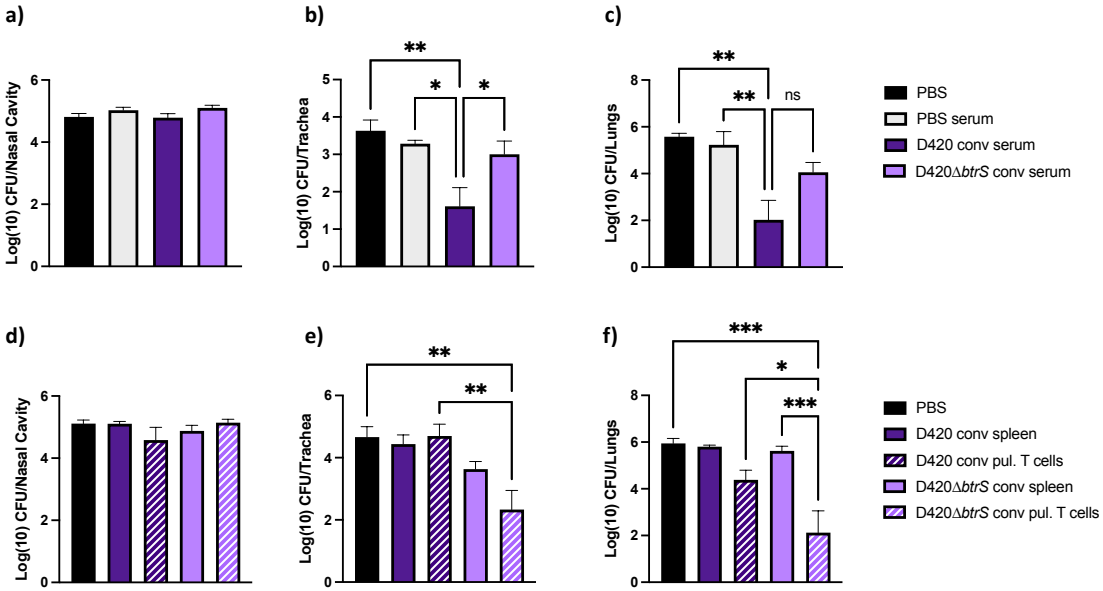


Figure 3.8: Transfer of T cells isolated from D420Δ*btrS*-convalescent mice enables faster clearance of WT *Bp* in Rag1$^{-/-}$ mice.

Rag-1$^{-/-}$ mice with adoptively transferred PBS, serum, splenocytes, or pulmonary T cells isolated from either naïve (PBS Serum), D420-convalescent, or D420Δ*btrS*-convalescent C57BL/6J mice. Data represents CFU recovered from respiratory tissues on day 14 post-challenge with D420. Statistical analysis was calcualted *via* One-way ANOVA. Error bars show standard error of the mean. n= 5 [ns p> 0.0332, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

PRIMER	SEQUENCE
BtrS_flank_R_Bp	5'- CAG GCC AAT ATC CTG TCT CAG CCG – 3'
BtrS_flank_F_Bp	5' – CTG CGC CAA GGA GCT CGA TCT GCA – 3'
YS009_PIPEve_pSS4245_F	5' – CTAGTGCGGCCGCCCTAGCATAGG - 3'
YS0010_PIPEve_pSS4245_R	5' – CCTAGGTGTACAGATCCGGACCTGC – 3'

Table S3.1 Primers used to for PIPE cloning & generation of mutant *B. pertussis* strains.

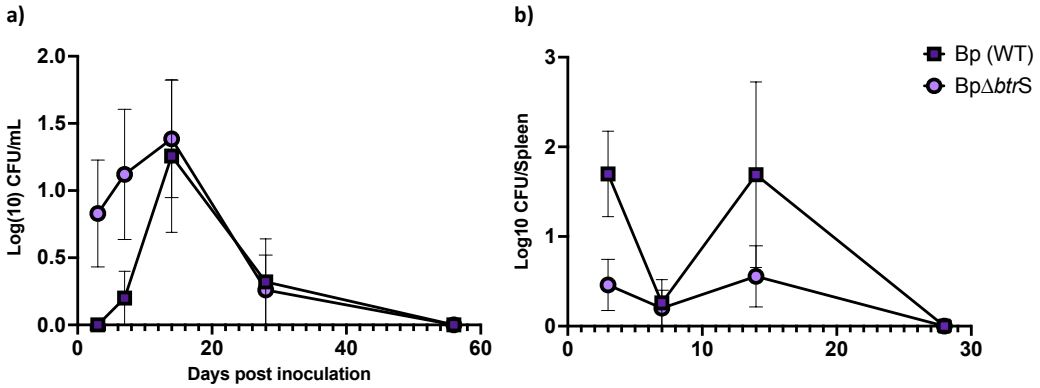


Figure S3.1 Recovery of wild type *B. pertussis* (Bp) or an isogenic mutant lacking BtrS (*BpΔbtrS*) from the (a) trachea and (b) spleen of C57BL/6J mice following primary nasopharyngeal challenge. (n=5, experiment conducted three times). Statistical significance was calculated using Two-Way ANOVA. Error bars show the standard error of the mean. n=5 per group.

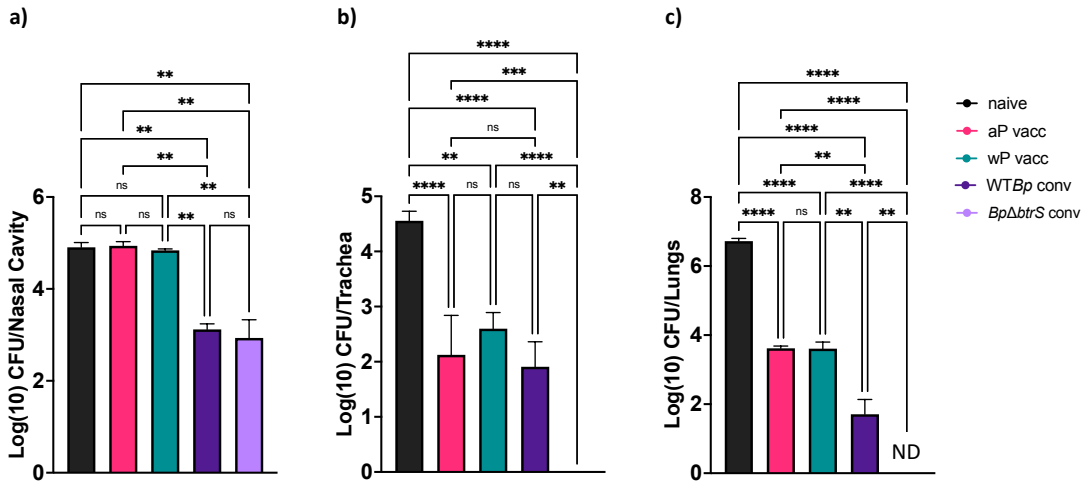


Figure S3.2 *Bp*Δ*btrS*-convalescent immunity enables complete clearance of pneumonic challenge from the lungs by D3pi (all statistical comparisons). Number of CFU recovered from the (a) nasal cavity, (b) trachea, and (c) lungs of mice on D3 post-secondary challenge. Statistical significance was calculated *via* Two-way ANOVA. Error bars show the standard error of the mean. ND= not detected. n=5 per group. [ns p> 0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001].

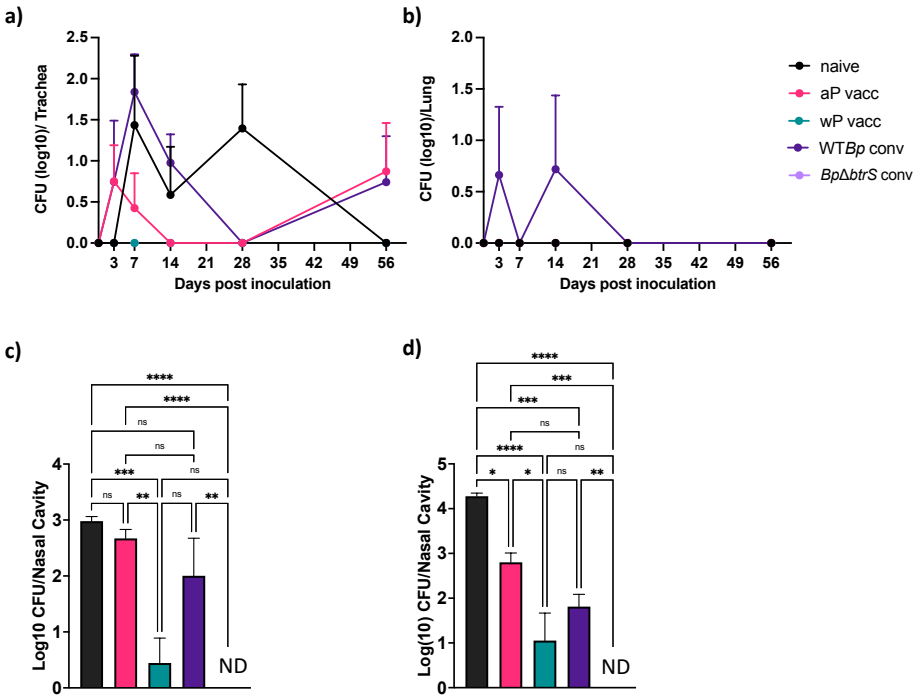


Figure S3.3: *Bp*Δ*btrS*- convalescent immunity enables complete clearance of nasopharyngeal challenge by D3pi (all statistical comparisons). Number of CFU recovered from the (a) trachea and (b) lungs of mice on days 3, 7, 14, 28, and 56 post-secondary challenge. Isolated comparison and statistics of CFU recovered on (b) D7pc and (c) D14pc from the nasal cavities. Statistical significance was calculated *via* Two-way ANOVA. Error bars show the standard error of the mean. ND= not detected. n=5 per group. [ns $p > 0.0332$, *$p < 0.0332$, **$p < 0.0021$, ***$p < 0.0002$, ****$p < 0.0001$].

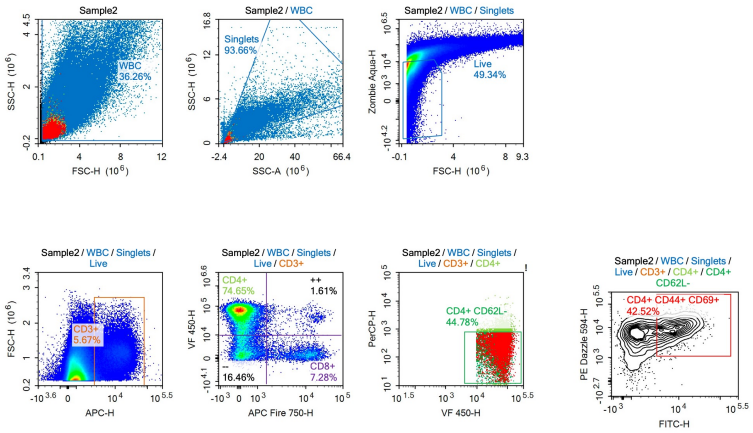


Figure S3.4 Gating strategy for the enumeration of T cell subsets in *B. pertussis* challenge experiments.

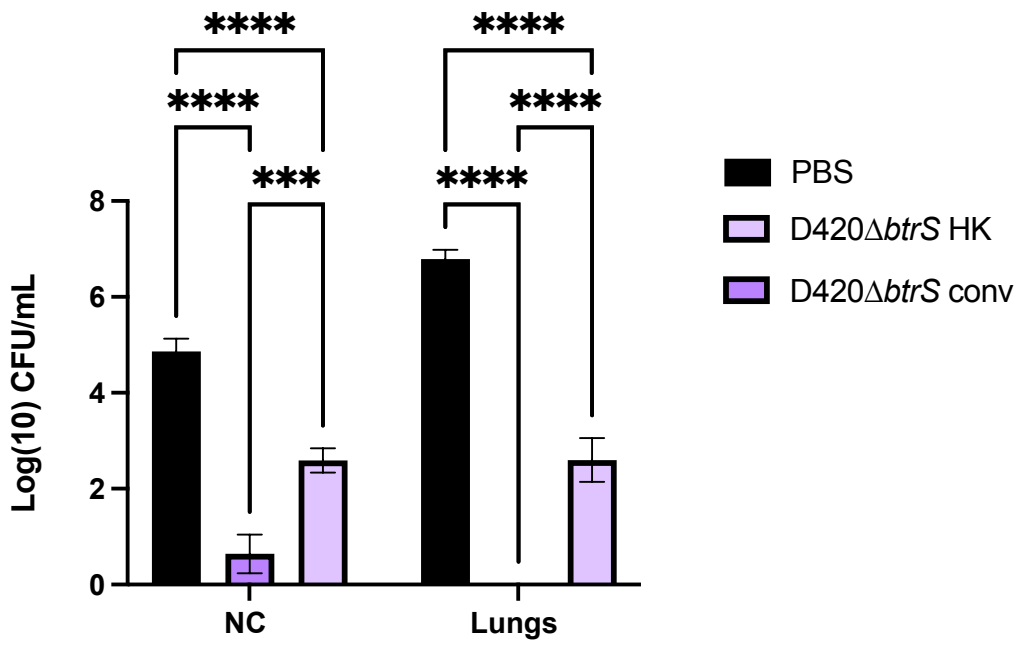


Figure S3.5

Total CFU recovered from naïve, D420Δ*btrS*-convalescent, and heat killed D420Δ*btrS*-immunized mice following high dose challenge ($5X10^5$ CFU in 50µl PBS). Statistical significance was calculated *via* One-way ANOVA. Error bars show the standard error of the mean. n=5 per group. [***p<0.0002, ****p<0.0001].

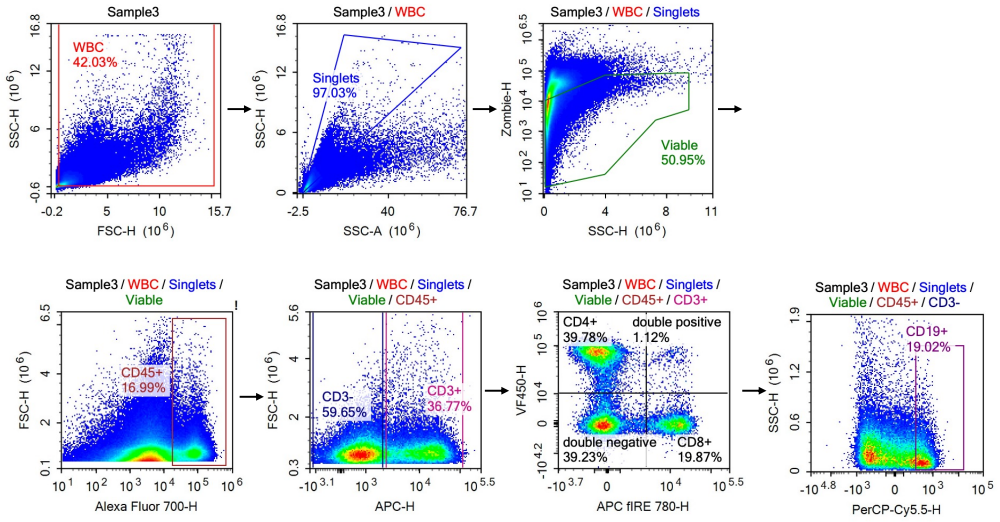


Figure S3.6 Gating strategy for RB50 experiments.

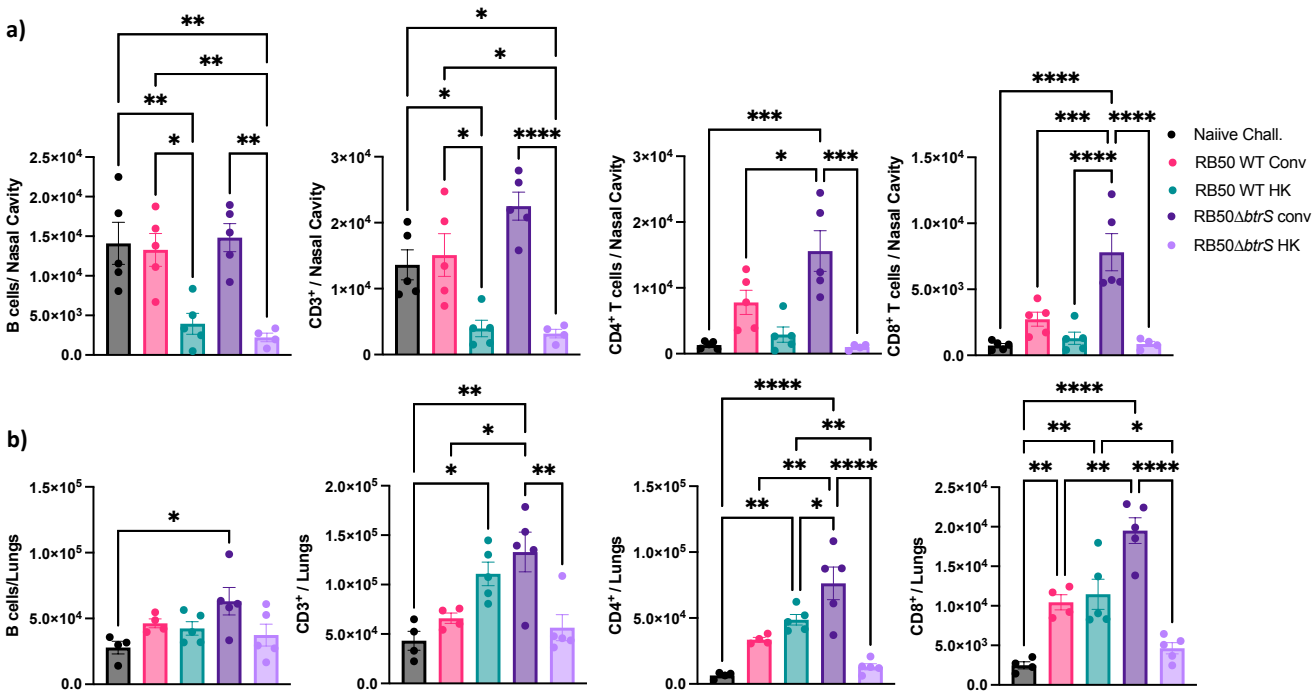


Figure S3.7 Total $CD4^+$ and $CD8^+$ T cells recovered from mice. (a) nasal cavities, (b) lungs of naïve, RB50-convalescent, or RB50-vaccinated C57BL6/J mice following high dose challenge ($5X10^5$ CFU in 50µl PBS). Naïve (PBS inoculated) mice, 56 d.p.i. convalescent mice following a low dose inoculum of *B. bronchiseptica* (500 CFU in 5µL of PBS), or mice intranasally vaccinated with $5x10^7$ CFU of heat killed *B. bronchiseptica*. Statistical analysis was calcualted *via* one-way ANOVA. Error bars show standard error of the mean (n= 5, experiment conducted once). [*p<0.05 and **p<0.002].

REFERENCES

1. Roush SW, Murphy TV, Vaccine-Preventable Disease Table Working G. Historical comparisons of morbidity and mortality for vaccine-preventable diseases in the United States. *JAMA*. 2007;298(18):2155- 63. Epub 2007/11/15. doi: 10.1001/jama.298.18.2155. PubMed PMID: 18000199.
2. Jackson, D. W., & Rohani, P. (2014). Perplexities of pertussis: Recent global epidemiological trends and their potential causes. *Epidemiology and Infection*.
3. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, et al. (2010) Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet 375: 1969–1987.
4. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proc Natl Acad Sci U S A*. 2014;111(2):787-92. Epub 2013/11/28. doi: 10.1073/pnas.1314688110. PubMed PMID: 24277828; PMCID: PMC3896208.
5. Jackson, D. W., & Rohani, P. (2014). Perplexities of pertussis: Recent global epidemiological trends and their potential causes. *Epidemiology and Infection*.
6. Safarchi, A., Octa*via*, S., Luu, L. D. W., Tay, C. Y., Sintchenko, V., Wood, N., … Lan, R. (2015). Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. *Vaccine*.
7. Ryan, M., Gothefors, L., Storsaeter, J., & Mills, K. H. (1997). *Bordetella pertussis*-specific Th1/Th2 cells generated following respiratory infection or immunization with an acellular vaccine: comparison of the T cell cytokine profiles in infants and mice. *Developments in Biological Standardization*, *89*.

8. Marcellini, V., Mortari, E. P., Fedele, G., Gesualdo, F., Pandolfi, E., Midulla, F., Leone, P., Stefanelli, P., Tozzi, A. E., Carsetti, R., Agricola, E., Ausiello, C. M., Buttinelli, G., Campagna, I., Concato, C., Del Chierico, F., Di Mattia, G., Ferretti, B., Frassanito, A., … Villani, A. (2017). Protection against pertussis in humans correlates to elevated serum antibodies and memory B cells. *Frontiers in Immunology*, *8*(SEP).
9. Mills, K. H. G., Ryan, M., Ryan, E., & Mahon, B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell- mediated immunity in protection against *Bordetella pertussis*. *Infection and Immunity*, *66*(2).
10. Wilk, M. M., Borkner, L., Misiak, A., Curham, L., Allen, A. C., & Mills, K. H. G. (2019). Immunization with whole cell but not acellular pertussis vaccines primes CD4 TRM cells that sustain protective immunity against nasal colonization with *Bordetella pertussis*. *Emerging Microbes and Infections*, *8*(1).
11. Borkner, L., Curham, L. M., Wilk, M. M., Moran, B., & Mills, K. H. G. (2021). IL-17 mediates protective immunity against nasal infection with *Bordetella pertussis* by mobilizing neutrophils, especially Siglec-F+ neutrophils. *Mucosal Immunology*, *14*(5).
12. Brummelman, J., Wilk, M. M., Han, W. G. H., van Els, C. A. C. M., & Mills, K. H. G. (2015). Roads to the development of improved pertussis vaccines paved by immunology. In *Pathogens and disease* (Vol. 73, Issue 8).
13. Wilk, M. M., & Mills, K. H. G. (2018). CD4 TRM cells following infection and immunization: Implications for more effective vaccine design. In *Frontiers in Immunology* (Vol. 9, Issue AUG).

14. Gestal, M. C., Whitesides, L. T., & Harvill, E. T. (2019). Integrated Signaling Pathways Mediate *Bordetella* Immunomodulation, Persistence, and Transmission. In *Trends in Microbiology* (Vol. 27, Issue 2).

15. Solans, L., Debrie, A. S., Borkner, L., Aguiló, N., Thiriard, A., Coutte, L., Uranga, S., Trottein, F., Martín, C., Mills, K. H. G., & Locht, C. (2018). IL-17-dependent SIgA-mediated protection against nasal *Bordetella pertussis* infection by live attenuated BPZE1 vaccine. *Mucosal Immunology*, *11*(6). https://doi.org/10.1038/s41385-018-0073-9

16. Locht, C., Papin, J. F., Lecher, S., Debrie, A. S., Thalen, M., Solovay, K., Rubin, K., & Mielcarek, N. (2017). Live Attenuated Pertussis Vaccine BPZE1 Protects Baboons Against *Bordetella pertussis* Disease and Infection. *Journal of Infectious Diseases*, *216*(1). https://doi.org/10.1093/infdis/jix254

17. Gestal MC, Howard LK, Dewan KK, Harvill ET. Bbvac: A Live Vaccine Candidate That Provides Long-Lasting Anamnestic and Th17-Mediated Immunity against the Three Classical *Bordetella* spp. *mSphere*. 2022 Feb 23;7(1):e0089221. doi: 10.1128/msphere.00892-21. Epub 2022 Feb 23. PMID: 35196124; PMCID: PMC8865921.

18. Gestal, M. C., Whitesides, L. T., & Harvill, E. T. (2019). Integrated Signaling Pathways Mediate *Bordetella* Immunomodulation, Persistence, and Transmission. In *Trends in Microbiology* (Vol. 27, Issue 2).

19. Gestal, M. C., Howard, L. K., Dewan, K., Johnson, H. M., Barbier, M., Bryant, C., Soumana, I. H., Rivera, I., Linz, B., Blas-Machado, U., & Harvill, E. T. (2019). Enhancement of immune response against *Bordetella* spp. by disrupting immunomodulation. *Scientific Reports*, *9*(1).

20. Ahuja U, Shokeen B, Cheng N, Cho Y, Blum C, Coppola G, Miller JF. Differential regulation of type III secretion and virulence genes in Bordetella pertussis and *Bordetella bronchiseptica* by a secreted anti-σ factor. *Proc Natl Acad Sci U S A*. 2016 Mar 1;113(9):2341-8. doi: 10.1073/pnas.1600320113. Epub 2016 Feb 16. PMID: 26884180; PMCID: PMC4780644.T3SS

21. Inatsuka CS, Xu Q, Vujkovic-Cvijin I, Wong S, Stibitz S, Miller JF, Cotter PA. Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. *Infect Immun*. 2010 Jul;78(7):2901-9. doi: 10.1128/IAI.00188-10. Epub 2010 Apr 26. PMID: 20421378; PMCID: PMC2897405.

22. Soumana, I. H., Linz, B., Dewan, K. K., Sarr, D., Gestal, M. C., Howard, L. K., Caulfield, A. D., Rada, B., & Harvill, E. T. (2021). Modeling immune evasion and vaccine limitations by targeted nasopharyngeal *Bordetella pertussis* inoculation in mice. *Emerging Infectious Diseases*, *27*(8).

23. Wilk, M. M., Borkner, L., Misiak, A., Curham, L., Allen, A. C., & Mills, K. H. G. (2019). Immunization with whole cell but not acellular pertussis vaccines primes CD4 TRM cells that sustain protective immunity against nasal colonization with *Bordetella pertussis. Emerging Microbes and Infections*, *8*(1).

24. Dewan KK, Taylor-Mulneix DL, Campos LL, Skarlupka AL, Wagner SM, Ryman VE, Gestal MC, Ma L, Blas-Machado U, Faddis BT, Harvill ET. A model of chronic, transmissible Otitis Media in mice. *PLoS Pathog.* 2019 Apr 10;15(4):e1007696. doi: 10.1371/journal.ppat.1007696. Erratum in: PLoS Pathog. 2019 Jun 28;15(6):e1007916. PMID: 30970038; PMCID: PMC6476515.

25. Ahuja, U., Shokeen, B., Cheng, N., Cho, Y., Blum, C., Coppola, G., & Miller, J. F. (2016). Differential regulation of type III secretion and virulence genes in *Bordetella pertussis* and *Bordetella bronchiseptica* by a secreted anti-σ factor. *ProcNat Acad Sci USA*, *113*(9).

26. Gestal, M. C., Blas-Machado, U., Johnson, H. M., Rubin, L. N., Dewan, K. K., Bryant, C., Tiemeyer, M., & Harvill, E. T. (2020). Disrupting *Bordetella* immunosuppression reveals a role for eosinophils in coordinating the adaptive immune response in the respiratory tract. *Microorganisms*, *8*(11).

27. Marcellini, V., Mortari, E. P., Fedele, G., Gesualdo, F., Pandolfi, E., Midulla, F., Leone, P., Stefanelli, P., Tozzi, A. E., Carsetti, R., Agricola, E., Ausiello, C. M., Buttinelli, G., Campagna, I., Concato, C., Del Chierico, F., Di Mattia, G., Ferretti, B., Frassanito, A., … Villani, A. (2017). Protection against pertussis in humans correlates to elevated serum antibodies and memory B cells. *Frontiers in Immunology*, *8*(SEP).

28. Mills, K. H. G., Ryan, M., Ryan, E., & Mahon, B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell- mediated immunity in protection against Bordetella pertussis. *Infection and Immunity*, *66*(2).

29. Wilk, M. M., Borkner, L., Misiak, A., Curham, L., Allen, A. C., & Mills, K. H. G. (2019). Immunization with whole cell but not acellular pertussis vaccines primes CD4 TRM cells that sustain protective immunity against nasal colonization with *Bordetella pertussis. Emerging Microbes and Infections*, *8*(1).

30. Borkner, L., Curham, L. M., Wilk, M. M., Moran, B., & Mills, K. H. G. (2021). IL-17 mediates protective immunity against nasal infection with *Bordetella pertussis* by mobilizing neutrophils, especially Siglec-F+ neutrophils. *Mucosal Immunology*, *14*(5).
31. Brummelman, J., Wilk, M. M., Han, W. G. H., van Els, C. A. C. M., & Mills, K. H. G. (2015). Roads to the development of improved pertussis vaccines paved by immunology. In *Pathogens and Disease* (Vol. 73, Issue 8).
32. Wilk, M. M., & Mills, K. H. G. (2018). CD4 TRM cells following infection and immunization: Implications for more effective vaccine design. In *Frontiers in Immunology* (Vol. 9, Issue AUG).
33. Ross, P. J., Sutton, C. E., Higgins, S., Allen, A. C., Walsh, K., Misiak, A., Lavelle, E. C., McLoughlin, R. M., & Mills, K. H. G. (2013). Relative Contribution of Th1 and Th17 Cells in Adaptive Immunity to *Bordetella pertussis*: Towards the Rational Design of an Improved Acellular Pertussis Vaccine. *PLoS Pathogens*, *9*(4).
34. Chasaide, C. N., & Mills, K. H. G. (2020). Next-generation pertussis vaccines based on the induction of protective t cells in the respiratory tract. In *Vaccines* (Vol. 8, Issue 4).
35. Lee, A. D., Cassiday, P. K., Pawloski, L. C., Tatti, K. M., Martin, M. D., Briere, E. C., Lucia Tondella, M., & Martin, S. W. (2018). Clinical evaluation and validation of laboratory methods for the diagnosis of *Bordetella pertussis* infection: Culture, polymerase chain reaction (PCR) and anti-pertussis toxin IgG serology (IgG-PT). *PLoS ONE*, *13*(4).
36. Yu, Y. R. A., O'Koren, E. G., Hotten, D. F., Kan, M. J., Kopin, D., Nelson, E. R., Que, L., & Gunn, M. D. (2016). A protocol for the comprehensive flow cytometric analysis of immune cells in normal and inflamed murine non-lymphoid tissues. *PLoS ONE*, *11*(3).

CHAPTER 4

ADAPTIVE IMMUNE PROTECTION OF THE MIDDLE EARS DIFFERS FROM THAT OF THE RESPIRATORY TRACT

Amanda D. Caulfield, Kalyan K. Dewan, Yang Su, Collen J. Sedney, Maiya Callender, Jillian Masters, Eric T. Harvill. 2023. *Front. Microbiol.*

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ABSTRACT

The efficacy of the adaptive immune system in the middle ear (ME) is well established, but the mechanisms are not as well defined as those of gastrointestinal or respiratory tracts. While cellular elements of the adaptive response have been detected in the MEs following infections (or intranasal immunizations), their specific contributions to protecting the organ against reinfections are unknown. How immune protection mechanisms of the MEs compares with those in the adjacent and attached upper and lower respiratory airways remains unclear. To address these knowledge gaps, we used an established mouse respiratory infection model that we recently showed also involves extremely efficient ME infections. *Bordetella bronchiseptica* delivered to the external nares of mice in tiny numbers very efficiently infects the respiratory tract and ascends the Eustachian tube to colonize and infect both MEs of all animals, where it consistently causes severe but acute inflammation resembling human acute otitis media (AOM). Since this AOM naturally resolves, we here examine the immunological mechanisms that clear infection and protect against subsequent infection, to guide efforts to induce protective immunity in the ME. Our results show that once the MEs are cleared of a primary B. bronchiseptica infection, the convalescent organ is strongly protected from reinfection by the pathogen despite its persistence in the upper respiratory tract, suggesting important immunological differences in these adjacent and connected organs. CD4+ and CD8+ T cells trafficked to the MEs following infection and were necessary to robustly protect against secondary challenge. Intranasal vaccination with heat killed B. bronchiseptica conferred robust protection against infection to the MEs, even though the nasopharynx itself was only partially protected. These data establish the MEs as discrete effector sites of adaptive immunity and shows that effective protection in the MEs and the respiratory tract is significantly different. This model system allows the dissection

of immunological mechanisms that can prevent bacteria in the nasopharynx from ascending the ET to colonize the ME, providing critical knowledge to guide the development of vaccines against AOM.

INTRODUCTION

Certain pathogens and commensals of the upper respiratory airways can ascend the Eustachian tube (ET) to reach, colonize and infect the middle ears (MEs) (1-3). This often happens during transient disruptions of the innate immune barriers to the MEs caused by infections of the upper respiratory tract and is particularly prevalent among infants (4, 5). The resulting inflammatory response in the ME, otitis media (OM), involves pain and fever (6) and can be traumatic for both patient and caregiver. Although most infections are acute (Acute Otitis Media: AOM) and resolve within a fortnight, the sheer volume of cases (7, 8) make AOM amongst the leading causes of urgent pediatric clinic visits, ambulatory surgery and prescribing of antibiotics [9]. In addition, the "proneness" to suffer OM varies (10, 11), and in an estimated 8% of cases the infections can progress to several chronic forms of ear diseases (Chronic Otitis Media, COM) where the outcomes are more severe, being the leading causes of hearing impairment and deafness (6, 12). The societal impact of preventing OM would indeed be profound. However, controlling the burden of disease using vaccines targeted against upper respiratory tract pathogens remains challenging due to our relatively poor understanding of adaptive immunity in the ME (1-3, 13,14). There are limitations to OM infection models using human otopathogens, which alone do not generally infect the MEs of healthy mice, limiting our ability to study and understand the generation of effective acquired immune protection in the ME. A particular knowledge gap lies in understanding how immune components might contribute differently to protection of the ME and the connected respiratory airways (19-21).

Over the course of conducting studies on the respiratory tract pathogens belonging to the genus *Bordetella* (22), we observed and reported that two mouse pathogens, *Bordetella pseudohinzii* (23) and *Bordetella bronchiseptica* (24) can rapidly and efficiently ascend the ET

from the nasopharynx to colonize and infect the MEs of mice. While infections by *B. pseudohinzii* are chronic and persist in the MEs, eventually leading to the deafness of the mice, *B. bronchiseptica* is controlled and subsequently cleared from the MEs over a period of convalescence coincident with the generation of a robust immune response. Here we use the *B. bronchiseptica*-mouse infection model in which bacteria in the nasopharynx naturally and efficiently ascend the ET to colonize and transiently infect the ME but are cleared by adaptive immunity. We identify the immune parameters induced by infection and vaccination and define their roles in protecting the ME, and how those roles differ from that of the adjacent and contiguous respiratory organs.

MATERIALS & METHODS

Bacterial cultures

Bordetella bronchiseptica RB50 and its gentamicin derivative resistant strain were maintained on Bordet-Gengou agar (DIFCO) supplemented with 10% sheep blood (Hema Resources) and 20 μg/mL streptomycin or gentamicin (Sigma) incubated for 36 hours at 37°C. Liquid cultures of the bacteria for inoculations were prepared in antibiotic-supplemented Stainer-Scholte broth at 37°C with shaking (200 RPM). Bacterial numbers were estimated spectrophotometrically at OD of 600 nm ($0.1 \cong 2 \times 10^8$ CFU/mL).

Heat-killed bacteria used in intranasal vaccination was prepared by heating the bacteria ($\sim 1 \times 10^9$ CFU/mL) at 65^0C for 45 minutes in PBS. Aliquots of the samples were plated on BG agar to confirm loss of bacterial *via*bility following treatment. Mice were immunized with 50μL of heat killed bacteria (5×10^7 CFU).

Mouse infections

The mouse strains, C57BL/6J, and derivative CD45.1 (B6.SJL-*Ptprca Pepcb*/BoyJ) and the *Rag-1-/-,* used in this study were obtained from Jackson laboratories and housed in SPF facilities at the University of Georgia throughout the experiments. Experiments were conducted with an n= 3-5 mice. Low dose nasopharyngeal infections were initiated by delivering ~ 500 CFU of the bacteria in 5 μL of PBS onto the external nares of lightly anesthetized mice (5% isoflurane). High dose bacterial challenges were conducted by droplet inhalation of ~ 5 x 10^5 CFU of the bacteria suspended in 50 μ L of PBS and delivered onto the external nares of anesthetized mice. To assess the colonization load, respiratory organs (nasal cavities, trachea lungs and middle ears) were carefully dissected out from mice that had been euthanized by CO_2 inhalation (1.5 L/min). For establishing bacterial loads, the organs were homogenized in a bead mill beater 1 mL of PBS and CFU numbers enumerated by dilution plating aliquots of the homogenized samples on Bordet-Gengou agar with appropriate antibiotics. Where flow cytometric analysis was conducted, organs were collected in ice-cold DMEM and carefully ground over 70-micron filter. Cellular content of the filtrate was gathered by centrifugation at 4000g for 5 minutes at 4^0C and resuspended in Dulbecco modified Eagle medium (DMEM) for subsequent flow analyses.

Flow cytometry

Nasal cavities (NALT), lungs, and/or middle ears were processed and stained as previously described. *Via*ble cells were identified with Zombie Aqua (Biolegend). Antibodies to identify T cell populations were (Biolegend, Invitrogen) included CD45.2 (AF 700), CD45.1 (PE Dazzle 594), CD3 (APC), CD4 (VF450), CD8 (APC Fire 750). Acquisition of the data was

performed using the Novocyte Quanteon (Agilent) and analyzed with NovoExpress software (Agilent) following standard gating strategies (Supplementary Figures 4.4 and 4.5).

Histology

Histopathology of the middle ears was assessed (n= 4 - 5) following fixation of mice in neutral-buffered, 10% formalin solution and subsequent decalcification in Kristensen's solution. Tissues were embedded in paraffin, sectioned at approximately 5 μm. Coronal sections through the nose and brain, and transverse sections at the level of the middle and inner ear were collected and stained with hematoxylin and eosin. Histopathological exam consisted of evaluation of the nose for the incidence (presence or absence), severity, and distribution of inflammation.

Statistical analysis

Data generated was statistically evaluated by Student's t-test and 2-way ANOVA using the statistical analyses package of GraphPad prism (V2.0)

RESULTS

B. bronchiseptica follows a course of primary infection in the middle ears distinct from that of the upper and lower respiratory tract

Respiratory pathogens associated with AOM in humans can often be detected persisting within the nasopharynx after OM resolves (25-27), suggesting immune responses may function more robustly and/or effectively in the ME. To define how an infection of the MEs proceeds, and how it might differ from that of the respiratory tract, we used *B. bronchiseptica* to follow the course of infection in groups of wild type (C57BL/6J) mice. The mice were inoculated intranasally with a low number of *B. bronchiseptica* in a small volume of PBS (~500 CFU in 5mL PBS) to localize the initial infection to the nasal cavities. Bacterial load in respiratory

organs (nasal cavities, trachea, lungs) and MEs were then monitored over days 1, 2, 3, 7, 14, 28 and 56 post inoculation (p.i.) (Figure 4.1A). As expected, *B. bronchiseptica* grew rapidly (~1000-fold) in the nasal cavity to very high numbers (>100,000 cfu) in the first week, gradually declining thereafter but persisting for 8 weeks here, and for life in every mouse in many prior experiments, as expected for a highly efficient nasopharyngeal commensal/pathogen. Also as expected, *B. bronchiseptica* remained mostly undetected in the lungs (limit of detection 10 CFU). However, although *B. bronchiseptica* was not initially delivered to the ME, both MEs of all animals were colonized by day 2 p.i. and high numbers (~10,000 CFU) were recovered at day 7 p.i. These numbers then dropped close to detection limits by day 28 p.i. and no bacteria were recovered from the MEs by day 56 p.i., indicating complete clearance. Histological examination of H/E-stained sections showed evidence of inflammation and hyperplasia at day 7 p.i, (peak of infection), but by day 56 inflammation had largely subsided (Figure 4.1B). These results show that despite being contiguous, the course of infection of the MEs differs from that of either the upper or the lower respiratory tract. The results also show that *B. bronchiseptica*, generally considered a respiratory pathogen, can rapidly overcome the substantial physical (cilia and mucus entrapment) and immunological (various innate responses) barriers of the Eustachian tube to efficiently reach and multiply within the MEs but does not effectively reach the lower respiratory tract. This is an important concern for conventional experimental studies of *Bordetella* spp. virulence mechanisms, which generally involve delivering *B. bronchiseptica* deep into the lungs, as discussed below.

MEs of convalescent mice are robustly protected against secondary challenge

The observation that B. bronchiseptica efficiently colonized the ME but is cleared over the space of about four weeks implicates adaptive immune functions in providing sterilizing

immunity in the ME. To determine whether mice that cleared *B. bronchiseptica* from the ME have effective anamnestic immunity, and contrast immunity in the ME to that in respiratory organs, we tested how well convalescent mice responded to a secondary challenge. We inoculated mice (n=5) with the low dose *B. bronchiseptica* as above and allowed the infection to progress for 56 days. On day 56 p.i, the convalescent mice and an age- matched naïve group, were challenged with the conventional high dose-high volume inoculum ($5x10^5$ CFU delivered in 50 μL PBS) of a gentamicin resistant derivative of wild type *B. bronchiseptica*, to distinguish the very high dose/volume secondary challenge from remnants of the initial low dose/volume inoculation (primary challenge). The strength of the high dose and volume challenge is that it ensures that the entire respiratory tract receives a substantial challenging load of the pathogen, although its delivery of bacteria to the ME is less well known. 3 days post challenge mice were sacrificed and bacterial loads in the respiratory organs and MEs were enumerated. The low dose primary (gentamicin *sensitive*) and high dose challenge (gentamicin *resistant*) bacteria were differentiated based on their ability to grow on the gentamicin supplemented agar plates.

As shown in Figure 4.2, the naïve control group had high numbers of CFU in all respiratory organs and MEs (between 10,000 and 1,000,000 CFU). The convalescent group retained relatively small numbers of the primary challenge inocula in the nasal cavity and trachea but had nearly or completely cleared this from the MEs and lungs. Following the secondary challenge, bacterial numbers in the nasal cavities of these convalescent mice were reduced by 100-fold relative to naïve mice, indicating partial but incomplete protection. In the lungs, robust protection was observed against the secondary challenge, demonstrating effective protective immunity despite almost no detected bacteria colonizing the lower respiratory tract over the course of the initial 56 day infection (Figure 4.1A). Interestingly, *B. bronchiseptica* was nearly or

completely cleared from the ME, reflecting a level of protection similar to that of the lungs, and substantially more complete than that of the nasal cavity. We also observed similar protection when convalescent mice were rechallenged with low numbers of *B. bronchiseptica* (500 CFU delivered in 5 μL PBS) (Figure S4.1). These results demonstrate that the ME of convalescent mice are robustly protected from reinfection and show that adaptive immunity in the MEs is as effective as that of the lower respiratory tract, and significantly more protective than that of the nasal cavity.

Immune cells from the spleens traffic to the middle ears

We next turned our attention to examining the contributions of humoral and cellular components of adaptive immunity on convalescent protection. We had previously observed (24) that when immune serum collected from convalescent WT mice (day 56 p.i.) was intraperitoneally injected into naive *Rag-1*$^{-/-}$ mice and challenged, bacterial CFUs in the MEs were reduced by ~ 100-fold compared to untreated *Rag-1*$^{-/-}$ mice. This indicated that serum IgG antibodies can substantially protect the MEs, though serum antibodies alone do not account for the near sterilizing immunity seen in the MEs of convalescent WT mice. To examine the contribution of cellular immunity to protection of the MEs, we examined whether immune cells from secondary lymphoid organs (SLOs) would traffic to the MEs in response to infections. We prepared single cell suspensions from spleens isolated from naive 6-7 week old C57BL/6J-CD45.1 (B6.SJL-*Ptprca Pepcb*/BoyJ) mice and adoptively transferred them into *Rag-1*$^{-/-}$ mice, which lack mature T- and B-cells. 3 hours after this transfer we intransally inoculated the treated mice with a low dose inoculum of *B. bronchiseptica* (500 CFU/ 5uL PBS) to initiate nasopharyngeal infections. As controls we included a group of splenocyte transferred, but uninfected *Rag-1*$^{-/-}$ mice.

At day 21 p.i, we sacrificed the mice and enumerated lymphocytes trafficked to the nasal cavities (NC) and MEs by flow cytometry. *Rag-1*$^{-/-}$ mice not given wild type spleen cells sucumb to infection before day 21, so could not be included for comparison. As shown in Figure 4.3, both CD4^{+} and CD8^{+} T cells CD45.1 (confirming donor origin) T-cells were detected in the nasal cavities and ME samples of uninfected control *Rag-1*$^{-/-}$ mice. *B. bronchiseptica* infection induced > 2-fold increase in numbers of CD4^{+} and CD8^{+} cells in both the nasal cavities and the MEs, demonstrating similar recruitment and/or expansion of lymphoid cells in response to infections at these sites.

Recruited T-cells complement the intraepithelial mucosa of the ME

As mentioned above, even low dose, low volume *B. bronchiseptica* inoculations are lethal to of *Rag-1*$^{-/-}$ mice within weeks, but adoptively transferring wild type splenocytes rescued them from this lethality (Figure S4.2). Although the MEs of the *Rag-1*$^{-/-}$ mice transferred wild type splenocytes did not clear the primary infections even at day 56 p.i. (Figure S4.3), they allowed long term experiments and observations to be made. To examine where the recruited T-cells localized in the convalescent MEs, we conducted a histological study of splenocyte-transferred and *B. bronchiseptica*-infected *Rag-1*$^{-/-}$ mice at day 56 p.i. As shown in Figure 4.4 (and in Fig 4.1B), H&E-stained sections of the day 56 p.i. MEs showed no gross pathology, though limited cellular infiltrates were observed (4B and 4C).

To localize T-cells in the MEs, H/E stained of sections prepared from of the same ME sample block were incubated with HRP-conjugated anti-CD3^{+} antibodies. Developed sections showed aggregates of stained T-cells dispersed in the mucosa (inset Figs 4.4D and 4.4E), clearly demonstrating that T-cells are recruited to the ME in response to *B. bronchiseptica* and occupy the intraepithelial regions of the ME mucosa.

Recruited immune cells protect the ME against secondary challenge

We next examined whether recruited splenocytes protect the MEs of *Rag-1*$^{-/-}$ mice from secondary (high dose) challenges. As schematically outlined in Figure 4.5, splenocytes from naïve WT mice were adoptively transferred into two groups of *Rag-1*$^{-/-}$ mice (n=4) that were then inoculated with low dose inocula 3 hours later. On day 56 p.i. one group was challenged with the high dose inoculum of the gentamicin resistant *B. bronchiseptica* strain, while the second group (n=3) was left uninfected to determine the numbers of bacteria persisting from the primary low dose challenge (Figure S4.3). A third group of naïve *Rag-1*$^{-/-}$ mice were challenged with the high dose of the gentamicin resistant pathogen on day 56 p.i. to serve as non-protected (not transferred splenocytes) controls. After 3 days of the secondary high dose challenge, mice were sacrificed and bacterial loads in the nasal cavity, MEs and lungs were enumerated.

In the untreated (no splenocytes transferred) *Rag-1*$^{-/-}$ mice challenged with high dose inoculum (top panel), uniformly high bacterial CFUs were recovered from the nasal cavities (>1,000,000), MEs (>100,000) and lungs (> 100,000). If left untreated these mice succumb to infection in about a month. In contrast, mice that received the splenocytes (middle panel) and a low dose of *B. bronchiseptica* 56 days earlier had moderate numbers (~100,000) in the nasal cavities. This (> 10-fold reduction relative to the untreated mice suggests partial protection of the nasal cavities. In contrast, the bacterial numbers in MEs and lungs of splenocyte transferred and challenged mice were >99% reduced, relative to controls, indicating that, unlike in the nasopharynx, the transferred splenocytes were able to confer robust protective immunity. It is important to highlight that we still recovered gentamicin sensitive *B. bronchiseptica* in the nasal cavities and MEs (albeit only hundreds of CFUs in the lungs) indicating that the transferred/recruited splenocytes are by themselves not sufficient to completely clear of primary

ME infections. This is confirmed by the control mice (lower panel, n=3) receiving the splenocytes and the low dose inoculum. In this group, substantial bacterial CFUs were recovered from the nasal cavities (~10^5 CFUs) while 100s of CFUs were also found in the MEs, but not in the lungs, indicating that the transferred/recruited splenocytes are by themselves sufficient to clear the lower respiratory tract, but are not sufficient to clear the ME. These results indicate that the anamnestic responses in the three contiguous compartments, i.e the MEs, lower and upper respiratory airways, are qualitatively distinct from each other. Furthermore, these data show that recruited immune cells from secondary lymphoid organs are insufficient to clear primary infections in the upper respiratory tracts but are necessary and sufficient to confer robust protection against subsequent challenges in the lungs and MEs.

Intranasal immunization with heat-killed B. bronchiseptica robustly protects the MEs.

We next examined whether vaccination induces similar protection in the MEs and respiratory organs. We used heat-killed whole cell *B. bronchiseptica* ($5x10^7$ CFU/mL in 50μL of PBS) delivered intranasally, where mucosal stimulation against respiratory pathogens is known to be strong [28] and is the recommended method of delivery for non-human animal vaccines to protect against *B. bronchiseptica* [29, 30]. To evaluate the levels of protection conferred by the vaccine relative to convalescent immune protection, we also included a group of mice that had been inoculated eight weeks earlier with a low dose of *B. bronchiseptica* (streptomycin resistant). Protection was assessed 3 days following high does challenge (Figure 4.6).

Naïve (PBS vaccinated) mice showed high bacterial loads in the MEs and respiratory organs. The convalescent group of mice showed strong adaptive immune protection in the MEs and lower respiratory tract while the nasal cavities were only partially protected. Interestingly, protection conferred by the intranasal vaccinations was very similar to that of the convalescent

mice, successfully protecting the MEs and lower respiratory tract from high-dose challenge of *B. bronchiseptic*a. However, the nasal cavities remained only partially protected, indicating that immune mechanisms more efficiently protect the MEs than they do the upper respiratory tract, despite being the actual site of vaccination.

Differential T-cell responses generated in middle ears relative to respiratory tract.

Flow cytometric analysis conducted on cells prepared from nasal, lung and ME samples from the above experiment did not indicate any expansion of B-cell populations on challenge in either the convalescent or vaccinated groups (Figure S4.6). This has precluded any definitive conclusion on their contributions. However, we observed greater numbers of $CD4^+$ and $CD8^+$ T cells recovered from these immune animals relative to naïve mice in all these organs (Figure 4.7). Interestingly, though higher numbers of $CD4^+$ T cells and differential $CD4^+/CD8^+$ ratios correlated with robust protection in the MEs (Figure 4.7c) and lungs (Figure 4.7a) of immune mice, these appear to be insufficient to facilitate clearance of the nasal cavity (Figure 4.7b) by 3 days post-challenge. This further supports the observation that robust protective T cell-mediated immunity in the MEs and lungs can be generated *via* upper respiratory infections *and* vaccination, yet the level of protection generated by either means is less complete in the nasopharynx.

DISCUSSION

Despite a compelling need to understand acquired immunity within the MEs, this has been particularly difficult to study using human otopathogens owing to their inability to naturally colonize the MEs of experimental mice that can be manipulated immunologically. A natural respiratory/middle ear infection model in mice is especially relevant to the study and understanding of adaptive immunity of the MEs given the connection of the organ, *via* the ET, to

the upper respiratory airways from which most ME infections originate and where the early stages of cognate immunity in a naïve host are likely to be established. The mammalian upper respiratory tract commensal/pathogen, *B. bronchiseptica,* appears to be a natural otopathogen of mice, highly efficient in causing infections resembling human AOM. This experimental system is well-suited to investigate how AOM is cleared and how long-term immune protection of the MEs may be generated. As we have shown, *B. bronchiseptica* efficiently establishes inflammatory infections in the MEs of nasally inoculated mice that are eventually controlled and subsequently cleared. As observed for the prevalent human otopathogens [28-30], *B. bronchiseptica* is cleared from the ME but persists in the nasopharynx indefinitely, underscoring the particulars of the protective immunological environment that surveils and protects the MEs from potential otopathogens that persist in the nasopharynx. Our assessment of the levels of protection in convalescent mice showed near sterilizing anamnestic immunity in the MEs and lungs, but only partial protection of the nasal cavities, highlighting the distinct environment of the MEs compared to the nasopharynx.

Adoptively transferring naïve wild type splenocytes into *Rag-1*$^{-/-}$ mice allowed peripheral T-cellsto be recruited to the MEs in response to infection. This influx of lymphocytes from secondary lymphoid organs was further confirmed by the histopathological detection of $CD3^{+}$ populations ofcells complementing the intraepithelial mucosa (or mucoperiosteum) of the infected mice at day 56 p.i.

Prior studies [15-17] established that the MEs exhibit the features of an effector site of adaptive immunity, with increase in antigen specific secretory IgA titers and Th2 cytokines on antigen restimulation. Intranasal immunization also partially reduced the bacterial burden of experimentally injected non-typeable *H. influenzae* in the MEs of BALB/c mice compared to

uninfected controls. However how this control relates to infection in the nasopharynx was not possible to assess due to the need to directly introduce the pathogen into the ME [17]. We see here that intranasal immunizations using heat-killed *B. bronchiseptica* provide strong protection to the MEs, but only partially protect the nasopharynx. These results clearly indicate that intranasal vaccine-induced protection of the MEs can be very effective even against a commensal/pathogen that is highly adapted to successfully colonize and persist in the upper respiratory airways and establishes that in resisting such an efficient colonizer the ME is an independent and vaccine-targetable organ.

This initial investigation of adaptive immunity of the MEs using a natural model of AOM in mice demonstrates an experimental system that can address several pressing questions. Where along the Eustachian tube-ME mucosal geography is protection conferred by antibodies and immune cells? How might protective immunitybe established to protect against other otopathogens and/or polymicrobial infections? Do viral infections or allergic immune disturbances compromise induced protection to facilitate (re)infections of the MEs? How might deficiencies in immune signaling compromise ME protection in naïve or convalescent hosts? Finally, since "nothing in biology makes sense except in light of evolution", it is worth consideringwhat possible evolutionary advantages to a pathogen there might be in infecting the ME, which is essentially a "dead end" site, only connected to the outside world *via* the ET and nasopharynx. *Prima facie,* colonizing the ME does not appear to contribute to transmission to new hosts. Are pathogens of the upper respiratory tract exploiting the ME as a reservoir from which to reseed the respiratory tract [31], from which they can then transmit to other hosts? Or is the ability to infect the ME not itself an adaptive trait, but simply a side-effect of adaptation to infect deep into the upper respiratory tract? Considering these evolutionary questions may help guide studies to deepen our

understanding of the infectious process of AOM and the complex interactions with the immune system that protects the ME.

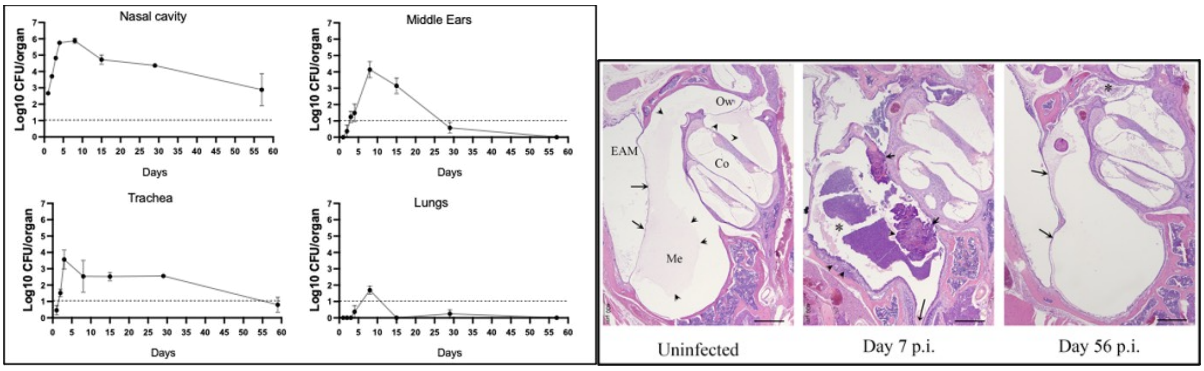


Figure 4.1: *B. bronchispetica* causes acute middle ear infections in mice

A) Graph shows the CFU numbers of *B. bronchiseptica* recovered from the respiratory tract (nasal cavities, trachea and lungs) and middle ears of C57BL/6J mice over 56 days p.i. Dotted line represent the limit of detection (n=5, experiment conducted 2 times) .

B) Representative Hematoxylin and eosin (HE) stained sections of the middle ears of uninfected (left panel), 7 days (central panel) and 56 days post inoculated mice(n = 4 mice). Left panel: Section of the middle ear from an uninfected mouse. Arrows point to the tympanic membrane. Within the middle ear (Me) and cochlea (Co), there is a pale eosinophilic (proteinaceous) material (arrowheads). Bar = 400 µm. Central panel: At the peak of infection on day 7, an inflammatory cellular infiltrate fills the middle ear (*) and expands the hyperplastic mucoperiosteum lining the inferior tympanic bulla (arrowheads). The long arrow points to the Eustachian tube. Right panel: By day 56 p.i., the degree of inflammation has subsided. In the current image, there is a small amounts of inflammatory infiltrate mixed with cholesterol clefts obscure the oval window (*). Arrows point to the tympanic memebrane. Abbre*via*tions: EAM = External Auditory Meatus; Ow = Oval window.

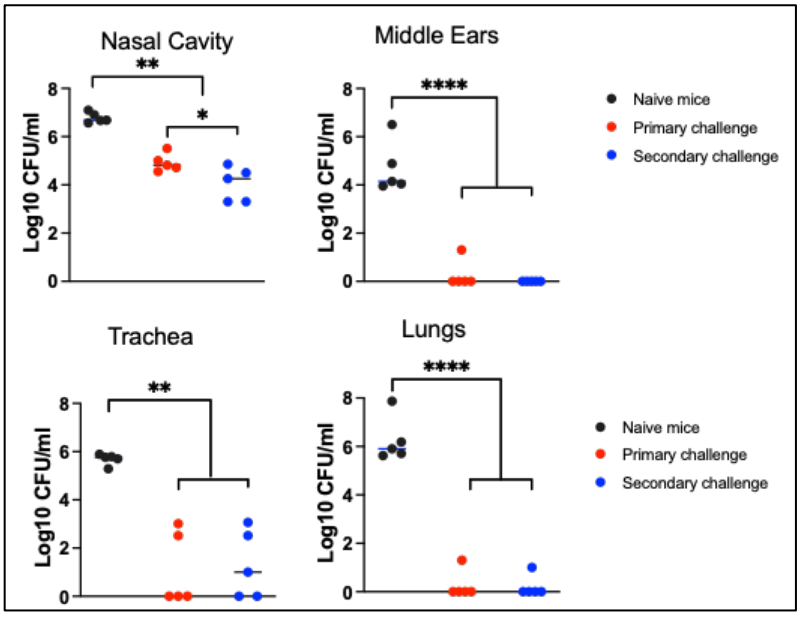


Figure 4.2: Robust convalescent immune protection in the middle ears

Graph shows bacterial CFU recovered from the nasal cavity, middle ears, trachea and lungs of mice after they had been intransally inoculated (1^0 challenge) with 500 CFU (5µL of PBS) of *B. bronchiseptica* and re-challenged (2^0 challenge) 56 days later with $5x10^5$ CFU (50 µL of PBS) of a gentamicin resitant isogenic strain of the bacteria. The bacterial CFU shown are from 3 days following the 2^0 challenge. The number of gentamicin-resitant CFU (red circles) and gentamicin-sensitive CFU (blue circles) are independently displayed to distinguish the bacterial populations between the 1^0 and 2^0 challenges. Dashed line represents limit of detection Statistical analysis was calcualted *via* one-way ANOVA. [** = P> 0.001: *** = P> 0.0001] (n=5, experiment conducted twice).

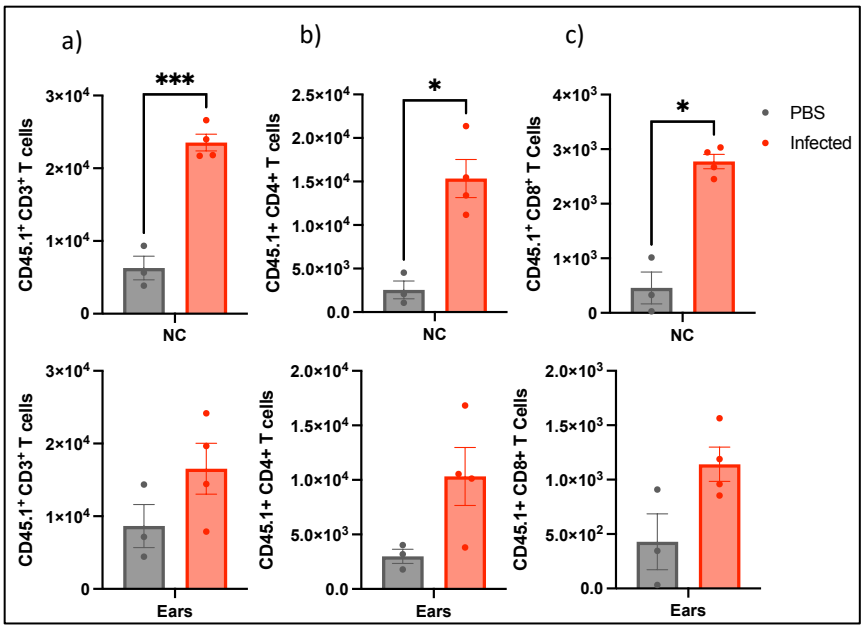


Figure 4.3: Lymphoid cells traffic to the ME in response to infection

Graphs show the number of CD45.1 marked (a) $CD3^+$, (b) $CD4^+$(b), and $CD8^+$ (c), cells isolated from *Rag-1*$^{-/-}$ mice that had been adoptively transferred with splenocytes from C57BL/6J (CD45.1) mice that were either left uninfected (grey-left columns), or inoculated 3 hours later with 500 CFU of *B. bronchiseptica* delivered in 5 µL of PBS (red-right columns). All mice were examined at 21 d.p.i. and cells recovered and enumerated from nasal cavity (NC) and ME (n=4 infected, 3 uninfected, experiment conducted once). Statistical analysis was calculated *via* one-way ANOVA. [*$p<0.05$, ***$p<0.001$].

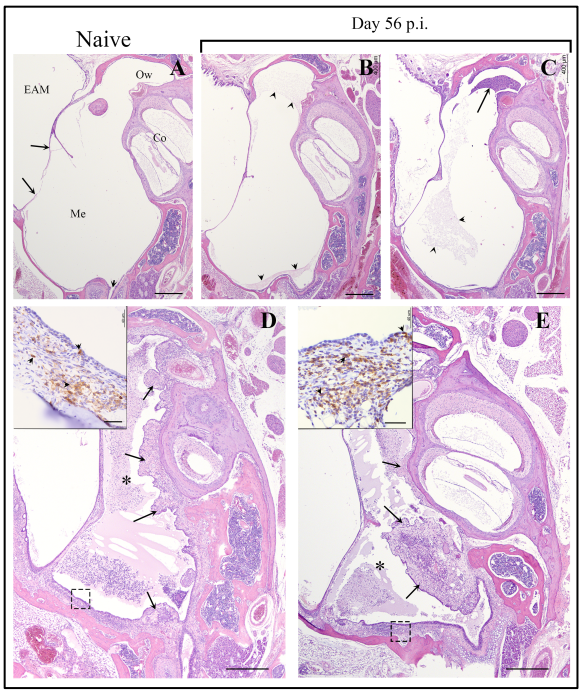


Figure 4.4: *B. bronchiseptica* middle ear infections in *Rag-1*$^{-/-}$ mice

Representative hematoxylin and eosin (HE) stained sections from the middle ears of uninfected/naïve (A) and 56 days post inoculated (p.i.) *Rag-1*$^{-/-}$ mice (B-E).

Top panel, A: Section of the middle ear from an uninfected C57BL/6J mouse. Long arrows point to the tympanic membrane. Short arrow points to the Eustachian tube. Bar = 400 µm.

Top panel, B and C: Representative sections of the middle ear from two infected Rag-1$^{-/-}$ mice with transferred splenocytes that were inoculated with 500 CFU of *B. bronchiseptica* and euthanized on Day 56 p.i. There is minimal accumulation of eosinophilic proteinaceous material (arrowheads) or inflammatory cellular infiltrate (arrow) within the middle ear of both mice. *Bottom panel,* D and E: Representative sections of the middle ear from two inoculated *Rag-1*$^{-/-}$ mice with transferred splenocytes, euthanized on Day 56. Moderate to marked inflammatory cellular infiltrates embedded in eosinophilic proteinaceous material obscure most of the middle ear both mice (*, asterisks). In both mice, the proliferative,

irregularly thickened mucoperiosteum is hypercellular (arrows). *Insets*: Higher magnification (Bar = 40 μm) of the mucoperiosteum outlined by a dash box with many CD3+ lymphocytes (brown; arrowheads), within the lamina propria and lining mucosa (arrows). Hematoxylin counterstain. Abbre*via*tions: EAM = External Auditory Meatus; Me = Middle ear; Co = Cochlea; Ow = Oval window.

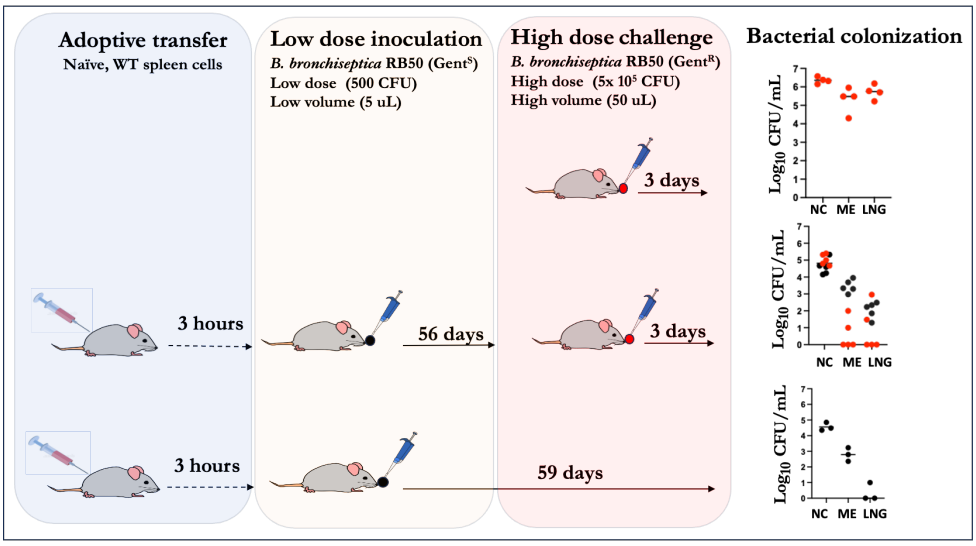


Figure 4.5:

Schematic outline showing how adoptively transferred splenocytes are sufficient to confer robust convalescent protection to the MEs and lungs of *Rag-1*$^{-/-}$ mice from a high dose challenge of the bacterium. *Top panel*: Naïve *Rag-1*$^{-/-}$ mice challenged with 5x10^5 CFU of a gentamicin derivative of *B. bronchiseptica* delivered in 50mL of PBS (*red* circles); *Middle panel*: *Rag-1*$^{-/-}$ mice given WT splenocytes, inoculated with low dose of *B. bronchisepica* RB50 (*black* circles) delivered in 5µL of PBS three hours later and then 56 days later challenged with 5x10^5 CFU of the gentamicin derivative of *B. bronchiseptica* RB50 (as in top panel) delivered in 50µL of PBS and assessed three days later. *Bottom panel*: *Rag-1*$^{-/-}$ mice adoptively transferred (i.p.) with splenocytes three hours prior to primary challenge with 500 CFU of *B. bronchisepica* (*black* circles) delivered in 5µL of PBS three hours later. Colonization assessed 59 days later. Corresponding graphs on the right show the bacterial CFU recovered from the nasal cavities (NC), middle ears (ME) and lungs (LNG). (Experiments for each group were conducted >2 times)

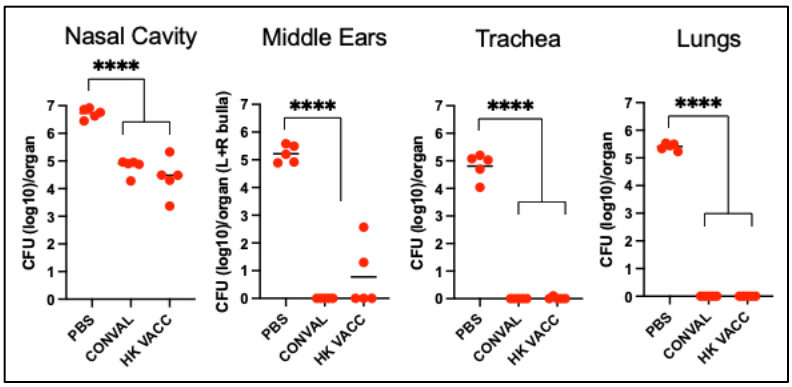


Figure 4.6:

Graph shows bacterial CFU recovered from the nasal cavity, trachea, lungs and middle ears of naïve, convalescent, and vaccinated groups of C57BL/6J mice following a high dose challenge ($5x10^5$ CFU in 50µL PBS) with *B. bronchiseptica* (circles) and analyzed 3 days later. Three groups of mice were examined: Naïve (PBS inoculated) mice, 56 d.p.i. convalescent mice following a low dose inoculum of *B. bronchiseptica* (500 CFU in 5µL of PBS), or mice intranasally vaccinated with $5x10^7$ CFU of heat killed *B. bronchiseptica*. (n=5; experiment conducted once). Statistical analysis was calcualted *via* one-way ANOVA. [** $p< 0.001$: *** = $p< 0.0001$]

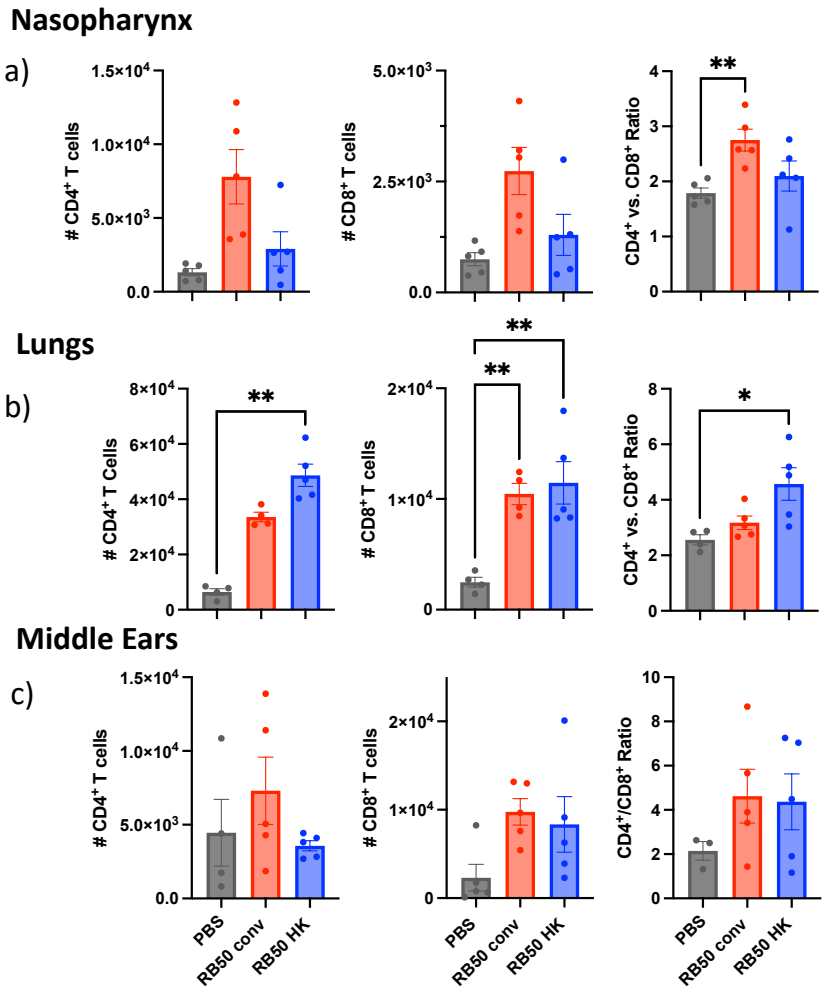


Figure 4.7: Total $CD4^+$ and $CD8^+$ T cells recovered from the mice in Figure 6. (a) nasal cavities, (b) lungs, and (c) middle ears of naïve, RB50-convalescent, or RB50-vaccinated C57BL6/J mice following high dose challenge ($5X10^5$ CFU in 50µl PBS). Naïve (PBS inoculated) mice, 56 d.p.i. convalescent mice following a low dose inoculum of *B. bronchiseptica* (500 CFU in 5µL of PBS), or mice intranasally vaccinated with $5x10^7$ CFU of heat killed *B. bronchiseptica*. Statistical analysis was calcualted *via* one-way ANOVA. Error bars show standard error of the mean (n= 5, experiment conducted once). [*$p<0.05$ and **$p<0.002$].

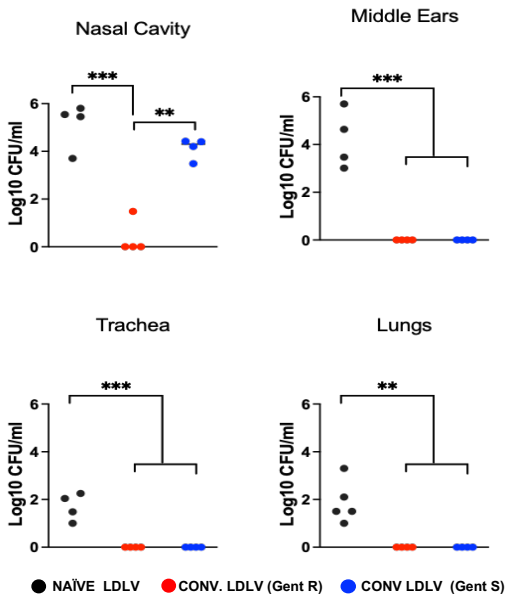


Figure S4.1: MEs of convalescent C57BL/6J mice protected against low dose low volume challenge of B. bronchiseptica Graph shows bacterial CFU recovered from the nasal cavity, trachea, lungs and middle ears of C57BL/6J mice that had been intransally inoculated with 500 CFU (5uL of PBS) of B. bronchiseptica (gentamicin sensitiveblue) and challenged on day 56 p.i. with low dose low volume (LDLV) inoculum: 500 CFU of a gentamicin resitant (red) isogenic strain of the bacteria delivered intranasally in 5 uL of PBS. Control group LDLV challenged non-convalescent naïve mice (black circles). Mice were sacificed 7 days following this challenge. Statistical analysis was calcualted *via* one-way ANOVA. Error bars show standard error of the mean (n=4) (experiment conducted once).

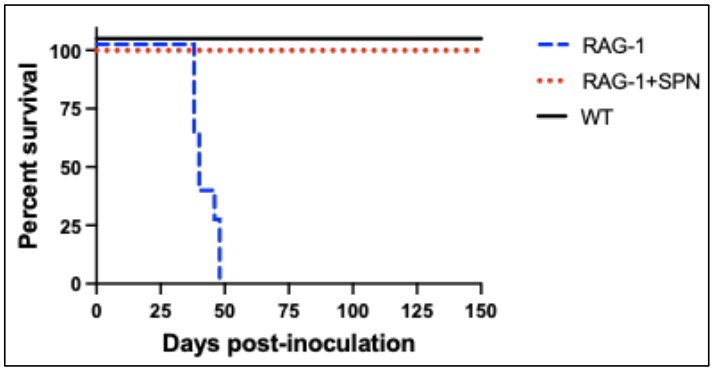


Figure S4.2: Adoptively transferred splenocytes protect Rag-1-/- mice against lethality from B. bronchiseptica Kaplan-Meier survival curves of C57BL/6J (WT), Rag-1-/- mice (blue dashed line) and Rag-1-/- mice with adoptively transferred splenocytes from C57BL/6J (red dashed line) inoculated with B. bronchiseptica (500 CFU delivered in 5uL PBS). B. bronchiseptica infections are lethal to Rag-1-/- mice within 7 weeks. However, Rag-1-/- mice with WT splenocytes survive.

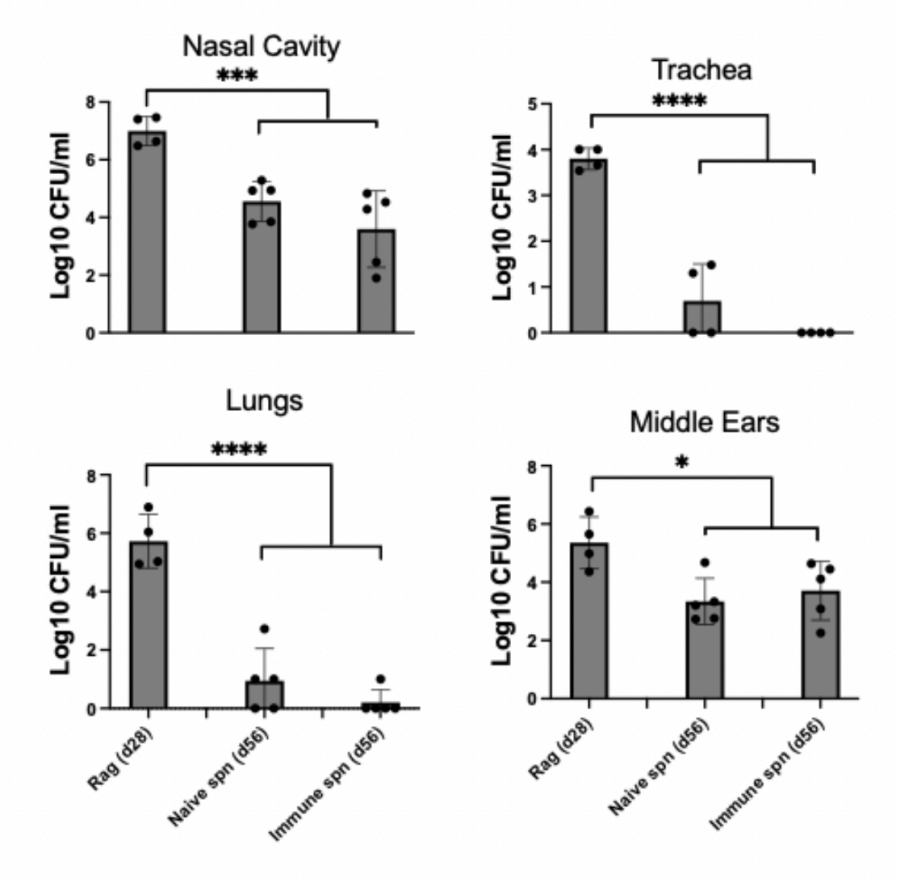


Figure S4.3: Adoptively transferred splenocytes protect Rag-1-/- mice fail to clear B. bronchiseptica from the middle ears Rag-1-/- mice with adoptively transferred splenocytes from either naïve (Naïve spn) or convalescent C57BL/6J mice (Immune spln) survived but, failed to clear B. bronchiseptica from the middle ears by day 56 p.i. when inoculated intranasally with 500 CFU suspended in 5uL PBS. (n=4 and 5) Statistical analysis was calcualted *via* one-way ANOVA. Error bars show standard error of the mean [p***<0.0001,p****<0.0001]. Rag-1-/- mice without splenocyte transfers do not survive beyond 5-6 weeks and data is shown for day 28 p.i. Experiment conducted once.

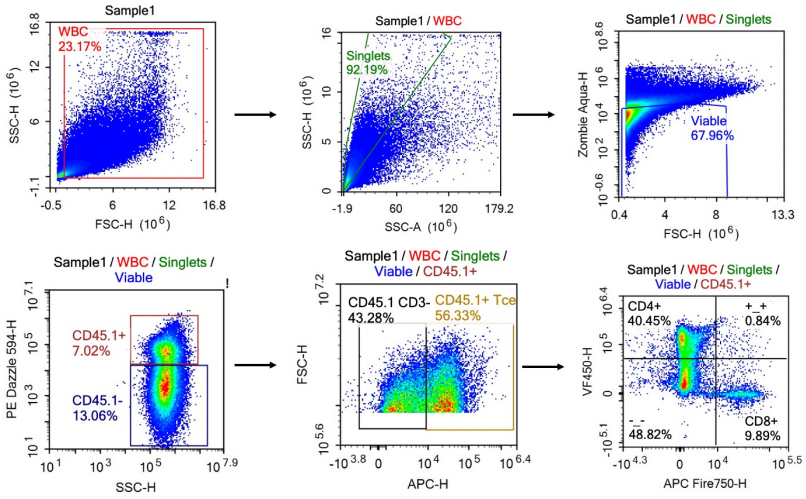


Figure S4.4: Gating strategy for the enumeration of transferred CD45.1^{+} CD4^{+}/CD8^{+} T cells trafficked to the middle ears of C57BL6/J mice.

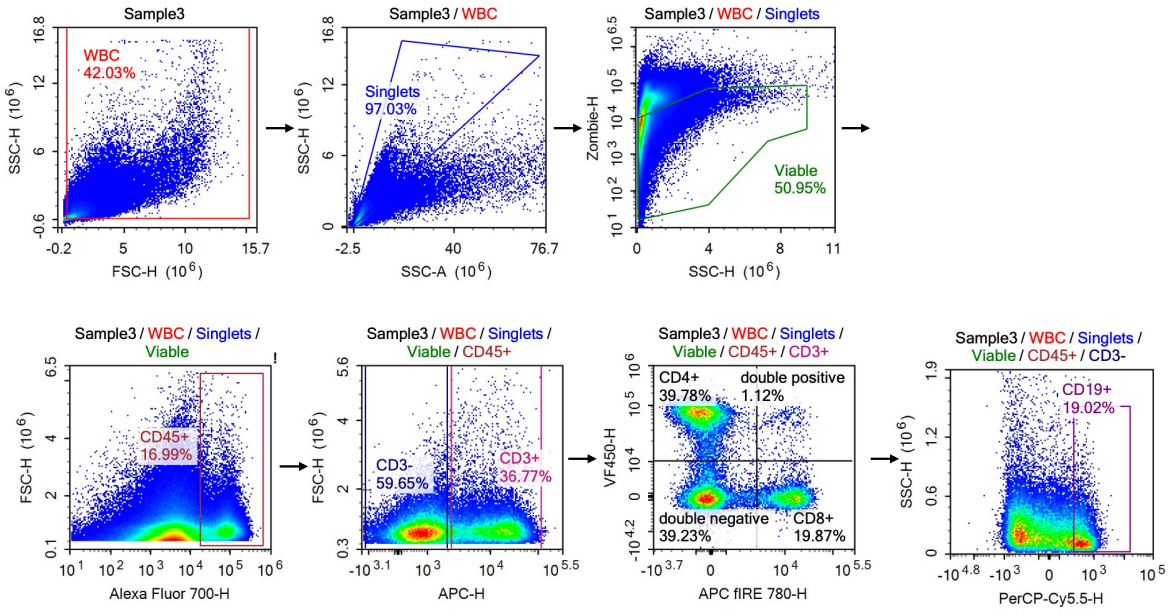


Figure S4.5: Gating strategy for the enumeration of $CD4^+/CD8^+$ T cells trafficked to the middle ears, lungs, and nasal cavities of C57BL6/J mice.

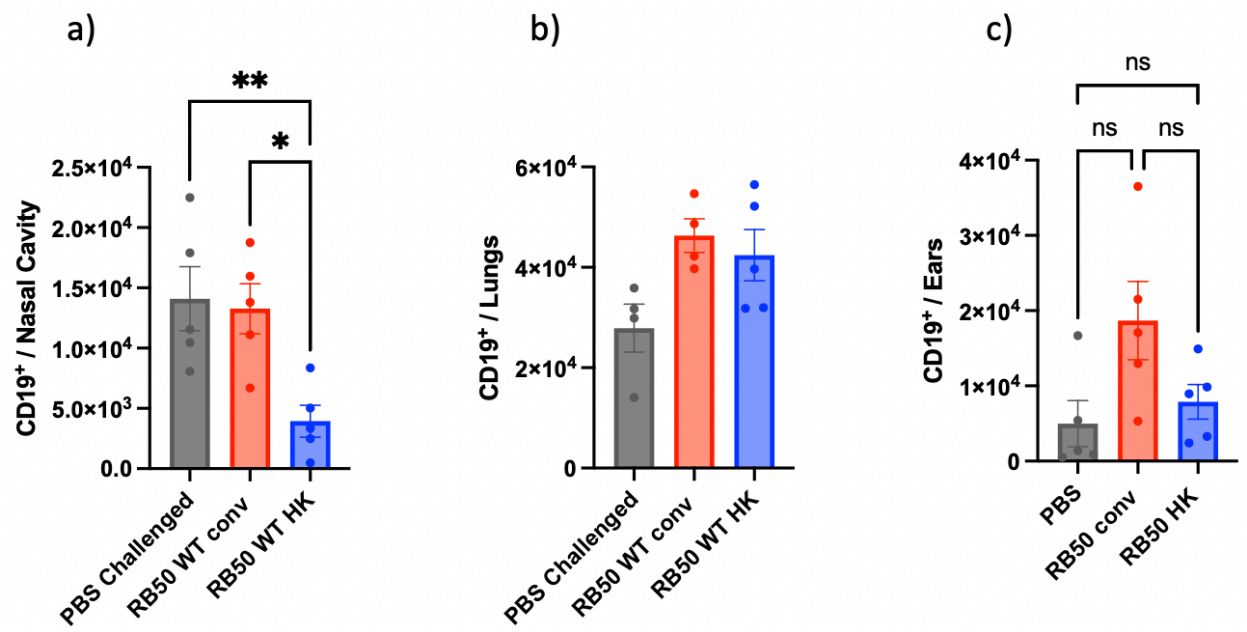


Figure S4.6: Total CD19+ cells recovered from the (a) nasal cavities, (b) lungs, and (c) middle ears of naïve, RB50- convalescent, or RB50 vaccinated C57BL6/J mice following high dose challenge (5X105 CFU in 50µl PBS). Naïve (PBS inoculated) mice, 56 d.p.i. convalescent mice following a low dose inoculum of B. bronchiseptica (500 CFU in 5µL of PBS), or mice intranasally vaccinated with 5x107 CFU of heat killed B. bronchiseptica. Statistical analysis was calculated *via* one-way ANOVA. Error bars show standard error of the mean (n= 4-5). [*p<0.002].

REFERENCES

1. Bluestone CD, Stephenson JS, Martin LM. Ten-year review of otitis media pathogens. *Pediatr Infect Dis J*. 1992; 11: S7-11. PMID:1513611.

2. Soriano F. Microbial etiologies of acute otitis media. *Clin Microbiol Infect.* 1997; 3: 3S23-25. PMID:11869224.

3. Ngo CC, Massa HM, Thornton RB, Cripps AW. Predominant Bacteria Detected from the Middle Ear Fluid of Children Experiencing Otitis Media: A Systematic Review. *PLoS One*. 2016; 11: e0150949. PMID:26953891.

4. Heikkinen T. Role of viruses in the pathogenesis of acute otitis media. *Pediatr Infect Dis J.* 2000 May;19(5 Suppl):S17-22; discussion S22-3. PMID: 10821468.

5. Chonmaitree T, Revai K, Grady JJ, Clos A, Patel JA, Nair S, Fan J, Henrickson KJ. Viral upper respiratory tract infection and otitis media complication in young children. *Clin Infect Dis*. 2008 Mar 15;46(6):815-23. doi: 10.1086/528685. PMID: 18279042; PMCID: PMC2744371.

6. Schilder AG, Chonmaitree T, Cripps AW, Rosenfeld RM, Casselbrant ML, Haggard MP, Venekamp RP. Otitis media. *Nat Rev Dis Primers*. 2016 Sep 8;2(1):16063. PMID: 27604644

7. Monasta L, Ronfani L, Marchetti F, Montico M, Vecchi Brumatti L, Bavcar A, Grasso D, Barbiero C, Tamburlini G. Burden of disease caused by otitis media: systematic review and global estimates. *PLoS One*. 2012;7(4):e36226.. PMID: 22558393

8. Schilder AG, Chonmaitree T, Cripps AW, Rosenfeld RM, Casselbrant ML, Haggard MP, Venekamp RP. Otitis media. *Nat Rev Dis Primers.* 2016 Sep 8;2(1):16063. PMID: 27604644

9. Rosenfeld RM, Tunkel DE, Schwartz SR, Anne S, Bishop CE, Chelius DC, Hackell J, Hunter LL, Keppel KL, Kim AH, Kim TW, Levine JM, Maksimoski MT, Moore DJ, Preciado DA, Raol NP, Vaughan WK, Walker EA, Monjur TM. Clinical Practice Guideline: Tympanostomy Tubes in Children (Update). *Otolaryngol Head Neck Surg*. 2022; 166(1_suppl): S1-S55. PMID: 35138954.

10. Alho OP, Koivu M, Sorri M. What is an 'otitis-prone' child? *Int J Pediatr Otorhinolaryngol*. 1991; 21(3): 201-9. PMID: 1869373.

11. Pichichero ME. Ten-Year Study of the Stringently Defined Otitis-prone Child in Rochester, NY. *Pediatr Infect Dis J.* 2016 Sep;35(9):1033-9. doi: 10.1097/INF.0000000000001217. PMID: 27273691.

12. Bhutta MF, Thornton RB, Kirkham LS, Kerschner JE, Cheeseman MT. Understanding the aetiology and resolution of chronic otitis media from animal and human studies. *Dis Model*

Mech. 2017 Nov 1;10(11):1289-1300. doi: 10.1242/dmm.029983. PMID: 29125825; PMCID: PMC5719252.

13. Pichichero M, Malley R, Kaur R, Zagursky R, Anderson P. Acute otitis media pneumococcal disease burden and nasopharyngeal colonization in children due to serotypes included and not included in current and new pneumococcal conjugate vaccines. *Expert Rev Vaccines.* 2023 Jan-Dec;22(1):118-138. doi: 10.1080/14760584.2023.2162506. Epub 2022 Dec 26. PMID: 36565291.

14. Bakaletz LO. Immunopathogenesis of polymicrobial otitis media. *J Leukoc Biol*. 2010 Feb;87(2):213-22. doi: 10.1189/jlb.0709518. Epub 2009 Oct 20. PMID: 19843575; PMCID: PMC2812561.

15. Kodama H, Faden H, Harabuchi Y, Kataura A, Bernstein JM, Brodsky L. Cellular immune response of adenoidal and tonsillar lymphocytes to the P6 outer membrane protein of non-typeable *Haemophilus influenzae* and its relation to otitis media. *Acta Otolaryngol.* 1999;119(3):377-83. PMID: 10380746.

16. Kodama S, Suenaga S, Hirano T, Suzuki M, Mogi G. Induction of specific immunoglobulin A and Th2 immune responses to P6 outer membrane protein of nontypeable *Haemophilus influenzae* in middle ear mucosa by intranasal immunization. *Infect Immun*. 2000; 68(4): 2294-300. doi: 10.1128/IAI.68.4.2294-2300.2000. PMID: 10722632.

17. Sabirov A, Kodama S, Hirano T, Suzuki M, Mogi G. Intranasal immunization enhances clearance of nontypeable *Haemophilus influenzae* and reduces stimulation of tumor necrosis factor alpha production in the murine model of otitis media. *Infect Immun*. 2001; 69(5): 2964-71. doi: 10.1128/IAI.69.5.2964-2971.2001. PMID: 11292713.

18. Basha S, Pichichero ME. Poor memory B cell generation contributes to non-protective responses to DTaP vaccine antigens in otitis-prone children. *Clin Exp Immunol*. 2015 Dec;182(3):314-22. doi: 10.1111/cei.12660. Epub 2015 Sep 30. PMID: 26138025; PMCID: PMC4636893.

19. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun*. 2002 Jun;70(6):2886-90. doi: 10.1128/IAI.70.6.2886-2890.2002. PMID: 12010976; PMCID: PMC128015.

20. Zohar T, Hsiao JC, Mehta N, Das J, Devadhasan A, Karpinski W, Callahan C, Citron MP, DiStefano DJ, Touch S, Wen Z, Sachs JR, Cejas PJ, Espeseth AS, Lauffenburger DA, Bett AJ, Alter G. Upper and lower respiratory tract correlates of protection against respiratory syncytial virus following vaccination of nonhuman primates. *Cell Host Microbe*. 2022 Jan 12;30(1):41-52.e5. doi: 10.1016/j.chom.2021.11.006. Epub 2021 Dec 7. PMID: 34879230.

21. Cho HJ, Ha JG, Lee SN, Kim CH, Wang DY, Yoon JH. Differences and similarities between the upper and lower airway: focusing on innate immunity. *Rhinology.* 2021 Oct 1;59(5):441-450. doi: 10.4193/Rhin21.046. PMID: 34339483.

22. Linz B, Ma L, Rivera I, Harvill ET. Genotypic and phenotypic adaptation of pathogens: lesson from the genus *Bordetella. Curr Opin Infect Dis*. 2019 Jun;32(3):223-230. doi: 10.1097/QCO.0000000000000549. PMID: 30921085; PMCID: PMC6658121.

23. Dewan KK, Taylor-Mulneix DL, Campos LL, Skarlupka AL, Wagner SM, Ryman VE, Gestal MC, Ma L, Blas-Machado U, Faddis BT, Harvill ET. A model of chronic, transmissible Otitis Media in mice. *PLoS Pathog*. 2019 Apr 10;15(4):e1007696. doi: 10.1371/journal.ppat.1007696. Erratum in: PLoS Pathog. 2019 Jun 28;15(6):e1007916. PMID: 30970038; PMCID: PMC6476515.

24. Dewan KK, Sedney C, Caulfield AD, Su Y, Ma L, Blas-Machado U, Harvill ET. Probing Immune-Mediated Clearance of Acute Middle Ear Infection in Mice. *Front Cell Infect Microbiol.* 2022; 11:815627. PMID: 35141173.

25. King P. *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). *Clin Transl Med*. 2012 Jun 14;1(1):10. PMID: 23369277.

26. Weiser JN. The pneumococcus: why a commensal misbehaves. *J Mol Med (Berl).* 2010 Feb;88(2):97-102. doi: 10.1007/s00109-009-0557-x. Epub 2009 Nov 7. PMID: 19898768.

27. Aebi C. *Moraxella catarrhalis* - pathogen or commensal? *Adv Exp Med Biol*. 2011;697:107-16. PMID: 21120723.

28. Kurono Y. The mucosal immune system of the upper respiratory tract and recent progress in mucosal vaccines. *Auris Nasus Larynx*. 2022 Feb;49(1):1-10. doi: 10.1016/j.anl.2021.07.003. Epub 2021 Jul 23. PMID: 34304944.

29. Ellis JA, Gow SP, Lee LB, Lacoste S, Ball EC. Comparative efficacy of intranasal and injectable vaccines in stimulating *Bordetella bronchiseptica*-reactive anamnestic antibody responses in household dogs. *Can Vet J*. 2017 Aug;58(8):809-815. PMID: 28761185; PMCID: PMC5508940.

30. Ellis JA, Gow SP, Waldner CL, Shields S, Wappel S, Bowers A, Lacoste S, Xu Z, Ball E. Comparative efficacy of intranasal and oral vaccines against *Bordetella bronchiseptica* in dogs. *Vet J.* 2016 Jun;212:71-7. doi: 10.1016/j.tvjl.2016.04.004. Epub 2016 Apr 12. PMID: 27256028.

31. Hardison RL, Harrison A, Wallace RM, Heimlich DR, O'Bryan ME, Sebra RP, Pinkett HW, Justice SS, Mason KM. Microevolution in response to transient heme-iron restriction enhances intracellular bacterial community development and persistence. *PLoS Pathog*. 2018; 14(10):e1007355. PMID: 30332468

CHAPTER 5

PERTACTIN-DEFICIENT *BORDETELLA* PERTUSSIS STRAINS: VACCINE-DRIVEN EVOLUTION AND THE RE-EMERGENCE OF PERTUSSIS

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ABSTRACT

The recent re-emergence of pertussis (whooping cough) in highly vaccinated populations and the rapid expansion of *Bordetella pertussis* strains lacking pertactin, a common acellular vaccine antigen, have raised the specter of vaccine-driven evolution and the potential return of what was once the major killer of children. Indeed, the discovery that most circulating *B. pertussis* strains in the USA have acquired new and independent disruptive mutations in pertactin is compelling evidence of strong selective pressure. Interestingly, the other four antigens included in acellular vaccines do not appear to be selected against so rapidly. In this perspective, we consider three aspects of pertactin that distinguish it from other vaccine antigens, which may, individually or collectively, explain why only this antigen is being precipitously eliminated. An understanding of the rise of pertactin-deficient strains should inform the current search for new protective antigens and provide broader lessons for the design of improved subunit vaccines.

INTRODUCTION

Bordetella pertussis, the causative agent of pertussis (whooping cough), continues to reemerge in countries that have high vaccine coverage, such as the United States, and has accelerated since the switch during the mid-1990s from whole cell formulations comprising many partially characterized bacterial proteins to the less reactogenic 1–5 component acellular pertussis (aP) vaccines (*1,2*). These aP vaccines, including DTaP and Tdap, protect against disease, but this protection wanes rapidly, and does not prevent colonization or transmission of the pathogen (*3– 5*). In this background of suboptimally performing aP vaccines, many countries have noted the emergence and expansion of strains specifically lacking pertactin (PRN), a membrane bound autotransporter, and 1 of up to 5 *B. pertussis* protein antigens included in the vaccines (*6–11*).

PRN-deficient *B. pertussis* strains have recently been reported in countries using aP vaccines, including the United States, Australia, Sweden, Italy, Norway, the United Kingdom, France, Belgium, Finland, the Netherlands, and Japan. The frequency of PRN-deficient strains has been variable but has risen to dominance in the United States (85%), Australia (>80%), Sweden (69%), and Italy (55%) (*7–11*). Lower frequencies were reported from Japan, which showed a major decrease from a prevalence of 41% during 2008–2010 to 8% during 2014–2016 and correlated with a change to aP vaccine formulations that exclude pertactin (*6,11–12*). Denmark, which uses the monocomponent pertussis toxin (PT) vaccine, had no reports of pertactin-deficient isolates before 2012, and the 4 PRN-deficient strains detected since have been associated with human migration from countries with PRN in their vaccines (*6*). There is limited data available from the predominantly developing countries that use whole-cell vaccines to

enable a robust comparison between the effects that aP and wP have on the selection of PRN. A sequencing study of the only 2 clinical isolates reported from India, which still uses wP, showed that the isolates still retained the broadly encountered PRN gene allele *prn-1* (*13*). Considered together, these observations provide a strong correlation between the use of aP vaccines containing PRN and the appearance and increase to prominence of PRN-deficient strains.

Lineages of all bacteria are constantly evolving, but increasing to dominance alone is not conclusive evidence of a causal relationship between use of PRN-containing aP vaccines and loss of PRN. A PRN mutation could be carried along with a strain that is increasing in dominance because of 1 or many other mutations. However, the appearance of a wide variety of PRN mutations, each arising from a diversity of *B. pertussis* lineages over time, provides additional strong evidence in favor of vaccine-driven selection on PRN in particular. Although insertions of IS481 at multiple genomic locations are the most common PRN mutation, there is a large diversity of disruptions to PRN expression, including deletions within the signal sequence, promoter inversion, transversions resulting in a stop codon, deletions resulting in a stop codon, and full-gene deletion (*2,6,12*). The variety of genomic lesions that have led to loss of PRN indicates that numerous independent selection and expansion events have occurred in most of the lineages now circulating in many countries using aP vaccines. Providing more direct experimental data, such as murine models aimed at investigating pertactin-deficient *B. pertussis* infection, have demonstrated an overall defect in colonization in unvaccinated mice, but advantages in both colonization and competition in assays using aP-vaccinated mice (*14–16*).

Together, these observations strongly support the hypothesis that loss of PRN confers a fitness advantage over wildtype *B. pertussis* particular to the aP vaccinated populations in which

they are arising. However, there are 4 other antigens included in aP vaccines that are not being disrupted or lost. Why are the other vaccine antigens not being mutated at similar rates? What are the characteristics of PRN that might lead to the loss of this antigen in particular? Understanding multiple possible explanations, and distinguishing between them where possible, will be useful for ongoing efforts to improve vaccines to control *B. pertussis* spread and disease.

Role of Pertactin

Pertactin is an autotransporter protein located on the surface of *B. pertussis* (*17*). Similar to all autotransporters found in gram-negative pathogens, PRN has 3 functional domains: the N terminal signal sequence, the passenger domain, and a C-terminal autotransporter domain. The signal sequence guides the passenger and transporter domains into the periplasm, enabling the transporter domain to form a pore in the outer membrane for translocation of the passenger domain to the cell surface. The protein is then cleaved by an outer membrane protease, with the passenger domain remaining in contact with the surface by noncovalent interactions (*18,19*).

The function of PRN is only partially understood. PRN is considered one of several virulence factors found in *B. pertussis* and has been shown to serve as an adhesin, facilitating attachment to various mammalian epithelial cells (*20,21*). The 3-dimensional structure of pertactin (PDB no. 1DAB) shows 16 right-handed parallel β-helixes, the largest β-helix structure recorded to date. Two Arg–Gly–Asp tripeptide motifs within the helical structure appear to be potential attachment sites to many mammalian adhesion proteins (*22–24*). PRN is reported to be essential for resisting neutrophil-mediated clearance and possesses additional immunomodulatory abilities that aid *B. pertussis* in suppressing the production of proinflammatory cytokines (*25,26*). The benefits of functional expression of PRN in

pathogenesis are consistent with its conservation in *B. pertussis* and other pathogenic *Bordetella* species. Loss of such a factor would be expected to be costly to the organism, yet pertactin-deficient strains appear to be rapidly expanding in aP vaccinated populations, suggesting a recent rebalancing of fitness costs and benefits.

Functional Redundancy of Pertactin

PRN is conserved in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* under the same *Bordetella* virulence gene regulatory system, suggesting that PRN has a more general role in pathogenesis that is not restricted to the human-specific *B. pertussis*. However, there are 16 other autotransporter genes identified in the genome of *B. pertussis*, 5 of which are disrupted by frameshift mutations (*27*). Park et al. (*28*) observed that autotransporters, as a group, are much more highly mutated or lost than other virulence factors. There appears to be no major loss in *in vitro* growth or *in vivo* fitness that prevents the expansion of *B. pertussis* strains lacking PRN, agreeing with the notion that the functional contributions of PRN in pathogenesis might be redundant or that complementary functions, potentially mediated by other autotransporters, might be compensating for any deficiencies caused its loss. This finding can be contrasted with pertussis toxin, which requires a complex operon to assemble and another to export and has a central and nonredundant role in the pathogenesis of *B. pertussis* and has no paralogs in the genome that can replace it. This finding is consistent with the rarity of clinical isolates lacking PT, compared with the abundance of PRN-deficient strains (*29–31*). For these and potentially other reasons, it might be that loss of PRN can be tolerated, but loss of PT would result in a more serious fitness defect.

Examination of the location and conformation of the different acellular vaccine components showed that most antibody functions directed against these antigens occur away from the bacterial surface. Both PT and the mature surface-associated filamentous hemagglutinin (FHA) (*32*) are secreted and diffuse away into the surrounding host environment. Therefore, antibodies directed against released FHA and PT would primarily neutralize toxin function by binding to the secreted molecules in the surrounding milieu, or to molecules in the process of being shed, mitigating potential surface-directed antibody effects. These antibodies do not effectively localize to the bacterial surface, and thus do not facilitate complement activation or FcR-mediated phagocytosis that would result in direct bacterial killing (Figure 5.1, panel A) (*33,34*). Fimbriae (FIM), although generally anchored to the bacteria, might extend more than a cell length away from the cell surface. Because FIM are composed of repeated structures with many copies of the same antigenic molecule, most FIM antibodies would be bound to parts of the structure extended >1 micron from the bacterial membrane, which is an enormous distance for highly reactive complement components to travel. Antibodies bound to fimbria would not be arrayed on a 2- dimensional surface required to optimally bind and activate complement or FcRs (Figure 5.1, panel B) (*35*).

However, pertactin is the only aP vaccine antigen that remains closely associated with the outer membrane. When cognate antibodies bind PRN, they become arrayed on the bacterial surface in a conformation that is particularly effective in binding and activating complement component C1q. The subsequent complement cascade that is activated rapidly deposits component in the adjacent membrane that opsonizes the cell and assembles into a membrane attack complex to lyse the bacterial membrane (*33–36*). The combination of the array of antibodies, bolstered by the complement components cleaved and activated in the immediate

vicinity, would effectively opsonize the bacteria for efficient phagocytic killing (Figure 5.1, panel C).

The model (Figure 5.1) we provide, although largely hypothetical, is consistent with evidence that aP vaccination is effective in preventing severe disease (by binding and neutralizing a key factor that mediate aspects of disease), but is much less effective in preventing nasopharyngeal colonization. Most antibodies, similar to those directed against secreted PT/FHA or distal FIM, do not effectively target and kill the bacteria. This model would also explain why loss of PRN might enable partial evasion of aP-induced immunity, and is also consistent with human surveys that showed that 3-component aP vaccines containing PT, FHA, and PRN were more efficacious than 2-component vaccines lacking PRN (*37,38*).

Persistence of Pertactin Antibodies

Despite the shortcomings of aP vaccines in generating a strong memory response, and the resulting waning immunity of these vaccines against disease (*39,40*), aP vaccines induce robust IgG titers against most of its component antigens. However, this strong humoral response, although protecting against symptoms of pertussis, does not prevent the pathogen from colonizing the upper respiratory tract or from transmitting between hosts (*3*). Furthermore, although initially induced at high levels, circulating antibodies decay relatively rapidly across all age groups (*4,40– 43*). Studies evaluating dynamic levels of specific antibodies across time have consistently show differential rates of decay for the various antigen-specific antibodies, with PTX antibody titers decaying more rapidly than antibodies to FHA and PRN (Figure 5.2). Similarly, FIM2/3 have been reported to poorly stimulate generation of protective antibodies postinfection and postvaccinations (*4,43*), although these data conflict with those of other reports

that show higher levels of FIM antibodies, even at 10 years postvaccination. However, these reports, similar to PTX, show a sharp decrease in antibody titers that might decrease below protective levels over time. This rapid waning immunity against PTX and FIM would be expected to narrow the window of selective pressure against these antigens.

In contrast to antibodies against PTX, those against PRN and FHA are relatively more persistent (Figure 5.2) (*38,44,45*), suggesting that there is a longer period after vaccination when there are effective titers of antibodies against FHA and PRN. This finding would be expected to result in strong pressure against PRN and FHA. However, in a search for serologic correlates of immunity to pertussis, Le et al. (*4*) noted that FHA provides relatively little contribution to protection but pertactin had a higher protective role.

These observations have recently been validated in studies by Lesne et al., who used human serum bactericidal assays to determine that antibodies to PRN, but no other aP component, are bactericidal in *in vitro* complement killing assays (*46*). These findings somewhat conflict with those of previous studies and testing methods, which often prioritize PT IgG as an indicator for protection against pertussis (*4,45,47*). However, the short period during which levels of neutralizing antibodies against PT remain elevated, in contrast to bactericidal antibodies against PRN, suggests that PRN antibodies might be a more appropriate measure for pertussis immunity.

The short period during which antibodies to PTX remain at elevated levels indicates that there is a longer period when antibodies to PRN remain at high levels, but levels of antibodies to PTX have decreased. Le et al. also noted that a much larger proportion of enrolled patients tested before aP vaccination already had high titers of antibodies to PRN, and quite few had antibodies

to PTX (*4*). Therefore, in addition to its surface localization and strong opsonizing potential, the persistence of the PRN antibodies is likely to contribute to prolonged selection against this antigen in particular.

DISCUSSION

The rapid reemergence of pertussis noted in the early 2000s brought much initial speculation. Factors such as human migration, increased sensitivity of testing, increased volumes of testing, and reporting through heightened surveillance have been proposed to contribute to the observed resurgence (*1–3*). However, the collective experience in countries switching from whole cell to acellular vaccines strongly suggests that these safer, but less effective vaccines have contributed to the resurgence of pertussis. In addition, the way multiple pertactin-deficient strains have swept across countries that use acellular vaccines presents a strong case in favor of vaccine- driven selection against pertactin (*6–12*). What has remained unclear is what will be the consequences of these changes. Will PRN-deficient strains continue to evolve, loosing other vaccine antigens until they escape vaccine effects completely? Or will the loss of these factors result in strains attenuated in virulence such that they become more like commensals than pathogens? Or is PRN really the only antigen that can be lost without serious fitness costs?

The 3 aspects of PRN that have been highlighted in this article for selective loss of PRN (i.e., its functional redundancy, the relatively longer functional persistence of antibodies against it, and its close location to the surface membrane for productive complement fixation) are not an exhaustive list of all possibilities and are not mutually exclusive. Sufficient sustained selective pressure against any particular antigen is likely to lead to its loss or change. Efforts to improve the current vaccine should be informed by a careful consideration of the lessons learned from

this instance. If PRN is being lost because it is the only surface antigen, then should we replace it with 1 or more new surface antigens? If it is lost because its function is at least partly redundant then should we select some molecule(s) that is less likely to be redundant? In designing new vaccines, it would be prudent to carefully consider the issues that appear to be enabling potential vaccine escape mutants, such as PRN-deficient strains, to rapidly expand and rise to prominence.

This study was supported by the National Science Foundation (grant no. DGE-1545433) and NIH (grant nos. 1R21AI156293-01 and R56 A1149787).

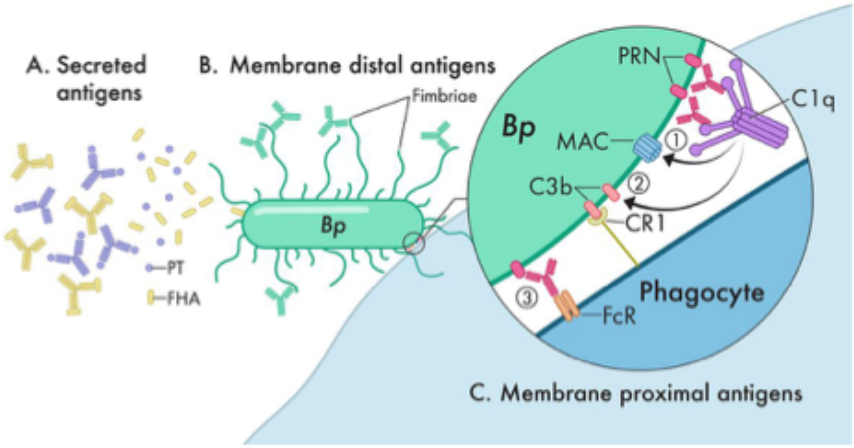


Figure 5.1: The various roles of antibodies against antigens in acellular pertussis vaccine.

(A) Antibodies against PT and FHA neutralize secreted virulence factors and mitigate disease progression but are not targeted to bacterial surface. (B) Antibodies attaching to fimbriae poorly activate the complement system far from the bacterial membrane. (C) Antibody-pertactin complexes induce strong bactericidal activity *via* multiple synergistic functions: 1. Activate complement to form a membrane attack complex. 2. Activate complement to deposit components like C3b that opsonize the bacterial surface. 3. Bind FcRs on phagocytes to activate phagocytosis.

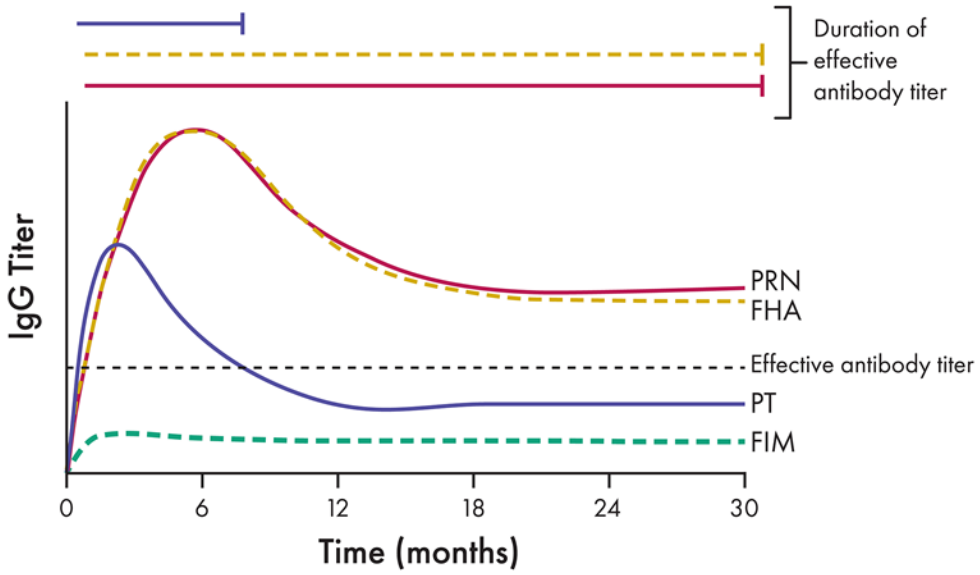


Figure 5.2: The differential decay of antibodies against acellular vaccine antigens and their effective capacity for protection. Antibodies against PRN and FHA remain at relatively higher titers for a longer period. However, PT specific antibodies decrease to very low titers rapidly. A consistently low-level of antibodies against FIM are induced. Solid lines indicate antibodies with high protective capacity, while dotted lines indicate antibodies with low protective capacity. Only anti-PRN antibodies are both highly protective and persist at high titers for years.

REFERENCES

1. Jackson DW, Rohani P. Perplexities of pertussis: recent global epidemiological trends and their potential causes. *Epidemiol Infect*. 2014;142:672–84.

2. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the United States. *Clin Vaccine Immunol*. 2014;21:119–25.

3. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proc Natl Acad Sci U S A*. 2014;111:787–92.

4. Le T, Cherry JD, Chang SJ, Knoll MD, Lee ML, Barenkamp S, et al.; APERT Study. Immune responses and antibody decay after immunization of adolescents and adults with an acellular pertussis vaccine: the APERT Study. *J Infect Dis*. 2004;190:535–44.

5. Breakwell L, Kelso P, Finley C, Schoenfeld S, Goode B, Misegades LK, et al. Pertussis vaccine effectiveness in the setting of pertactin-deficient pertussis. *Pediatrics.* 2016;137:e20153973.

6. Barkoff AM, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Pertactin-deficient *Bordetella pertussis* isolates: evidence of increased circulation in Europe, 1998 to 2015. *Euro Surveill.* 2019;24:1700832.

7. Martin SW, Pawloski L, Williams M, Weening K, DeBolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. *Clin Infect Dis.* 2015;60:223–7.

8. Byrne S, Slack AT. Analysis of *Bordetella pertussis* pertactin and pertussis toxin types from Queensland, Australia, 1999-2003. *BMC Infect Dis*. 2006;6:53.

9. Weigand MR, Williams MM, Peng Y, Kania D, Pawloski LC, Tondella ML; CDC Pertussis Working Group. Genomic survey of *Bordetella pertussis* diversity, United States, 2000–2013. *Emerg Infect Dis.* 2019;25:780–3.

10. Hiramatsu Y, Miyaji Y, Otsuka N, Arakawa Y, Shibayama K, Kamachi K. Significant decrease in pertactin-deficient *Bordetella pertussis* isolates, Japan. *Emerg Infect Dis*. 2017;23:699–701.

11. Zomer A, Otsuka N, Hiramatsu Y, Kamachi K, Nishimura N, Ozaki T, et al. *Bordetella pertussis* population dynamics and phylogeny in Japan after adoption of acellular pertussis vaccines. *Microb Genom.* 2018;4:e000180.

12. Otsuka N, Han HJ, Toyoizumi-Ajisaka H, Nakamura Y, Arakawa Y, Shibayama K, et al. Prevalence and genetic characterization of pertactin-deficient *Bordetella pertussis* in Japan. *PLoS One.* 2012;7:e31985.

13. Alai S, Ghattargi VC, Gautam M, Patel K, Pawar SP, Dhotre DP, et al. Comparative genomics of whole-cell pertussis vaccine strains from India. *BMC Genomics*. 2020;21:345.

14. van Gent M, van Loo IH, Heuvelman KJ, de Neeling AJ, Teunis P, Mooi FR. Studies on Prn variation in the mouse model and comparison with epidemiological data. *PLoS One*. 2011;6:e18014.

15. Safarchi A, Octavia S, Luu LD, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. *Vaccine.* 2015;33:6277–81.

16. Hegerle N, Dore G, Guiso N. Pertactin deficient *Bordetella pertussis* present a better fitness in mice immunized with an acellular pertussis vaccine. *Vaccine*. 2014;32:6597–600.

17. Brennan MJ, Li ZM, Cowell JL, Bisher ME, Steven AC, Novotny P, et al. Identification of a 69- kilodalton nonfimbrial protein as an agglutinogen of *Bordetella pertussis*. *Infect Immun.* 1988;56:3189–95.

18. Henderson IR, Navarro-Garcia F, Nataro JP. The great escape: structure and function of the autotransporter proteins. *Trends Microbiol*. 1998;6:370–8.

17. Brennan MJ, Li ZM, Cowell JL, Bisher ME, Steven AC, Novotny P, et al. Identification of a 69- kilodalton nonfimbrial protein as an agglutinogen of *Bordetella pertussis*. *Infect Immun.* 1988;56:3189–95.

19. Wells TJ, Tree JJ, Ulett GC, Schembri MA. Autotransporter proteins: novel targets at the bacterial cell surface. *FEMS Microbiol Lett*. 2007;274:163–72.

20. Everest P, Li J, Douce G, Charles I, De Azavedo J, Chatfield S, et al. Role of the *Bordetella pertussis* mammalian cells. *Microbiology (Reading).* 1996;142:3261–8.

21. Leininger E, Roberts M, Kenimer JG, Charles IG, Fairweather N, Novotny P, et al. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc Natl Acad Sci U S A.* 1991;88:345–9.

22. Emsley P, McDermott G, Charles IG, Fairweather NF, Isaacs NW. Crystallographic characterization of pertactin, a membrane-associated protein from *Bordetella pertussis*. *J Mol Biol.* 1994;235:772– 3.

23. Emsley P, Charles IG, Fairweather NF, Isaacs NW. Structure of *Bordetella pertussis* virulence factor P.69 pertactin. Nature. 1996;381:90–2.

24. Junker M, Schuster CC, McDonnell AV, Sorg KA, Finn MC, Berger B, et al. Pertactin β-helix folding P.69/pertactin protein and the P.69/pertactin RGD motif in the adherence to and invasion of mammalian cells. *Proc Natl Acad Sci U S A*. 2006;103:4918–23.

25. Inatsuka CS, Xu Q, Vujkovic-Cvijin I, Wong S, Stibitz S, Miller JF, et al. Pertactin is required for *Bordetella species* to resist neutrophil-mediated clearance. *Infect Immun*. 2010;78:2901–9.

26. Hovingh ES, Mariman R, Solans L, Hijdra D, Hamstra HJ, Jongerius I, et al. *Bordetella pertussis* pertactin knock-out strains reveal immunomodulatory properties of this virulence factor. *Emerg Microbes Infect*. 2018;7:39.

27. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet*. 2003;35:32–40.

28. Park J, Zhang Y, Chen C, Dudley EG, Harvill ET. Diversity of secretion systems associated with virulence characteristics of the classical Bordetellae. *Microbiology (Reading).* 2015;161:2328–40.

29. Linz B, Ivanov YV, Preston A, Brinkac L, Parkill J, Kim M, *et al.* Acquisition and loss of virulence-associated factors during genome evolution and speciation in three clades of *Bordetella* species. *BMC Genomics*. 2016;17:767.

30. Bouchez V, Hegerle N, Strati F, Njamkepo E, Guiso N. New data on vaccine antigen deficient *Bordetella pertussis* isolates. *Vaccines (Basel).* 2015;3:751–70.

31. Bouchez V, Brun D, Cantinelli T, Dore G, Njamkepo E, Guiso N. First report and detailed characterization of *B. pertussis* isolates not expressing pertussis toxin or pertactin. *Vaccine.* 2009;27:6034–41.

32. Nash ZM, Cotter PA. *Bordetella* filamentous hemagglutinin, a model for the two-partner secretion pathway. *Microbiol Spectr.* 2019;7:319–28.

33. Hellwig SM, Rodriguez ME, Berbers GA, van de Winkel JG, Mooi FR. Crucial role of antibodies to pertactin in *Bordetella pertussis* immunity. *J Infect Dis*. 2003;188:738-42.

34. Weiss AA, Mobberley PS, Fernandez RC, Mink CM. Characterization of human bactericidal antibodies to *Bordetella pertussis*. *Infect Immun*. 1999;67:1424–31.

35. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol.* 2008;6:132–42.

36. Jongerius I, Schuijt TJ, Mooi FR, Pinelli E. Complement evasion by *Bordetella pertussis*: implications for improving current vaccines. *J Mol Med (Berl).* 2015;93:395–402.

37. Greco D, Salmaso S, Mastrantonio P, Giuliano M, Tozzi AE, Anemona A, et al.; Progetto Pertosse Working Group. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. *N Engl J Med*. 1996;334:341–8.

38. Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. *N Engl J Med.* 1996;334:349-55.

39. van Twillert I, Han WG, van Els CA. Waning and aging of cellular immunity to *Bordetella pertussis*. *Pathog Dis*. 2015;73:ftv071.

40. Tomovici A, Barreto L, Zickler P, Meekison W, Noya F, Voloshen T, et al. Humoral immunity 10 years after booster immunization with an adolescent and adult formulation combined tetanus, diphtheria, and 5-component acellular pertussis vaccine. *Vaccine.* 2012;30:2647–53.

41. Taranger J, Trollfors B, Lagergård T, Sundh V, Bryla DA, Schneerson R, et al. Correlation between pertussis toxin IgG antibodies in postvaccination sera and subsequent protection against pertussis. *J Infect Dis*. 2000;181:1010–3.

42. Munoz FM, Bond NH, Maccato M, Pinell P, Hammill HA, Swamy GK, et al. Safety and immunogenicity of tetanus diphtheria and acellular pertussis (Tdap) immunization during pregnancy in mothers and infants: a randomized clinical trial. *JAMA*. 2014;311:1760-9

43. Abu Raya B, Srugo I, Kessel A, Peterman M, Vaknin A, Bamberger E. The decline of pertussis- specific antibodies after tetanus, diphtheria, and acellular pertussis immunization in late pregnancy. *J Infect Dis.* 2015;212:1869–73.

44. Heininger U, Cherry JD, Stehr K. Serologic response and antibody-titer decay in adults with pertussis. *Clin Infect Dis*. 2004;38:591–4.

45. Storsaeter J, Hallander HO, Gustafsson L, Olin P. Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. *Vaccine.* 1998;16:1907–16.

46. Lesne E, Cavell BE, Freire-Martin I, Persaud R, Alexander F, Taylor S, et al. Acellular pertussis vaccines induce anti-pertactin bactericidal antibodies which drives the emergence of pertactin- negative strains. *Front Microbiol.* 2020;11:2108.

47. Lee AD, Cassiday PK, Pawloski LC, Tatti KM, Martin MD, Briere EC, et al.; Clinical Validation Study Group. Clinical evaluation and validation of laboratory methods for the diagnosis of *Bordetella pertussis* infection: culture, polymerase chain reaction (PCR) and anti-pertussis toxin IgG serology (IgG-PT). *PLoS One*. 2018;13:e0195979.

CHAPTER 7

CONCLUSIONS

Overview & Significance

In these studies, we utilize multiple murine models of infection to evaluate tissue-specific and systemic immune responses following primary and secondary infections, vaccinations, adoptive transfer experiments and other treatments relevant to respiratory responses aimed at elucidating determinants of site-specific protective immunity against *Bordetella spp.* The secondary goal of this dissertation was to utilize these innovative models combined with techniques in genetics, immunology, histopathology, and microbiology to probe mechanisms by which we may enhance or utilize these immune responses to inform our understanding of the complex host-pathogen interactions occurring within the airways (and connected tissues).

In Chapter 3 we describe the first experiments aimed at understanding how the immune responses in the upper and lower respiratory tracts are affected by BtrS-mediated regulation of immunomodulatory factors. Our data supports the observation that enhanced convalescent immunity is induced by *Bp*Δ*btrS* mutant strains, allowing complete clearance of the nasal cavity and lungs within 3 days post-secondary challenge: more effective protective immunity than that induced by wildtype *Bp* infection, or vaccination with wP or aP.

We also report that infection with two isogenic mutant strains (*Bp*Δ*btrS* and D420Δ*btrS*) results in enhanced convalescent immunity, characterized by significantly higher recovery of $CD4^+$ T cells with indications of increased $CD4^+$ T_{RM} cells and elevated nasopharyngeal IgA titers relative to wildtype-convalescent mice. These data indicate that BtrS modulates an aspect of $CD4^+$ T cell responses. Given that BtrS exerts substantial control over T3SS expression, we can surmise that a proportion of BtrS-mediated immunomodulation is attributable to T3SS

activity, which has been shown to suppress certain innate pro-inflammatory responses (IL-1β, IL-12p40, MIP-1α, and MIP-2 during early infection), and adaptive responses (*Bp*-specific IFNγ, IL-17 and IgG2a levels during late infection). There is additional evidence that the T3SS in *Bp* may stimulate anti-inflammatory IL-10 responses, which is suspected to impair dendritic cell maturation as it does in *Bb.* Together, these effects of the T3SS are suggested to enable persistence by delaying/suppressing innate inflammatory responses and some, likely dependent, adaptive responses. Therefore, it is likely that the decreased $CD4^+$ populations we recover from BtrS-intact infections (WT) results from a complex mechanism whereby the T3SS and other BtrS-dependent factors may affect dendritic cell-dependent activation of $CD4^+$ T cells in the lymph nodes, and/or suppression of host factors required for effective activation and/or recruitment or expansion of T cells in infected tissues. It is also plausible that disrupted $CD4^+$ T cell activity may affect maturation of IgA-committed B cells in the lymph nodes, which could result in our observation that BtrS seems to decrease IgA titers in the nasal cavity. Importantly, our data suggest this activity to be dependent on active expression and/or secretion of bacterial factors, however we do not yet know the mechanism, or whether these effects stem from T3SS-disruption or other unknown BtrS-dependent mediators.

Resolution of these queries would require an in-depth characterization of the host immune response to WT*Bp* vs. *Bp*Δ*btrS* primary infection and secondary challenge models, as informed by our current observations and previous reports on T3SS-mediated immunomodulation. Possible avenues include multiplex cytokine kits (targeting IL-1β, IL-12p40, IL-17, IFNγ, etc), ELISAs differentiating IgG subtypes, as well as experiments in targeted immunodeficient mice. However, we have previously evaluated the individual contributions of T cells, B cells, eosinophils/basophils and IFNγ to the differential immune

response to WT*Bp/Bp*Δ*btrS* using immunodeficient mice (T cell$^{-/-}$, B cell$^{-/-}$, and GATA1$^{-/-}$, IFNy$^{-/-}$, respectively), and, of those evaluated (B cell$^{-/-}$, and GATA1$^{-/-}$, IFNy$^{-/-}$ *data not shown*), have found T cells to be the primary target of BtrS-mediated modulation.

Complimentary findings in alternative mouse models to assess immunity following low-dose nasopharyngeal infection and vaccination validates the need to use different experimental model systems to inform our understanding of the immune responses that are required to elicit protection against natural infection. Excitingly, data from these experiments also challenges the long-standing belief that natural convalescent immunity should be held as the highest standard of immune protection we can achieve and suggests that we might be able to utilize disruption of immunomodulation as a tool to induce and evaluate more robust unimpeded immunity against *Bp*. Since many well adapted pathogens modulate the host immune response, this concept may have broad applications to the study and control of other pathogens.

However, given that these were the *first* experiments evaluating the role of BtrS in *Bp* infection and immunity, we are left with an abundance of questions and possible research directions including: What specific-bacterial factors contribute to immunomodulation? What is the specific mechanism of action? Can we utilize this platform (or similar approaches) to establish an improved "gold standard" for vaccine design/development?

Due to the function of BtrS as a sigma factor that is suspected to regulate a variety of unknown genes, we cannot easily identify the specific bacterial factor(s) that contribute to our phenotypes. However, we could generate a *Bp*Δ*T3SS (bscN)* mutant and compare the resultant immune responses to primary/secondary infection with our Δ*btrS* strains to determine the relative contributions of T3SS-mediated immunomodulation to our observed BtrS-mediated effects. In addition, given that some of our *Bp*Δ*btrS* induced phenotypes overlap with those reported from

*Bb*Δ*btrS* (enhanced $CD4^+$ responses, increased IgA titers), we may wish to generate a list of overlapping genes regulated by BtrS in both species, and systematically generate *Bp* knockout-mutants for individual bacterial factors for further testing. Finally, while we also do not understand the mechanism that disrupts $CD4^+$ T cell-mediated immunity; or whether BtrS-mediated factors inhibit signaling, proliferation, or perhaps direct *via*bility, we could use a variety of experimental systems to determine the specific effects BtrS exerts on $CD4^+$ T cells as a starting point (trafficking & adoptive transfer experiments, *in vitro* assessment of BtrS-induced cytotoxicity, etc.,) and further utilize the methods mentioned above to tease apart a potential mechanism of action.

Importantly, chapter 4 (Otitis Media) provides additional support for the expanded use of novel experimental systems capable of elucidating determinants of site-specific protective immunity against *Bordetella spp.* This particular system utilizes a recently developed murine model of *B. bronchiseptica* infection, aimed at colonizing the middle ears *via* typical ascension of microbes from the nasal cavity, through the Eustachian tube, followed by acute colonization of the middle ears. The resultant infection and pathology resemble human otitis media. Interestingly, we report the middle ears to be discrete effector sites of adaptive immunity, which can be primed *via* intranasal vaccination. This paper supports the uncoupling of middle ear and nasopharyngeal immunities, as we report intranasal vaccination to provide poor immunity in the nasopharynx, but robust protection in the middle ears. Thus, intranasal vaccination is suggested as a potential strategy for vaccines against pathogens that cause otitis media, however, additional experiments should be performed to evaluate whether addition of specific adjuvants (or live-attenuated vaccines like *Bp*Δ*btrS)* may provide dual protection in the upper respiratory tract and the middle ears. Experiments aimed at generating sufficient protection against otopathogens in

the middle ears *and* nasopharynx could have the added benefit of preventing respiratory infections with pathogens including Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis, all of which cause clinically relevant disease in addition to OM.

Summary & Conclusions

The problematic resurgence of pertussis has prompted investigators to evaluate the flawed efficacy of current acellular vaccines, and their inability to confer complete sterilizing immunity against *Bordetella pertussis.* Of major concern, aP vaccines fail to provide long-term protection and permit the asymptomatic carriage of *B. pertussis* in the nasal cavity of vaccinated individuals. This sub-clinical infection poses a direct transmission risk to unvaccinated neonates and immunocompromised persons, who may develop symptomatic pertussis, thereby negating the protective effects of herd immunity.

Problematically, the true incidence and prevalence of *Bp* infection appears to be much higher than the elevated rates already being reported due to undiagnosed mild or sub-clinical infections, as clinicians (justly) rely on the presentation of classic whooping-cough symptoms before suspecting infection with *B. pertussis*. Given that pertussis is a notifiable disease in most countries, this results in incidence rates that stem almost entirely from reports of **full** symptomatic pertussis. Waning immunity in aP vaccinated populations is believed to further increase transmission and **real** incidence rates by enabling the subclinical carriage of *Bp,* which is likely exacerbated by the precipitous loss of pertactin from circulating strains around the globe.

The development of improved vaccines to combat waning immunity and asymptomatic carriage is complicated by our current limited understanding of the host immune response to *natural B. pertussis* infection, the development of accessible animal models capable of

effectively simulating sub-clinical nasal colonization, and the broad immunomodulatory abilities of *B. pertussis.* Collectively the work performed in this dissertation utilized multiple murine models of infection to evaluate tissue-specific and systemic immune responses to *Bordetella pertussis* and *Bordetella bronchiseptica.* Our data indicate broad applicability for use of such models in *Bordetella* research, including novel observations on immunomodulation and generating enhanced convalescent immune responses to *Bp*, modeling vaccine and pathogen-induced immunity in the nasopharynx, and describing the middle ears as discrete effector sites of adaptive immunity that are targetable *via* intranasal vaccination. Given that these chapters describe some of the earliest applications of these murine systems (nasopharyngeal *Bp* and otitis media), we enthusiastically support use and development of additional *in vitro* and *in vivo* systems that may continue to advance our understanding of the complex host-pathogen interactions occurring in the respiratory tract. These dynamics in *Bp* infection have broad implications at the population level and should be considered as we move forward toward more effective research, management, and prevention of pertussis in the future.

The increased risk for transmission of *Bp* to vulnerable adults and children who may develop severe disease warrants the prioritization of actionable research initiatives to include establishing universal standards in improved correlates of protection, instituting more effective and universally applicable aP immunization schedules, and a better understanding of how immunomodulation, waning immunity, and sub-clinical *Bp* infection contribute to rising incidence rates in vaccinated populations.