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# Characterization Of The Immune Stimulating Properties Of Type III Secretion System Needle Protein Bscf From Bordetella Pertussis: Towards The Development Of A New Acellular Pertussis Vaccine

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# CHARACTERIZATION OF THE IMMUNE STIMULATING PROPERTIES OF TYPE III SECRETION SYSTEM NEEDLE PROTEIN BSCF FROM *BORDETELLA PERTUSSIS*: TOWARDS THE DEVELOPMENT OF A NEW ACELLULAR PERTUSSIS VACCINE

by

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This dissertation, submitted by Travis Douglas Alvine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Travis Alvine
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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

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ABSTRACT

Despite widespread vaccination, *Bordetella pertussis*, the causative agent of whooping cough, is still a threat to global health. One cause of pertussis reemergence observed in many countries is ineffective immunity generated by the current acellular pertussis (aP) vaccines. Interestingly, recent studies have shown that TLR stimulating agents can enhance aP vaccine induced immunity. Type III secretion (T3S) system needle proteins from many gram-negative bacteria have been shown to be strong TLR agonists that induce NF-$\kappa$B/AP-1 signaling and promote inflammatory cytokine release from innate cells *in vitro*. In this study, we investigated the immune modulating properties of BscF, a purified T3S system needle protein from *B. pertussis*. In addition, we characterized the ability of BscF to enhance aP vaccine induced immunity. In the current study, we demonstrated that BscF is a strong TLR2 and TLR4 agonist that induced NF-$\kappa$B/AP-1 activation and promoted inflammatory cytokine release, augmented by clathrin-mediated endocytosis. *In vivo*, BscF immunization induced robust antibody responses, strong Th1 and Th17 responses from stimulated splenocytes, and provided modest protection against *B. pertussis* challenge. BscF also enhanced aP induced immunity and reduced lung bacterial burden in mice challenged with *B. pertussis*. These results demonstrate that BscF has considerable potential to be included in a next-generation *B. pertussis* aP vaccine.

# CHAPTER I

# INTRODUCTION

## *Bordetella* Microbiology and History

The genus *Bordetella*, belonging to the *Alcaligenaceae* family, are comprised of 10 genetically distinct species (1-3). *B. pertussis* is a Gram-negative, non-motile, aerobic coccobacillus that is typically grown at 37 ℃ on special Bordet-Gengou agar supplemented with blood and other growth factors. *B. pertussis*'s growth on blood supplemented medium is slow, requiring at least 3 days for colonies to appear. Within the genus *Bordetella*, the species can be differentiated, in part, by their hosts they infect as well as the symptoms reported during infection. *B. pertussis* is strictly a human pathogen (4-5) and was first thought as the sole cause of the prototypical whooping cough in humans. More recently, *B. parapertussis*, and *B. holmesii* have been identified to cause the prototypical whooping cough symptoms (6-11). While primarily thought of as a domestic animal pathogen i.e., cats and dogs (12-13), *B. bronchiseptica* has been isolated in rare events from immunocompromised or traumatized humans (14-16). Despite the ability of many *Bordetella* species to infect humans, *B. pertussis* still remains the most well characterized, and possess the greatest risk to overall global health.

When compared to many other infectious diseases, whooping cough is a fairly newly discovered pathogen. Pertussis-like symptoms and illness go back roughly 1,500

years when it was described by a Chinese medical scholar as “the cough of 100 days” (17-18). Fast forward to 1578 when Guillaume de Baillou characterized what was thought of today as the oldest pertussis outbreak among children in Paris (19). Recent evidence suggests that 3 epidemics of whooping cough occurred in Persia (present-day Iran) in the 15$^{th}$ and 16$^{th}$ century, likely indicating the earliest recorded epidemics of whooping cough in the world (20). Outbreaks of pertussis have also been reported in Europe during the 16$^{th}$ century, however the causative agent was not identified until much later. In 1906, *B. pertussis* was first identified as the causative agent of whooping cough by Jules Bordet and Octave Gengou (21), leading to Bordet winning the 1920 Nobel Prize in Physiology or Medicine for his extensive body of work from developing culture medium necessary to grow *B. pertussis* to further characterization of *B. pertussis* as the causative agent of whooping cough. *B. pertussis* as it’s named today, was originally named *Haemophilus pertussis*, but the name was changed to honor one of its discoverers (22).

## ***Bordetella pertussis*** **pathogenesis**

As previously mentioned, *B. pertussis* is strictly a human pathogen (4-5). *B. pertussis* is pathogen that targets the upper respiratory tract is classically considered an extracellular pathogen. Despite its extracellular location within the respiratory tract, *B. pertussis* has been shown to invade ciliated epithelial cells as well as alveolar macrophages (23-25). *B. pertussis* is passed from human to human through inhalation of infected respiratory droplets (26-30). Upon inhalation, *B pertussis* enters and adheres to ciliated epithelial cells of the upper respiratory tract (26-31). *B. pertussis* is classified as a

toxin mediated disease that requires a coordinated effort from a number of virulence factors expressed by the bacteria. This coordinated virulence factor activation is initiated upon *B. pertussis* attachment, and further allows for *B. pertussis* dissemination to the lower respiratory tract (26-31). These virulence factors include toxins: pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), and tracheal cytotoxin (TCT), as well as other structures including filamentous hemagglutinin (FHA), fimbriae (FIM), pertactin (PRN), the type three secretion (T3S) system, and lipopolysaccharide (LPS). These *bvg*AS virulence genes are controlled by the BvgAS two-component regulatory system of *B. pertussis* and allows the bacteria to respond to changing environments (32-37). The importance of these virulence factors during infection are highlighted in a number of studies indicating that these virulence factors not only promote adhesion and invasion of ciliated epithelial cells, but also strongly influence the host's innate and adaptive immune system often times at the expense of the host and to the benefit of the pathogen.

## ***B. pertussis*** **virulence factors and host immune modulation**

### **pertussis toxin (PT)**

PT is the most well characterized toxin and is the only *B. pertussis* antigen that is included in all formulations of current licensed acellular pertussis (aP) vaccines. PT is classified as an A-B toxin consisting of 5 subunits (S1 through S5). The A subunit (S1) is an ADP-ribosyltransferase while the B subunit is a pentameric ring structure (S2, S3, S5, and two S4 subunits) that mediates toxin binding to target cells through binding to glycosylated receptors (38-39). Once inside the cell, the holotoxin undergoes retrograde transport to the endoplasmic reticulum and the S1 subunit (the catalytic domain of PT)

is released into the cytosol (40). Once inside the cytosol, PT modifies intracellular signaling cascades through its ribosylation of the $\alpha$ subunit of heterotrimeric G proteins. PT has been implicated in many secondary systemic complications that arise from *B. pertussis* infections because of its ability to bind glycosylated receptors, as well as its ability to modify G proteins; both glycosylated receptors and G proteins are expressed in many different tissues. The pleiotropic affects seen by PT range from paroxysms and neurological disturbance (41), lymphocytosis (42), hyperinsulinemia, hypoglycemia, as well as histamine sensitization (43) are the result of its enzymatic activity. In addition, PT as well as detoxified PT (dPT) can act as pattern associated molecular patterns (PAMPs) that bind and activate a group of pattern recognition receptors (PRRs) called toll-like receptors (TLRs), specifically TLR2 and TLR4 (44-46).

In addition to its enzymatic and immune stimulating activities, PT can modulate the host innate and adaptive immune response further limiting the ability of the host to respond to, and clear, a *B. pertussis* infection, as well as influence the surrounding environment by either promoting pro- or anti-inflammatory mechanisms. Many of these studies have been completed in PT-deficient *B. pertussis* strains. The contribution of PT to early stages of infection were elucidated as infection with a PT-deficient *B. pertussis* strain resulted in lower bacterial colonization as early as 24 hours post infection (47). In addition, it appears that PT suppresses early neutrophil influx into the lungs during *B. pertussis* infection through inhibition of neutrophil attracting chemokine release from resident cells within the lungs (47-50). PT has also been shown to target and inhibit airway macrophages in addition to neutrophils (51). PT also targets the adaptive

immune system by suppressing *B. pertussis* specific serum antibody levels during infection (52-53). Interestingly, the role of PT appears to evolve as the infection progresses. Early in infection, the primary role of PT is to down regulate host immunity, primarily through innate cell inhibition. However, at the peak of infection PT induces robust lung inflammation and pathology in mice (54). From these studies we can appreciate the importance and complicated role that PT plays during *B. pertussis* infection.

**Adenylate cyclase toxin (ACT)**

ACT is another important *B. pertussis* specific toxin that is now recently been the focus of studies determining the usefulness of adding ACT to new aP vaccines. To date, ACT is not an antigen that is included in current aP vaccines. ACT is a member of the repeat in toxin family and is secreted from the cell via type I secretion. ACT possesses two functional C- and N-terminal domains that facilitate receptor binding on host cells and its catalytic adenylate cyclase activity, respectively (55). Once inside the host cell cytosol, the catalytic domain is activated by by calmodulin binding, facilitating the conversion of cellular ATP into cAMP and modifying intracellular signaling cascades (56). Given that both PT and ACT appear to modify intracellular signaling in similar manners, it was thought that these toxins may play redundant roles during infection. ACT-deficient *B. pertussis* strains showed reduced colonization ability; however, these strains were able to colonize the host for a short period of time but were unable to cause persistent infections (48). From these observations and the observations that PT-deficient strains showed reduced ability to colonize early during infection, it is likely that

these two toxins have non-redundant roles in *B. pertussis* infections: PT facilitates early colonization while ACT is necessary for persistent infection (48).

Like PT, ACT also modulates the surrounding environment to facilitate infection. ACT has been shown to down regulate host immunity by inhibiting phagocytic cell trafficking, bactericidal activities, and pro-inflammatory cytokine release (57-58). ACT also has been shown to modulate the adaptive arm of the immune system during infection. T-helper (Th) cells, specifically Th1 and to a lesser extent Th17 cells, are important for resolution of *B. pertussis* infections. ACT directly targets T cell activation and differentiation to skew $CD4^+$ T cells to a Th2 type phenotype (59); a T cell response that has shown to be non protective both animal and human models. ACT also polarizes the adaptive immune system to a Th17 type response by NLRP3 inflammasome activation and subsequent IL-1$\beta$ production from murine dendritic cells (DC) (60). The pro-inflammatory role of ACT is controversial, but the importance of ACT in *B. pertussis* pathogenesis is highlighted in both murine and human studies.

**Tracheal cytotoxin (TCT)**

TCT is not currently used in aP vaccines and is not unique to *B. pertussis*. TCT is a disaccharide-tetrapeptide monomer of peptidoglycan that is present in all Gram-negative bacteria (61). ACT is released as part of normal bacterial cell growth as they remodel their cell wall. Due to the lack of the cytoplasmic membrane protein called AmpG, which normally participates in recycling of the peptidoglycan fragment, TCT is constitutively released into the environment and is not under control of the BvgAS two-component system controlling other virulence factor gene expression. Because TCT is

released from the bacteria it acts locally on cells of the respiratory system to promote *B. pertussis* infection. TCT acts directly on nonciliated respiratory cells to induce IL-1$\alpha$, leading to increased nitric oxide (NO) synthase activity and subsequent NO production (62-63). TCT's ability to induce NO production is dependent on LPS (62). The NO is then able to diffuse to neighboring ciliated epithelial cells further promoting respiratory tract inflammation.

**Filamentous hemagglutinin (FHA)**

FHA is one of the most important adhesions and like PT, is included in all of the current aP vaccines. FHA has binding domains specific for heparin sulfate, carbohydrate, and integrin binding via an Arg-Gly-Asp site (64). Due to the multiple binding sites, FHA mediates initial adhesion to ciliated epithelial cells of the upper respiratory tract. Dissemination of *B. pertussis* down to the lower respiratory tract is mediated by FHA. Secretion of FHA to the cell wall has been shown to be dependent on an outer membrane accessory protein named FhaC as FhaC is able to make channels within the outer membrane (65). In addition to its binding characteristics, FHA is also an immune modulator. In mice, FHA primarily acts an immune suppressor. Systemic administration of FHA suppressed pro-inflammatory cytokine and enhanced anti-inflammatory cytokine release from innate cells, in addition to generating regulatory T cells and reduced colitis induced intestinal inflammation (66). Infection studies in *B. bronchiseptica* further clarified FHA's immunosuppressive role (67-68). Interestingly, the role of FHA in a human context appears to be inflammatory rather than anti-inflammatory. FHA elicited pro-inflammatory cytokine release from human innate and epithelial cells (69-70).

## Fimbriae (FIM)

FIM is a surface exposed structure that has heparin binding activity. The major subunits of FIM, FIM2 and FIM3, are serotype specific and have been shown to be serologically distinct (71). FIM antigens have been included as antigens in current aP vaccines; however not to the same extent that PT and FHA have been. FIM were shown to important for colonizing the respiratory tract in *B. bronchiseptica* infections (72).

## Pertactin (PRN)

PRN is an auto transporter protein that mediates *B. pertussis* adhesion to eukaryotic cells via its Arg-Gly-Asp binding site; however, PRN also contains proline-rich regions and leucine-rich repeats (73). PRN contributes to *B. pertussis* pathogenesis by resisting neutrophil-mediated clearance (74). PRN antigens are commonly used in current aP vaccines. Because PRN is highly polymorphic, the circulating *B. pertussis* strains may have different PRN variants that are included in aP vaccines. In addition, PRN-negative strains are beginning to emerge across the globe. The first reported PRN-negative strain was in 2012 in France (75), with many other PRN-negative reports following (76-77). PRN appears to be dispensable to the bacteria as PRN-negative strains can evade aP generated immunity better than PRN-positive strains (78). The emergence of PRN-negative *B. pertussis* strains highlights the need for newly developed protective antigens in next-generation aP vaccines.

## Type III secretion (T3S) system

In comparison to other Gram-negative bacteria (i.e. *Yersinia* spp.), the T3S system of *B. pertussis* is not well characterized. Four *B. pertussis* proteins: Bsp22, BopN,

BopD, and BteA have been shown to be secreted. The structure and function of the *B. pertussis* is similar to other Gram-negative bacteria. This is a syringe-like structure that extends from the surface of the bacteria and facilitates direct translocation of effector molecules into host cells. The first reports of the contribution the T3S system plays during infection came from studies in *B. bronchiseptica*. These studies reveled that *B. bronchiseptica* uses T3S to persist within the murine respiratory tract (79). The ability of *B. bronchiseptica* to persist during infection is likely due to the ability of the T3S system to modulate innate immunity. Re-stimulated splenocytes from T3S mutant *B. bronchiseptica* produced more IFN-$\gamma$ and less of the immunosuppressive cytokine IL-10, suggesting that T3S down regulates host immunity (80). In addition, the T3S system from *B. bronchiseptica* has been shown to be important for modulating DC maturation (81-82). The first evidence of a functionally active T3S system in *B. pertussis* was reported in 2008 (83). Mutation of the effector molecule *bscN*, which abolished protein secretion, resulted in a reduced ability to colonize the respiratory tract of mice (83). Reduced colonization was the result of an increase in innate pro-inflammatory cytokine release in the lungs, elevated antigen specific IFN-$\gamma$ and IL-17, as well as increased antibody responses (83). These results confirm an immunosuppressive role of the T3S system in *B. pertussis* infections and provides evidence of the importance of T3S during *B. bronchiseptica* and *B. pertussis* infections. Another interesting finding from the work of Fennelly et al., (83) was that a number of laboratory-adapted strains of *B. pertussis* did not secrete the effector molecule Bsp22. This reversibility of T3S system expression was confirmed by Gaillard et al., (84). In addition to finding that laboratory-adapted

strains did not have a functional T3S system *in vitro*, they reported that the T3S system can become functional *in vivo* when the bacteria are put into a host; this was also true in the laboratory-adapted strains as well (84).

**Host immune responses to *B. pertussis* infection**

Our understanding of what protective immunity looks like comes from a large body of work examining the host immune response to *B. pertussis* infection. The first line of defense against *B. pertussis* infections occurs at the upper respiratory tract and involves cells of the innate immune system. These lung resident cells include airway mucosal dendritic cells (AMDCs) and alveolar macrophages (AMs). The innate immune system helps to control early infection, and polarizes the adaptive immune response necessary to clear the infection. AMDCs will take up antigen at the site of infection and migrate to the lymph nodes to prime an adaptive immune response (85-86). On the other hand, AMs residing at the site of infection uptake and kill *B. pertussis* directly (87). These resident cells respond to, and become activated by, the many PAMPs expressed by *B. pertussis*. Many of the virulence factors have been shown to stimulate PRRs of the innate immune system. These resident cells within the lung not only act as the first line of defense, they also orchestrate further immune responses that prime the adaptive immune response and facilitate the recruitment of other immune cells.

In addition to AMDCs and AMs, several other cell types contribute to *B. pertussis* clearance, especially during the early stages of infection. $\gamma\delta$ T cells, have been shown to be important in the early immune response. $\gamma\delta$ T cells release an early source of IL-17 which promotes cell trafficking into the lungs as well as antimicrobial peptide

production (88-89). IL-17 release in combination with the cytokine CXCL2 (MIP-2) secreted from macrophages and epithelial cells promotes neutrophil influx to the site of infection. Neutrophils have been shown to be an important cell type for controlling *B. pertussis* infections by antibody-mediated phagocytosis, intracellular killing of *B. pertussis*, and the production of neutrophil extracellular traps (81-82). Natural killer (NK) cells have also been shown to play an important role in early innate immune control of *B. pertussis* infection. NK cells primarily exert their protective effects through the release of IFN-$\gamma$. IFN-$\gamma$ activates macrophages and depletion of NK cells from mice infected with *B. pertussis* reduced IFN-$\gamma$ release resulting in bacterial dissemination from the respiratory tract to the liver (83). While these innate cells play a critical role in the early stages of infection, a polarized adaptive immune response is critical in controlling late stages of infection, primarily by augmenting neutrophil and macrophage bactericidal activity.

$CD4^+$ T cells have been shown to play an important role in *B. pertussis* clearance as adaptive transfer of *B. pertussis* specific $CD4^+$ T cells, but not *B. pertussis* specific $CD8^+$ T cells into immunosuppressed mice resulted in effective bacterial clearance (84). $CD4^+$ T cells mediate their immune activation through cytokine release. Th1 and Th17 $CD4^+$ T cells release IFN-$\gamma$ and IL-17, respectively. The role of Th1 and Th17 cells in protective immunity against *B. pertussis* was investigated in IFN-$\gamma^{-/-}$ and IL-$17^{-/-}$ mice which demonstrated a reduced ability to clear *B. pertussis* from the lungs (85-86). Moreover, in the case of the IL-$17^{-/-}$ mice, the inability to clear the pathogen was associated with a reduction in neutrophils within the lungs during infection (86). Once polarized by

AMDCs in the lymph nodes, primed T cells proliferate and differentiate into Th1 and Th17 cells. These cells then migrate back to the site of infection, and enhance macrophage and neutrophil bactericidal activity through IFN-$\gamma$ and IL-17 production, respectively.

## Pertussis Epidemiology

### Global burden and economic impact

For the past few decades, the use of either the whole cell *B. pertussis* vaccine (wP) or the currently aP vaccine has significantly reduced the global burden of the disease, although pertussis still remains endemic in both developed and developing countries. In addition, *B. pertussis* is undergoing a reemergence in many parts of the world. Estimates of the global burden of *B. pertussis* has been complicated by a number of issues from inadequate and/or underreporting, constant changes in surveillance and diagnostic protocols, as well as routine modifications to vaccine schedules as well as vaccine components (87). While the global incidence of pertussis is tricky, more recent statistics show that in a 2010 analysis, there were 16 million reported *B. pertussis* cases worldwide, with 195,000 deaths (88). A separate report completed in 2013 determined there were an estimated 136,000 cases worldwide that year (89). Given the great difficult in accurately reporting *B. pertussis* cases, underreporting is a significant concern. Specifically, in the United States there have been a number of reported *B. pertussis* outbreaks. Minnesota experienced a pertussis outbreak resulting in 4,144 reported cases (90). In the same year, there were a total of 4,918 cases in Washington State (91). During the same time period and into 2014, pertussis was widespread

throughout California. In 2014, 10,831 cases were reported, the highest number in any one year (92). In terms of the economic impact of pertussis, often times costs can be high due to hospitalization. Total societal costs due to pertussis was estimated to be approximately $800 for adolescents and $1,950 for adults per case (93). Clearly pertussis is still active globally and negatively impacts human health worldwide.

## Clinical presentation

Clinical progression of a *B. pertussis* infection is identified by 3 common stages: 1. Catarrhal 2. Paroxysmal 3. Convalescent. Each stage can vary in length, but typically can last 1-3 weeks, with complete recovery taking much longer. The course and duration of the disease is dependent on age of patient, vaccination history, infectious agent (i.e. *B. pertussis* versus *B. parapertussis*), and infectious dose. The three stages are most likely noticed and identified in infants and young children, while adolescents and adults usually present with milder symptoms and may go undiagnosed. The incubation period for B. pertussis is around 7-10 days. In the first stage, symptoms are often overlooked because they are similar to other viral infections (94). Interestingly, in the catarrhal stage, the bacterial burden is extremely high and the individual is most contagious at this stage. In the second stage, the paroxysmal stage, patients experience severe coughing bouts as well as the characteristic inspiratory whooping sound. Presently, it is not clear if one of the many toxins or virulence factors are responsible for the coughing. In the final stage, convalescence, coughing bouts are less in frequency and are not as severe.

## *B. pertussis* vaccines

To date, there have been 2 available vaccines for *B. pertussis*: the wP vaccine as well as the aP vaccine. The wP vaccine was first introduced in the United States in the late 1940s. the wP vaccine consisted of inactivated *B. pertussis* and has been given in combination with diphtheria and tetanus toxoids. The cases of *B. pertussis* in the United States plummeted to approximately 1,000 in 1976 after the implementation of the wP vaccine (95). Despite its effectiveness, the wP vaccine fell out of favor due to safety concerns of reported local and systemic reactions (96-97). In rare cases, serve neurological diseases such as encephalopathy, spasms, and sudden infant death syndrome were reported (96,98-99) The antigenicity and subsequent adverse reactions can likely be attributed to LPS and other innate stimulating agents on the bacterial surface. The continued adverse reactions from the wP vaccine prompted the development of less reactogenic vaccines consisting of purified antigens from *B. pertussis*. These vaccines, or derivations of these vaccines, are currently used today and are classified as aP vaccines. The first aP vaccine was developed in Japan in 1984 (100). Interestingly, the composition of the current aP vaccines are not uniform in the number and/or amount of antigens. Mutant PT and FHA are the only two antigens included in every aP vaccine. Others include PRN or fimbrial proteins. These new aP vaccines are given with diphtheria and tetanus toxoids and absorbed in alum as an adjuvant. Many different aP vaccines have undergone rigorous safety and toxicity testing and have been deemed safe and immunogenic. Edwards et al., (101-102) concluded that all 13 aP vaccines tested were found to be safer and produced less severe adverse reactions and were at least as immunogenic as compared to wP vaccines.

## Reemergence of *B. pertussis*

The reemergence of pertussis related diseases is a complex issue and is most likely the result of a combination of multiple factors. First, many circulating *B. pertussis* strains have undergone pathogen adaptation and genetic changes, especially in aP vaccine antigens (103-105). One explanation for this is vaccine induced selective pressure (106-107). Two of the major antigens that have undergone genetic changes are PRN and PT. PRN-negative strains have been identified in many different parts of the world (77,108-110). In the United States, more than 50% of the collected strains were PRN-negative in 2012 (108). Interestingly, PRN-negative strains appeared to have a competitive advantage over PRN-positive strains in mixed infections in mice (78). In addition, PRN protein variants found in circulating *B. pertussis* strains are not included in the vaccine strain. PRN2 and PRN3 protein variants have been found to outperform PRN1 by enhanced colonization and increased transmissibility (111). In the case of PT, strains with genetic changes within the PT promoter produce slightly more PT than previously circulating strains (112), and are circulating globally. Given that vaccine selection pressure is accelerating at an increased pace with the aP vaccines, and that current antigens included in the aP vaccine are no longer present on many isolates, the need for new vaccination strategies will be important moving forward.

Second, recent research indicates that the current aP vaccines fail to induce protective immunity and vaccine induced immunity may wane overtime. The data from both mouse and human studies indicate that $CD4^+$ Th1 and to a lesser extent $CD4^+$ Th17 cells are critical for protective immunity generated by vaccination. In animal studies, wP

vaccination conferred protective adaptive immunity mediated by Th1 and Th17 cells, while aP vaccination promoted non-protective Th2 type responses (84,86, 113). Th2 type responses have been shown to redundant in animal models (86). Human studies also highlight suboptimal immunity induced by aP vaccines. An analysis of T cell responses in children reveal that aP vaccination promotes Th2 type responses, while wP vaccination induces strong Th1 immune responses (114-115). In addition to suboptimal immunity induced by aP vaccines, the longevity of protective immunity has been shown to wane over time. In head to head comparisons of long term cellular immunity induced by aP or wP vaccination in children, Schure et al., (116) found that aP vaccination actually produced stronger *B. pertussis* specific $CD4^+$ T cell responses compared to wP vaccinated children. However, analysis of vaccine induced responses 5 years after primary pertussis vaccination levels of IL-17 production from aP vaccinated PBMCs was reduced compared to wP primed children (117). In addition, aP vaccination lead to significantly more end-stage differentiated $CD4^+$ T cells responses compared to wP vaccination, suggesting the memory capacity of the immune responses is reduced in the aP vaccinated children (117). Together, these reports highlight a major inadequacy with the aP vaccine, potentially contributing to the increased incidence of pertussis cases in many countries.

Finally, one of the hurdles pertussis researchers have had to overcome is that many of the animal models currently used to understand pertussis pathogenesis and vaccine induced immunity are not natural hosts to *B. pertussis*. For example, mice and other small mammals do not exhibit the classical whooping cough symptom. Recently, a

baboon model has been developed and has greatly enhanced our understanding of *B. pertussis* infection and transmission. One of the most striking finding from baboon studies is that while aP vaccination prevented clinical symptoms of B. pertussis, the baboons were highly colonized and were able to infect naïve baboons (113). This finding is significant in that in aP vaccinated areas, while clinical identification of whooping cough may be low, there may be a significant portion of the population that are colonized and able to transmit *B. pertussis* to susceptible newborns and infants who have not yet received aP vaccination. In addition, aP vaccination in the baboon model induced a mixed Th1/Th2 response and failed to prevent colonization while wP vaccination provided protection and induced a mixed Th1/Th17 response (113), confirming the importance of both Th1 and Th17 cells in protective immunity against *B. pertussis*.

**Approaches to improve aP vaccines**

Due to the number of shortcomings of current aP vaccines, the need for better vaccines has been recognized by a number of authorities in the field. Although a number of approaches have been suggested, a large amount of work has centered around developing new protective antigens or adjuvants that when added to current aP vaccines will skew vaccine induced immunity toward Th1/Th17 type responses. It is thought that the numerous PAMPs included in the wP vaccine are what contributed to long term protective immunity. These PAMPs activate the innate immune system and promote inflammatory cytokine release and DC maturation to induce Th1/Th17 cellular immunity (118). Current aP vaccines are absorbed in alum and it has been suggested

that there are no classical PAMPs included in the aP vaccines. Although PT has been shown to have immune stimulating properties, vaccine preparation strategies have destroyed PT's immune stimulating ability (119). To that end, replacement or supplementation with innate stimulating adjuvants is one strategy to enhance aP vaccine immunogenicity and efficacy.

A TLR2 agonist of *B. pertussis* when combined with the components of the aP vaccine enhanced protection from an aerosol *B. pertussis* challenge, induced robust IgG2a antibodies, and enhanced IL-17 and IFNy production from antigen stimulated splenocytes *ex vivo* compared with the aP vaccine in alum (120). A TLR4 agonist, monophosphoryl lipid A (MPL), when mixed with the aP vaccine increased protection against *B. pertussis* challenge when compared with the aP vaccine in alum adjuvant, while suppressing Th2 responses (121). A separate TLR agonist, LpxL1 (122), a genetically engineered LPS from *Neisseria meningitidis*, enhanced antigen specific IFNy and IL-17 $CD4^+$ T cells and increased the number of specific memory $CD4^+$ $T_{CM}$ cells when added to the aP vaccine (123). Finally, CpG oligonucleotides from bacterial DNA that activate TLR9 have been shown to induce antigen-specific IgG2a titers (124) and Th1 and Th17 cells (125) when added to the aP vaccine (86). These studies show the feasibility and effectiveness of incorporating novel TLR agonists into the aP vaccine to enhance pertussis specific immunity that will not only promote the proper adaptive immune responses, but could also augment the long-term efficacy of the aP vaccine.

The importance of stimulating the innate immune system during vaccination and the subsequent generation of protective immunity is highlighted by the efficacy of the

live-attenuated *B. pertussis* vaccine BPZE1. This vaccine is designed for intranasal administration and has been genetically modified to remove or inactivate DNT, TCT, and PT mice (126). BPZE1 has been found to be safe in mice (126), and in humans during a Phase I clinical trial (127). Importantly, BPZE1 induced strong *B. pertussis* specific Th1 responses and provided protection against *B. pertussis* in mice (128) as well as protected mice against *B. parapertussis* (129). The benefit of adding PAMPs to enhance protection either by the addition of TLR stimulating molecules to the aP vaccine or novel vaccines that seek to harness innate immune stimulation to promote protective cellular responses opens the door to more efficient approaches to significantly decrease pertussis cases globally.

**Rationale of current work**

Based on the current literature, the strategy of adding TLR stimulating agents to the aP vaccine to skew aP induced immunity has proven successful. Recent work in our laboratory has identified a number of novel TLR ligands purified from the T3S system of many Gram-negative bacteria (130). These molecules have been shown to activate TLR2 and TLR4, induce NF-$\kappa$B/AP-1 signaling, and promote inflammatory cytokine release *in vitro* (130-131). Interestingly, we have successfully demonstrated that the N-terminus from these proteins functions to modulate innate immune activation (131). This is unique in that not only have our proteins been shown to activate TLR2 and TLR4, but these proteins can be modified to enhance or reduce NF-$\kappa$B/AP-1 signaling. In addition to the immune stimulating properties of these proteins, we believe that given the extracellular location of the T3S system, BscF may also act as a protective antigen in *B.*

*pertussis* infections. YscF, a purified needle protein from *Y. pestis*, protected mice against a *Yersinia pestis* infection (132). In addition, a *Chlamydia* T3S system needle protein induced specific humoral and cellular responses, and decreased *Chlamydia* loads in mice (133). In the current study, we investigated the immune stimulating properties of a novel *B. pertussis* specific protein from the T3S system called BscF. Further, we assessed the ability of BscF to induce both humoral and cellular responses in mice necessary for vaccine induced protection, and characterized the contribution of BscF to a laboratory prepared aP vaccine.

## CHAPTER II

## CHARACTERIZATION OF THE IMMUNE RESPONSE INDUCED BY BSCF, A PURIFIED TYPE III SECRETION SYSTEM NEEDLE PROTEIN FROM *BORDETELLA PERTUSSIS*

### Introduction

*Bordetella pertussis* is a gram-negative bacterium and the causative agent of the vaccine preventable disease whooping cough (pertussis). With the development of a whole cell pertussis (wP) vaccine in the 1940s-1950s, cases of pertussis were dramatically reduced. The wP vaccine proved to be too reactogenic and was replaced with a less reactogenic vaccine: the subunit acellular pertussis (aP) absorbed in alum as the adjuvant in the 1990s. Despite continued widespread vaccination, whooping cough has again reemerged as a global health threat not only in newborns and infants, but surprisingly among adults as well (88). This resurgence has been linked to antigenic variation in many circulating *B. pertussis* strains (103,134,135,76,136), defective long-term immunological memory (137,138,139,140), and ineffective immune responses that are necessary for long-term protection (141,113). It has been demonstrated in both mice and humans that the aP vaccine induces robust Th2 responses, with a limited Th17 response (114,142,117,143). The aP vaccine has been shown to prevent clinical pertussis symptoms, but does not prevent bacterial colonization or transmission (113).

On the other hand, the wP vaccine has been shown to promote Th1 and Th17 responses and protective immunity in both mice and humans (114,144,86).

Effectiveness of the wP vaccine has been largely attributed to its many antigens and pathogen associated molecular patterns (PAMPs) that bind and activate innate pattern recognition receptors (PRRs) (27). Given the contribution of the many PAMPs to the effectiveness of the wP vaccine, we propose that the addition of PRR stimulating agents could enhance the effectiveness of the current aP vaccine.

Endogenous *B. pertussis* specific PAMPs activate the innate immune system through PRRs – including Toll-like receptors (TLRs), promote inflammatory cytokine release, and direct pertussis-specific adaptive immunity (118). Because PAMPs appear to be an important aspect of the wP vaccine, it has been suggested that the addition of TLR agonists to the aP vaccine could re-direct the immune response generated by the aP vaccine to a more wP-like immune response. A TLR2 agonist (lipoprotein BP1569 and its synthetic derivative, LP1569) of *B. pertussis* when combined with the components of the aP vaccine induced strong Th1 and Th17 responses and enhanced protection from an aerosol *B. pertussis* challenge (120). A TLR4 agonist, monophosphoryl lipid A (MPL), increased protection against *B. pertussis* challenge while suppressing Th2 responses (121). In addition, other TLR4 agonists have been shown to enhance immunity generated by the aP vaccine (123). Finally, CpG oligonucleotides from bacterial DNA that activate TLR9 have been shown to enhance aP-directed pertussis immunity. (86,124,125). These studies demonstrate the feasibility and effectiveness of incorporating novel TLR agonists into the aP vaccine to enhance pertussis specific immunity.

We have recently identified that the needle proteins from bacterial type III secretion (T3S) systems are novel TLR agonists (130,131]. Interestingly, these proteins activate TLR2 and TLR4, promote pro-inflammatory cytokine release, and can be modified to modulate TLR signaling (130,131). Immunization with T3S needle and translocon proteins have shown to produce protective immunity in mice against a number of gram-negative pathogens (145,132,146,147,148,149). In the current study, the immune stimulating properties of BscF, a purified T3S apparatus protein from *B. pertussis*, were examined. In addition, the ability of BscF to provide protective immunity against a sub-lethal *B. pertussis* challenge was also assessed. We demonstrate that BscF induces NF-kB and/or AP-1 signaling following TLR2 or TLR4 ligation. This activation promotes strong inflammatory cytokine release from both mouse and human innate cells. Furthermore, mice immunized with BscF produce robust BscF specific humoral and adaptive immune responses; contributing to modest protection against a sub lethal *B. pertussis* challenge. Our findings demonstrate that BscF from *B. pertussis* is a TLR agonist, like other T3S needle proteins, that could contribute to a next-generation pertussis vaccine through its innate immune stimulating properties or by acting as a protective antigen.

## Materials and Methods

### Bacterial strains and growth conditions

*E. coli* Novablue (EMDMIllipore, Billerica MA), BL21 (DE3) star (Invitrogen, Carlsbad, CA), LPS modified BL21 (DE3) (161), TOP10 (Invitrogen), and *Bordetella pertussis* (Tohama I, ATCC BAA-589) were stored at -80°C in 25% glycerol (vol/vol). *E. coli*

strains were grown at 37°C in LB broth (BD, Franklin Lakes, NJ) or on tryptose blood agar base (TBA, BD) plates, with kanamycin (50 µg/ml) as needed. *B. pertussis* was maintained as previously described (162). Briefly, *B. pertussis* was grown at 37°C on Bordet-Gengou (BG) solid medium (Remel$^{TM}$ Thermo Fisher Scientific, Lenexa, KS) supplemented with glycerol and 15% sterile sheep's blood (Lampire Biological Labs, Pipersville, PA). *B. pertussis* liquid cultures were grown in Stainer-Scholte broth supplemented with heptakis (2,6-di-*O*-methyl-ß-cyclodextrin; Sigma-Aldrich, St. Louis, MO) and Stainer-Scholte supplements at 37°C. In the case of the GFP-expressing *pertussis*, both solid and broth medium were supplemented with kanamycin (50 µg/ml) and gentamicin (30 µg/ml).

**Expression and purification of His-tagged recombinant proteins**

Template DNA for amplification was generated by using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Oligonucleotide primers (Eurofins MWG Operon, Inc. Huntsville, AL) were used to amplify BscF DNA from *B. pertussis* Tohama I strain: BscF forward (5'-CAC CAT GGC CAT TAA CCT GGG AGG-3') and BscF reverse (5'-TCA ACT CGC CTT CTG TAT GAC GCC C-3'). PCR was performed using Pfu Ultra polymerase (Agilent Technologies, Santa Clara, CA). The amplified DNA was cloned in frame with a N-terminal His-tag into pET200 by using a Champion TOPO expression kit (Invitrogen). Plasmid for protein expression was purified from *E. coli* TOP10 with a Qiaprep Miniprep kit (Qiagen). Purified plasmid DNA was then transformed into chemically competent *E. coli* BL21 (DE3) Star (Invitrogen). Plasmid constructs were verified by sequencing (Eurofins MWG Operon, Inc.).

Protein purification was performed as previously described (130,131). Briefly, *E. coli* BL21 (DE3) Star (Invitrogen) was grown overnight at 37°C in a shaking water bath in non-inducing medium (50X M, 1 M $MgSO_4$, 40% glucose, 5% aspartic acid (163)) supplemented with antibiotic. Bacteria were then inoculated into auto-inducing medium (50X M, 1 M $MgSO_4$, 50X 5052, NZ-amine S, yeast extract, distilled water (163)) supplemented with antibiotic and grown to an $A_{620}$ of 0.6 to 0.8. Cells were harvested by centrifugation at 4,000 x *g* for 10 min at 4°C and resuspended with wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10% glycerol (wt/vol)). The bacterial suspension was then French pressed at 20,000 lb/in$^2$ twice to lyse cells. The lysate was centrifuged at 10,000 x *g* for 20 min at 4°C. The supernatant was collected and diluted with 1,000 ml of wash buffer before application to a pre-equilibrated TALON metal affinity resin (Clontech, Mountain View, CA) column. The lysate was applied to the column twice followed by washing the column with 15 bed volumes of wash buffer. Bound protein was eluted in elution buffer (50 mM $NaH_2PO_4$, 200 mM NaCl,150 mM imidazole, and 20% glycerol (wt/vol)). Purified protein was concentrated by centrifugation (Amicon Ultra centrifugal filters, Millipore, Billerica, MA), and dialyzed against phosphate-buffered saline (PBS) plus 10% glycerol (wt/vol) in Slide-A-Lyzer dialysis cassettes (Pierce, Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Bradford protein assay (Pierce, Thermo Fisher Scientific), and purified protein was stored at -80°C for future use. Purified BscF was shown to be > 95% pure by coomassie blue staining of 15% SDS-PAGE gels as previously described (130,131,132).

**Stimulation of SEAP reporter cell lines**

THP1-XBlue cells (InvivoGen, San Diego, CA) were maintained in RPMI 1640 (Gibco, Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum ((FBS); Atlanta Biologicals)), 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg/ml Pen-Strep at 37°C with 5% $CO_2$. HEK-Blue cells (InvivoGen) were maintained in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS, 2 mM L-gluatmine, 100 μg/ml Normocin (InvivoGen), HEK-Blue selection (InvivoGen), and 50 μg/ml Pen-Strep at 37°C with 5% $CO_2$. These cells contain the secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of NF-kB and AP-1. THP-1XBlue cells and HEK-Blue cells were seeded at 3 X $10^6$ cells/ml and 2.5 X $10^5$ cells/ml into 96-well plates, respectively. Cells were suspended in infection medium as described by the manufacturer. Proteins were added to a final concentration of 1 μg/ml. Cells were stimulated at 37°C with 5% $CO_2$ for 24 h. Quantification of SEAP from the supernatant was detected using Quanti-Blue reagent (InvivoGen) according to the manufacturer's protocol. SEAP activity was quantified by measuring the absorbance at 630 nm using a microplate reader (Synergy HT, BioTek, Winooski, VT) and was analyzed with KC4 v3.3 software (BioTek).

**Quality control for contamination of purified BscF protein**

Quality control of purified proteins was performed as previously described (130). Needle proteins and flagellin (*Salmonella* Typhimurium, InvivoGen) were incubated with 40 μg/ml of proteinase K at 37°C for 16 h to ensure activity from purified proteins was from protein. Proteinase K was inactivated with 1.6 mg/ml of phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). To check for lipopolysaccharide (LPS) contamination,

THP-1XBlue cells were pretreated with 20 μg/ml of polymyxin B (InvivoGen). Following enzymatic digestion or polymyxin B treatment, the THP-1XBlue cells were stimulated as indicated above. BscF was also expressed as indicated above in *E. coli* strains lacking LPS to further rule out LPS contamination. These protein preparations were compared directly to BscF expressed in *E. coli* BL21 (DE3) Star (Invitrogen) by measuring SEAP activity from THP-1XBlue cells.

**Cell line growth and bone marrow derived cell isolation and differentiation**

THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 (Gibco, Thermo Fisher Scientific) containing 10% heat inactivated FBS, 50 mM 2-mercaptoethanol, and 50 μg/ml Pen-Strep at 37°C with 5% $CO_2$. Mouse macrophage-like RAW 264.7 (ATCC TB-71) cells were maintained in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS at 37°C with 5% $CO_2$. Bone marrow cells were collected from femurs of the following C57BL/6 mice: WT, Toll-like receptor 2 (TLR2), and TLR4 Knockout, $Asc^{-/-}$, $Nlrp3^{-/-}$, $Nlrc4^{-/-}$, $Caspase11^{-/-}$, $Caspase1^{-/-}$ ($Caspase11^{Tg}$), and $Caspase1^{-/-}$ $Caspase11^{-/-}$. Femurs were aseptically removed from each hind leg, briefly soaked in 70% ethanol, and placed in fresh RPMI medium (10% heat inactivated FBS, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 50 μg/ml Pen-Strep). Both ends of the femur were cut and the bone was flushed with 10 ml of RPMI and the cells were collected in a 50 ml conical tube. The cell suspension was centrifuged at 400 x *g* for 10 min at 4°C. The cells were resuspended in RPMI supplemented with 40 ng/ml granulocyte-macrophage colony-stimulating factor (GMCSF; PeproTech, Rocky Hill, NJ), seeded at a density of 4 X $10^6$ in 20 ml of medium in a 150 x 20 mm round culture dish, and incubated at 37°C with

5% $CO_2$. On day 3, the cells were supplemented with 20 ml of fresh RPMI + 40 ng/ml GMCSF and incubated for an additional 3 days. At day 6, the non-adherent cells dendritic cells (DCs), and the adherent bone marrow derived macrophages (BMDM) were used for subsequent analysis.

**Innate cytokine analysis**

THP-1 cells and mouse RAW 264.7 cells were seeded in triplicate at 1 X $10^6$ cells/ml into 24-well plates and stimulated with 1 µg/ml of needle protein for 24 h at 37°C with 5% $CO_2$. PBS, 1 µg/ml LPS (E. coli K12; InvivoGen), and/or 1 µg/ml $Pam_3CSK_4$ (InvivoGen) were used as negative and positive controls. BMDM were seeded in triplicate at 5 X $10^5$ cells/ml into 24 well plates and stimulated with indicated concentrations of needle protein for 24 h at 37°C with 5% $CO_2$. Following stimulation, cells were centrifuged at 400 x *g* for 5 min at 4°C and the cellular supernatant was removed and stored at -20°C for future analysis. Numerous human and mouse innate cytokines were measured by DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) or mouse inflammation panel cytometric bead analysis (CBA) kit (BioLegend, San Diego, CA). CBA samples were measured by flow cytometery (LSR II, Becton Dickinson, San Jose, CA), and analyzed with software provided by the manufacturer (LEGENDplex v7.0).

**Phagocytosis inhibition and identification of intracellular pro-IL-1$\beta$**

To prevent phagocytosis, mouse DCs were seeded and pretreated with 0.25 $\mu$g/ml cytochalasin D (Sigma) for 1 h prior to stimulation with 10 $\mu$g/ml BscF. Following 24 h stimulation, cell culture supernatant was collected and levels of secreted IL-1$\beta$ was

measured via ELISA. Intracellular pro-IL-1$\beta$ was measured in stimulated DCs by flow cytometry. Cells were collected, permeabilized, and stained with anti-pro-IL-1$\beta$ PE conjugagted (Biolegend). Within the single cell population, MFI of PE was calculated as a measure of intracellular pro-IL-1$\beta$.

**Mouse immunization and intranasal challenge**

6-8 week old C57BL/6 male mice were immunized intra peritoneally with 40 µg of purified BscF in either PBS, or diluted 1:1 with aluminum hydroxide gel (Alhydrogel adjuvant 2%, InvivoGen). Mice were boosted with 20 µg of BscF at 2 and 4 weeks post vaccination. Mice that received PBS injections served as controls. Two weeks after the last immunization, mice were intra nasally challenged with 2 X $10^6$ CFU of *B. pertussis* Tohama I in a 25 µl inoculum. 7 d post infection, lungs were aseptically removed, and homogenized (Bullet blender, Next Advance, Averill Park, NY) in 1 ml of sterile PBS. Lung homogenate was centrifuged at 130 x *g* for 1 min at 4°C, serial dilutions were plated on 15% blood BG plates, and incubated at 37°C for 4 d. Lung bacterial burden was determined by counting CFUs. All animal experiments were approved by the IACUC at the University of North Dakota.

**ELISA assay of antibody levels in mouse serum**

ELISA plates (Costar EIA/RIA, Corning) were coated with 100 µl of 1 µg/ml BscF diluted in PBS and incubated overnight at 4°C. Plates were washed with wash buffer (1X PBS, 0.05% Tween-20), and blocked with blocking buffer (1X PBS, 1% BSA, 0.05% Tween-20) and incubated at room temperature for 1 h. Plates were again washed and incubated with serially diluted mouse serum for 1 h at room temperature. Following

incubation, plates were washed, blocked for 10 min at room temperature with blocking buffer, and incubated with rabbit anti-mouse IgG biotinylated (Invitrogen) antibody diluted 1:10,000 in blocking buffer for 1 h at room temperature. Both wash and blocking steps were repeated as indicated above, and plates were incubated with streptavidin-HRP (Invitrogen) diluted 1:2,000 in blocking buffer for 1 h at room temperature. For measuring IgG isotypes, isotype specific goat anti-mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3, IgM, and IgA (Sigma-Aldrich) diluted 1:1,000 in blocking buffer was incubated following serially diluted serum for 30 min at room temperature. Plates were washed and blocked as indicated above, and bound IgG was detected with biotinylated rabbit anti-goat IgG (Sigma-Aldrich) for 30 min at room temperature. Plates were again washed and blocked, and incubated with streptavidin-HRP (Invitrogen) diluted 1:2,000 in blocking buffer for 30 min at room temperature. Plates were washed with wash buffer and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 10 min at room temperature. The reaction was stopped by adding 50 µl of 1 M $H_2SO_4$. Optical densities (OD) were measured at 450 nm with a microplate reader (Synergy HT, BioTek) and were analyzed with KC4 v3.3 software (BioTek). End point titer was determined as the last dilution that gave an OD 2 times above the pre-immune serum.

**Analysis of cellular response elicited by needle protein vaccination**

At 2 weeks post last immunization, spleens were collected from mice receiving either PBS, BscF, or BscF diluted in alum, and processed to a single cell suspension. Splenocytes were suspended in RPMI (10% heat inactivated FBS and 50 µg/ml of Pen-Strep) and seeded at 1 X $10^6$ cells/ml into 24 well plates. Splenocytes were stimulated

with 1 μg/ml BscF or medium alone for 72 h at 37°C with 5% $CO_2$. Plates were centrifuged at 400 x *g* for 5 min at 4°C and cellular supernatant was removed. IFN-y, IL-17A, and IL-4 production was determined by DuoSet ELISA kits (R&D Systems).

**Opsonophagocytosis assay**

*B. pertussis* Tohama I was electroporated with plasmid pCW504 (provided by Dr. Allison Weiss, Univ of Cincinnati) generating a GFP-expressing *B. pertussis* strain. These bacteria were opsonized with 6-week mouse serum from BscF-immunized mice or with pre-immune serum as a negative control for 30 min at 37°C. Opsonized bacteria were incubated on a RAW 264.7 cell monolayer seeded in 24 well plates at a multiplicity of infection (MOI) of 50 for 30 min at 37°C to allow for binding and internalization. Prior to incubation, the plates were centrifuged at 800 x *g* for 5 min to facilitate cell contact. Non-attached bacteria were removed by washing the cell monolayer 5 times with pre-warmed PBS. Cells were scraped from the plate, Fc receptors were blocked, and incubated with 0.25 μg anti-CD11b antibody-PB conjugated (Biolegend) diluted in FACS buffer (1X PBS, 2% heat inactivated FBS) for 30 min at room temperature. Cells were washed with FACS buffer, and resuspended in FACS buffer. Single stained controls were used to facilitate analysis. Samples were collected by flow cytometery (BD LSR II) and data were analyzed with FlowJo (FlowJo LLC, Ashland, OR). Phagocytosis was estimated by mean fluorescence intensity (MFI).

**Statistical analysis**

Data were assembled into graphs using GraphPad Prism, version 5.0f (GraphPad Software, La Jolla, CA). Data were analyzed using one-way analysis of variance (ANOVA)

followed by Bonferroni's multiple comparison test. Differences were considered statistically significant when $p<0.05$.

## Results

### Protein in purified protein preparations is responsible for NF-κB/AP-1 activation in THP-1XBlue cells

To examine contamination in the BscF protein preparations, we first examined possible lipopolysaccharide (LPS) contamination. Pre-treating cells with polymyxin B significantly reduced LPS activation down to background levels (Fig 1A). Polymyxin B treatment of purified BscF resulted in a slight decrease in NF-κB and/or AP-1 activation in THP-1XBlue cells (Fig 1A). Although polymyxin B treatment resulted in a slight decrease in SEAP activation by BscF, the level of NF-κB and/or AP-1 activation was still significantly higher than LPS treated with polymyxin B (Fig 1A). Due to the slight decrease in NF-κB and/or AP-1 activation of BscF when pre-treated with polymyxin B, we expressed BscF in an LPS free *E. coli* strain. THP-1XBlue cells were stimulated with BscF expressed in BL21 (DE3) or LPS free *E. coli* BL21 (DE3) and NF-κB and/or AP-1 activation was measured 24 hours later. There was no difference in NF-κB and/or AP-1 activation between the two BscF protein preparations (Fig 1B); confirming LPS is not a significant contaminant in our purified T3S system needle protein preparations. This is consistent with our previous work demonstrating no LPS contamination in our protein preparations by *Limulus* Amebocyte Lysate (LAL) assay, as well as purifying LcrG in the same manner as BscF in the current study, and demonstrating no activation of NF-κB and/or AP-1 when THP-1XBlue cells were treated with purified LcrG (130,131).

To further demonstrate that protein in purified needle preparations is responsible for NF-$\kappa$B and/or AP-1 activation in THP-1XBlue cells, BscF or flagellin (TLR5 agonist used as positive control) were incubated with the serine protease, Proteinase K, for 16 h at 37°C prior to stimulation. As anticipated, Proteinase K treatment reduced NF-$\kappa$B and/or AP-1 activation by flagellin to background levels (Fig 1C). Proteinase K-treated needle proteins also failed to induce NF-$\kappa$B and/or AP-1 activation (Fig 1C), confirming that protein caused the cellular response seen in the THP1-XBlue and HEK-Blue cells. Together, these results demonstrate that BscF protein is responsible for inducing SEAP activity by THP-1Xblue and HEK-Blue cells.

**Purified BscF activates NF- $\kappa$B/AP-1 signaling through TLR2 and TLR4**

T3S system needle proteins from *Yersinia pestis* (YscF) and a number of other gram-negative pathogens act as pathogen associated molecular patterns (PAMPs) and activate Toll-like receptors 2 (TLR2) and 4 (TLR4) (130,131). *Bordetella pertussis* relies on a T3S system for virulence (83) and expresses a T3S needle protein (BscF) that shares low sequence similarity to YscF (23% identity). These observations led us to examine if BscF acts similarly to other needle proteins by inducing downstream signaling of TLR activation. Evaluation of TLR signaling was assessed using human THP-1XBlue and HEK-Blue cells engineered with a secreted embryonic alkaline phosphatase (SEAP) reporter system. SEAP expression is under control of the transcriptional activators NF- $\kappa$B and AP-1; which are critical transcriptions factors for innate immune responses. An increase in SEAP expression is indicative of increased TLR signaling through NF-$\kappa$B and/or AP-1. Treating THP-1XBlue cells with 1 µg/ml BscF significantly induced NF-$\kappa$B and/or AP-1

activation (Fig 2A) compared to the untreated controls. To confirm the TLRs that BscF is stimulating through, HEK-Blue cells transfected with either hTLR2 or hTLR4 were stimulated with 1 μg/ml of BscF and SEAP expression was measured 24 h after stimulation. BscF stimulation induced NF-$\kappa$B and/or AP-1 activation in both HEK-Blue hTLR2 and hTLR4 cells (Figs 2B and C). These results demonstrate that BscF acts similarly to other characterized T3S system needle proteins (130,131) by acting as a PAMP to activate NF-$\kappa$B and/or AP-1 signaling through TLR2 and TLR4 ligation.

**BscF induces inflammatory cytokines by innate immune cells**

We demonstrated that BscF activates NF-$\kappa$B and/or AP-1 activation with SEAP as a reporter gene. We next examined the possibility that BscF could induce inflammatory cytokines from both murine and human innate immune cells. Murine Raw 264.7 cells were treated with BscF for 24 h and several prototypical innate cytokines were measured in the supernatant. As expected, low levels of cytokines were detected in non-treated cells. Stimulation with either LPS or $Pam_3CSK_4$ as positive controls induced significant TNF-$\alpha$, IL-6, and IL-1$\beta$ production when compared to untreated levels (Figs 3A-C). BscF also stimulated TNF-$\alpha$, IL-6, and IL-1$\beta$ production by murine Raw 264.7 cells (Figs 3A-C). Because the reporter cell lines used are human cell lines, we confirmed that BscF could promote inflammatory cytokine release from non-transfected human THP-1 cells. BscF, in addition to other known TLR ligands, induced strong IL-12p40, TNF- $\alpha$, and IL-1$\beta$ production by human THP-1 cells (Figs 3D-F). These findings demonstrate that in addition to inducing TLR signaling, BscF activated both murine and human innate immune responses *in vitro*.

**BscF induction of proinflammatory cytokines is largely dependent on TLR4 signaling**

We have shown that BscF induces NF-$\kappa$B and/or AP-1 signaling downstream of TLR2 and TLR4 activation, and subsequent inflammatory cytokine release from murine and human innate cells. We next demonstrated that BscF more strongly activates TLR4 by using TLR2 and TLR4 knockout bone-marrow derived macrophage (BMDM) cells from mice. BscF induced significantly less TNF- $\alpha$, IL-6, and IL-12p40 in the TLR4 knockout BMDM cells when compared to wild type cells (Fig 4). Inflammatory cytokine release was also significantly lower in the TLR4 knockout cells when compared to TLR2 knockout cells. TLR2 knockout cells also resulted in a marked reduction in IL-6, and IL-12p40 production, although the reduction was not as great when compared to the TLR4 knockout cells (Fig 4). These findings demonstrate that although BscF induces NF-$\kappa$B and/or AP-1 signaling through both TLR2 and TLR4 in the human SEAP reporter cell line, BscF-induced inflammatory cytokine release from mouse BMDM cells is largely dependent on TLR4 activation.

**BscF induced IL-1$\beta$ through an NLRP3 Caspase 1 dependent mechanism requiring phagocytosis**

Having shown that BscF promotes IL-1$\beta$, among other inflammatory cytokines, by human THP-1 and mouse RAW 264.7 cells, BscF must also engage a cytosolic pattern recognition receptor (PRR) of the nucleotide-binding domain leucine rich repeat (NLR) family in addition to TLR signaling. It has been shown that T3S system components and bacterial flagellin are detected by the intracellular NLRC4 inflammasome (153,154,155,156,157). Using inflammasome deficient BMDMs and DCs, we demonstrated that BscF promotes IL-1$\beta$ in an NLRP3 caspase-1 dependent mechanism

(Fig 5A and B). We confirmed that there was no defect in NF-$\kappa$B signaling as levels of TNF-$\alpha$ were similar in all cell types, with a slight increase in the Caspase1$^{-/-}$ (Caspase11$^{Tg}$) cells (Fig 5C). We next investigated the role of phagocytosis in BscF induced IL-1$\beta$ production. Blocking phagocytosis by cytochalasin D pretreatment significantly reduced IL-1$\beta$ release from stimulated DCs (Fig 6A). Inhibition of IL-1$\beta$ production was coupled with an increase in intracellular pro-IL-1$\beta$ (Fig 6B and C). These data suggest that while normal NF-$\kappa$B signaling occurred, resulting in accumulating pro-IL-1$\beta$, internalization of BscF is required to activate the NLRP3 inflammasome to cleave pro-IL-1$\beta$ into mature IL-1$\beta$. Together, these results demonstrate that BscF induces IL-1$\beta$ by engaging the NLRP3 inflammasome upon internalization, in addition to its TLR activating properties.

**Characterization of BscF serum from immunized mice**

To examine the immune response generated by BscF immunization, total BscF-specific IgG and antibody isotyping was performed in the serum from mice vaccinated with BscF alone or BscF absorbed in aluminum hydroxide (alum). Immunization with purified BscF induced significant BscF-specific antibody responses (Fig 7A). The presence of alum enhanced the antibody response to BscF immunization (Fig 7A). Surprisingly, BscF immunization alone produced robust IgG1 and IgG2b responses, with modest IgG2c, IgG3, IgA, and IgM responses (Fig 7B); while BscF absorbed in alum produced higher titers to many IgG isotypes (Fig 7B). Given the extracellular location of BscF within the T3S, we assessed the ability of BscF-specific IgG to opsonize and enhance phagocytosis of GFP-expressing *B. pertussis*. Interestingly, serum from BscF immunized mice significantly enhanced bacterial uptake by Raw 264.7 cells compared to non-

immunized mouse serum (Fig 7C). These findings demonstrate that BscF immunization promotes the induction of a mixed Th1, Th17 response, evidenced by the IgG2 response and a Th2, Th17 response, demonstrated by the IgG1 response. Additionally, immunization produced BscF-specific antibodies that enhanced *B. pertussis* opsonization and phagocytosis.

**BscF vaccination indirectly promotes Th1 and Th17 immune responses in mice**

It is clear that protective immunity against *B. pertussis* is mediated by Th1 cells and Th17 cells (114,144,86). We have shown that BscF promotes inflammatory cytokine production by innate cells, specifically IL-12, IL-1$\beta$, and IL-6, which are associated with differentiation of Th1 and Th17 cells. We next characterized the adaptive immune response to BscF immunization. Spleens from mice immunized with either BscF alone or BscF absorbed in aluminum hydroxide gel were harvested 6 weeks post first vaccination, processed to a single cell suspension, and stimulated with BscF for 3 days. Stimulation of splenocytes with BscF induced strong production of IFN-$\gamma$ as measured by ELISA (Fig 8A). In addition, BscF stimulation induced IL-17A production (Fig 8B). BscF stimulation failed to elicit production of the Th2 cytokine IL-4 (Fig 8C). These findings indicate that BscF immunization indirectly promotes IFN- $\gamma$ and IL-17A production by T cells *in vitro*, suggesting BscF may either provide protective immunity by acting as a protective antigen *in vivo*, or could augment innate and adaptive immunity generated by an acellular vaccine.

**BscF vaccination reduces lung bacterial burden in a sub lethal infection with *B. pertussis***

Having shown that BscF stimulates innate cytokine release, produces robust antibody responses *in vivo*, and drives strong Th1 and Th17 T cell responses, we next characterized the ability of BscF to act as a protective antigen *in vivo*. Following vaccination with BscF alone or BscF absorbed in aluminum hydroxide gel mice were challenged with 2 X $10^6$ CFU of *B. pertussis* Tohama I in a 25 µl inoculum intranasally. Lungs were aseptically removed, homogenized in PBS, and serial dilutions of lung homogenate were plated on 15% blood BG plates. BscF absorbed in alum promoted a modest, but significant, reduction in bacterial colonization of the lungs 7 d post infection when compared to mock immunized mice (Fig 9). Immunization with BscF alone did not significantly reduce lung bacterial burden when compared to mock immunized mice (Fig 9). These data demonstrate that BscF can act as a protective antigen in a sub lethal *B. pertussis* infection model, possibly by the induction of Th1 and Th17 cells. Although the protective immunity generated by BscF immunization *per se* was very modest in our infection model, we provide persuasive evidence that BscF could be an ideal candidate to include in an acellular pertussis vaccine.

**Discussion**

Type III secretion (T3S) system needle proteins are pathogen-associated molecular patterns (PAMPs) that act as toll like receptor 2 (TLR2) and toll like receptor 4 (TLR4) ligands to induce innate inflammatory cytokine release in a MyD88-depedendent mechanism (130,131). In this study, we characterized the immunostimulatory properties of the T3S needle protein from *B. pertussis*, called BscF. It has been shown that TLR4

signaling is critical for generating protective cellular immunity by pertussis vaccination or natural infection (150,151).

Additionally, the efficacy of the whole cell pertussis (wP) vaccine is attributed to its numerous PAMPs that activate pattern recognition receptors (PRR) (27). These studies clearly highlight the important contribution PAMPs play in developing protective immunity against pertussis. The role of PAMPs in augmenting pertussis-specific cellular immunity is further highlighted by a number of studies using the current acellular pertussis (aP) vaccine (86,120,121,123,124,125). In the current study, we demonstrated that BscF, like other gram-negative needle proteins, is a strong PAMP leading to activation of NF-$\kappa$B and/or AP-1 signaling through TLR2 and TLR4 (Fig 2). Additionally, BscF stimulation of innate cells induced strong inflammatory cytokines that promoted protective adaptive immunity against *B. pertussis*.

Recently, it has been established that host immunity to *B. pertussis* involves cellular immunity, specifically Th1 and Th17 cells (114,143,86,141). The development of cellular immunity is influenced by activation of the innate immune system. Antigen presenting cells (APCs) that become activated through PAMP recognition, release polarizing cytokines IL-12, IL-6, and IL-1β, which dictate the differentiation of Th1 and Th17 cells. Our data demonstrates that BscF stimulation of murine and human innate cells induced robust IL-12, IL-6, and IL-1$\beta$ production, indicating the ability of BscF to induce Th1 and Th17 polarizing cytokines (Figs 3 and 4). Interestingly, the ability of BscF to induce these inflammatory cytokines appeared to be largely dependent upon TLR4 activation in mice, although the loss of TLR2 did result in decreased cytokine production

from murine BMDM cells (Fig 4). We believe that the ability of BscF to engage multiple PRRs is unique and noteworthy because many of the other PAMPs used in combination with the aP vaccine engage only a single TLR (e.g. TLR2, TLR4, or TLR9). In addition, the ability of BscF to activate both murine and human cells suggests that BscF could be included in an improved *B. pertussis* human aP vaccine.

Th17 cells have been shown to be important in immunity to *B. pertussis* (141). *B. pertussis* specific Th17 cellular protective immunity and effective bacterial clearance were shown to be dependent on stimulation of the NLRP3 inflammasome by adenylate cyclase toxin, and subsequent IL-1$\beta$ release (60). Interestingly, BscF stimulation resulted in the production of IL-1$\beta$ from murine and human innate cells (Fig 3). To investigate which inflammasome BscF signals through, inflammasome deficient BMDMs and DCs were stimulated with BscF. BscF induced the production of IL-1$\beta$ in an NLRP3 and caspase-1 dependent mechanism through the adaptor molecule ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) (Fig. 5). Additionally, inhibition of phagocytosis by cytochalasin D significantly reduced secreted IL-1$\beta$ while levels of intracellular pro-IL-1$\beta$ increased (Fig 6). These data suggest that BscF is a potent agonist for two unique receptors: TLRs and the NLRP3 inflammasome. We believe that BscF induces NF-$\kappa$B/AP-1 signaling downstream of TLRs leading to the production of pro-IL-1$\beta$ as well as other inflammatory cytokines. Internalization of BscF, and activation of the NLRP3 inflammasome then activates caspase-1 to cleave pro-IL-1$\beta$ into mature IL-1$\beta$ (Fig 10). This finding is in contrast to other reports that demonstrate

T3S system components and bacterial flagellin interact with the NLRC4 inflammasome (153,154,155,156,157).

The previous studies (153,154,155,156,157) differ significantly in a number of ways from the current study in terms of how the T3S system components or flagellin were administered to the innate cells *in vitro*: 1. recombinant flagellin was fused to the amino-terminal domain of anthrax lethal factor to target the protein to the cytosol through an endocytosis mediated pathway (156) 2. the T3SS rod protein PrgJ and the needle protein PrgI from *Salmonella typhimurium* were transfected directly into macrophages following lipopolysaccharide (LPS) stimulation (155) 3. Cells were treated with bacteria expressing intact T3S system to directly inject effector molecules into the cytosol (157). In the current study, BscF was added to the culture medium, not directly into the cell cytoplasm. It is possible that the differences in methodology among the studies contributes to the discrepancies in inflammasome activation. In support of that conclusion, bacterial amyloids produced in *Escherichia coli* and *Salmonella typhimurium* produce IL-1$\beta$ *in vitro* when administered in the culture medium of BMDM cells through cooperation of TLR2 and the NLRP3 inflammasome (152). In addition to the Th1 polarizing cytokines produced by BscF stimulation, the ability of BscF to activate intracellular inflammasomes and produce IL-1$\beta$ suggests that BscF may provide *B. pertussis* specific cellular immunity through Th17 cells.

In the current study we confirmed that BscF vaccination induced robust antibody responses in mice (Figs 7A and B). Interestingly, BscF immunized serum effectively opsonized *B. pertussis* compared to non-immunized serum (Fig 7C). One of the

insufficiencies of the aP vaccine is its inability to generate bactericidal antibodies (158,159,160). Due to the extracellular localization of BscF on *B. pertussis*, we anticipate that BscF might be a protective antigen, in part through the humoral response elicited by BscF. It has been demonstrated that T3S system needle and translocon proteins act as protective antigens, presumably through the generation of a robust antibody response (145,132,146,147,148,149). Presently, it is unclear how BscF antibodies might provide protection *in vivo*. We are currently addressing the ability of BscF-specific antibodies to enhance complement mediated bacterial killing, inhibit bacterial adherence, or augment opsonophagocytosis and subsequent bacterial uptake and killing.

Despite widespread vaccination, whooping cough still remains as a global health threat (88). This is due, in part, to the aP vaccine promoting robust Th2 responses (114,142,117,143). Although the aP vaccine can control pertussis related symptoms, it may not prevent bacterial colonization or transmission (113). An improved pertussis vaccine should elicit strong Th1 responses, in addition to Th17 responses. Here, we demonstrated that BscF immunization indirectly enhanced IFN$\gamma$ and IL-17A production in splenocytes from immunized mice (Fig 8). In addition, immunization with BscF absorbed in aluminum hydroxide gel provided modest protection in a sub lethal *B. pertussis* challenge (Fig 9). Although bacterial burden is still relatively high, these results demonstrate that BscF acts as a protective antigen; in addition to its innate stimulating properties. Modest protection is also seen with YscF-induced protection against *Yersinia pestis* (132). Taken together, these data demonstrate that BscF has the

immunomodulatory properties to be a beneficial vaccine candidate in an improved aP *B. pertussis* vaccine. BscF acts as a strong innate agonist, induces robust inflammatory cytokine release, and promotes protective cellular immunity critical to clear a *B. pertussis* infection. BscF immunization produced robust humoral responses, and acted as a modest protective antigen in a sub lethal *B. pertussis* infection. Studies are currently ongoing to investigate the contribution of BscF when added to the aP vaccine. Considering the shortcomings of the aP vaccine, BscF has significant potential to enhance a next generation subunit pertussis vaccine.

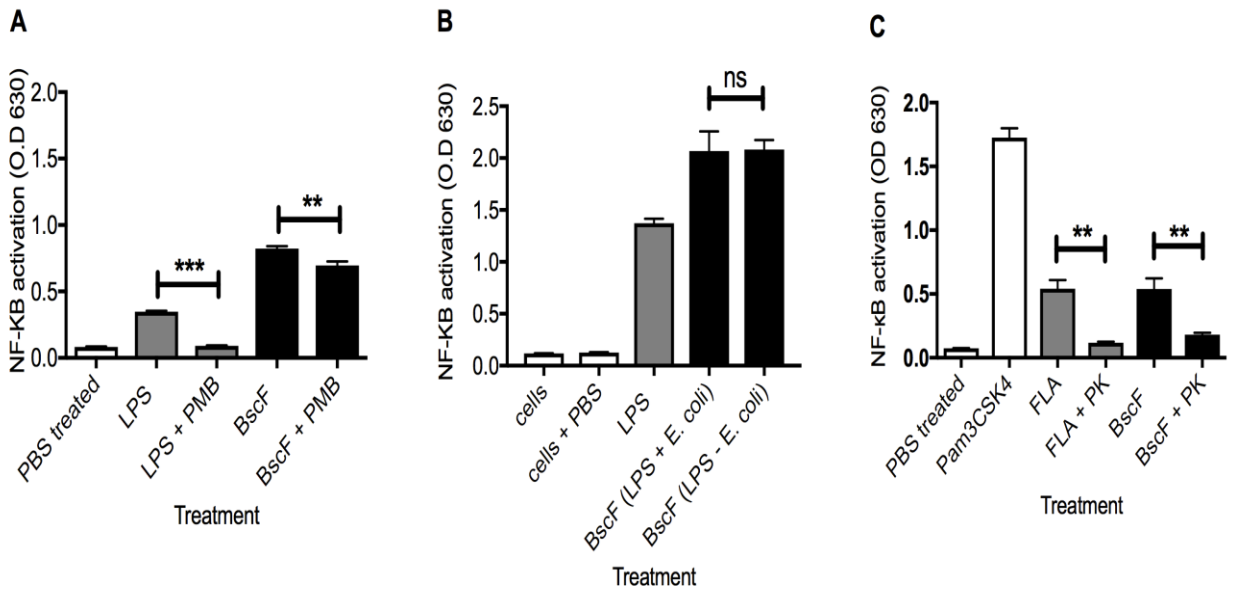


**Figure 1. Characterization of BscF protein preparation.** (A) BscF or LPS were treated with polymyxin B (PMB, 20 µg/ml) before addition to THP1-XBlue cells. (B) THP-1XBlue cells were treated with 1 µg/ml of BscF expressed in BL21 (DE3) or BscF expressed in an LPS free *E. coli* and activation of NF- $\kappa$B and AP-1 was measured after 24 hours. (C) BscF and Flagellin (FLA) were treated with proteinase K (40 µg/ml). Following treatment with polymyxin B or proteinase K, THP-1XBlue cells were stimulated and NF- $\kappa$B and AP-1 activation was measured by SEAP detection after 24 hours. Data are representative of three experiments. ** Indicates *p* is between 0.01 and 0.001; *** Indicates *p* is between 0.001 and 0.0001.

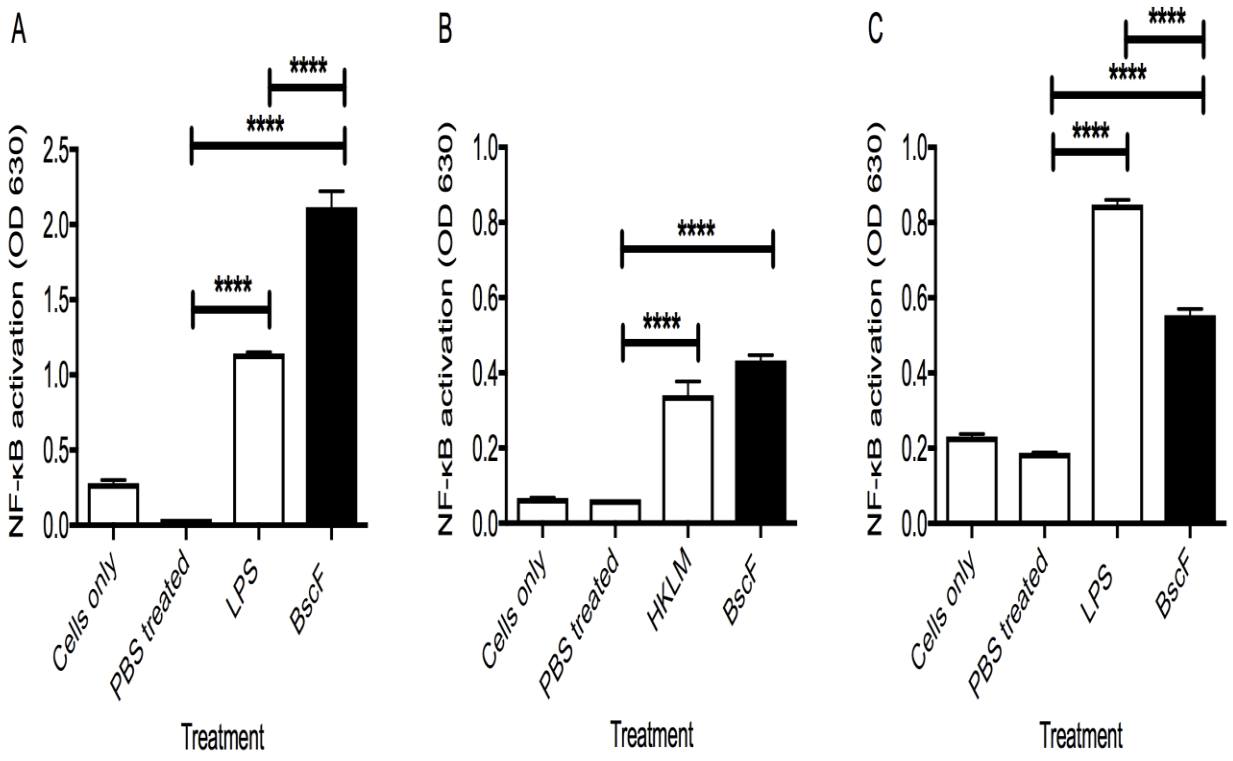


**Figure 2. BscF activates NF-$\kappa$B/AP-1 signaling in THP1-XBlue and HEK293 cells in a TLR2 and TLR4 dependent mechanism.** (A) THP-1XBlue reporter cells were treated PBS, 1 µg/ml of BscF, or 1 µg/ml of LPS dissolved in PBS. (B) HEK-Blue hTLR2 and (C) HEK-Blue hTLR4 reporter cells were treated with PBS, 1 µg/ml of BscF, 1 µg/ml of HKLM (Heat Killed *Listeria monocytogenes*; TLR2 agonist), or 1 µg/ml of LPS (TLR4 agonist) dissolved in PBS. SEAP levels were measured as an indirect measure of NF-$\kappa$B/AP-1 activation. Data are presented as mean ± standard error of triplicate wells. Data are representative of at least three experiments. **** Indicates $p < 0.0001$.

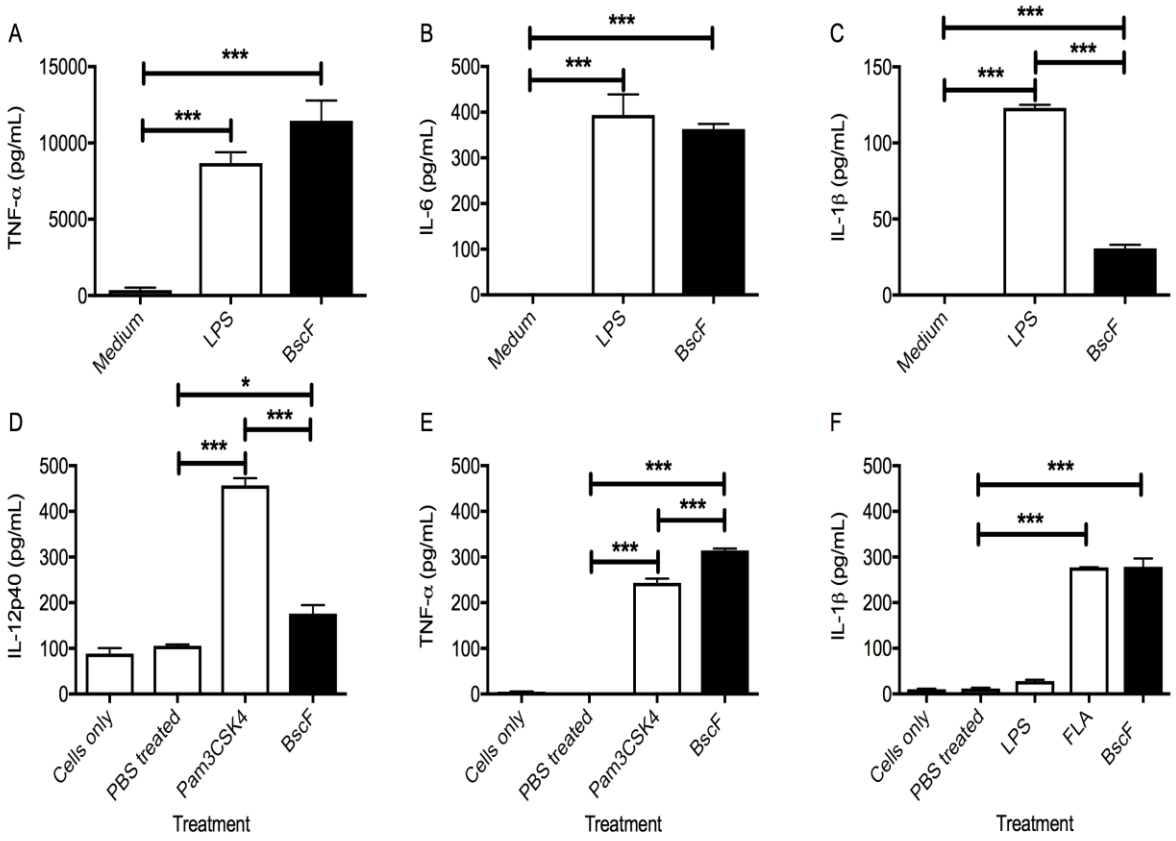


**Figure 3. BscF stimulation induces robust inflammatory cytokine release from murine and human innate cells.** Murine Raw 264.7 cells were treated with 1 µg/ml of BscF, 1 µg/ml of LPS, 1 µg/ml of $Pam_3CSK_4$, or medium. After 24 h stimulation, levels of (A) TNF-$\alpha$, (B) IL-6, and (C) IL-1$\beta$ release were measured in the supernatant by ELISA. Human THP-1 cells were treated with 1 µg/ml of BscF, 1 µg/ml of LPS, or 1 µg/ml of $Pam_3CSK_4$ for 24 h. Release of (D) IL-12p40, (E) TNF-$\alpha$, and (F) IL-1$\beta$ was measured in the supernatant by ELISA after 24 h stimulation. Data are presented as mean ± standard error of triplicate wells. Data are representative of three experiments. * Indicates *p* is between 0.05 and 0.01; *** Indicates *p* is between 0.001 and 0.0001; **** Indicates *p* < 0.0001.

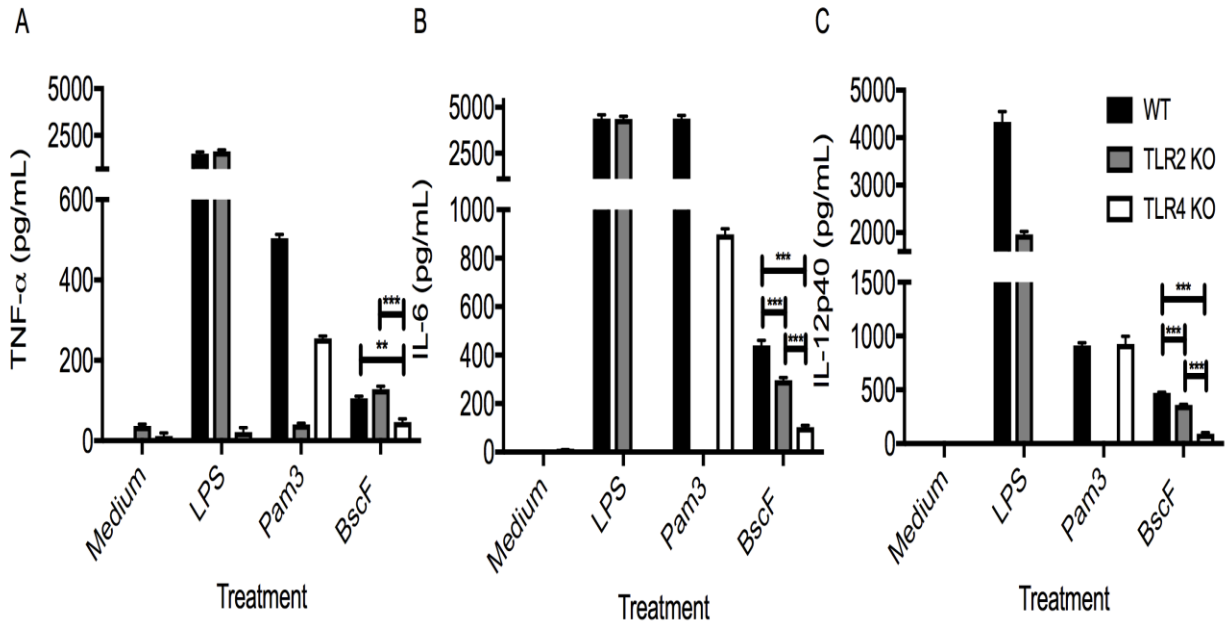


**Figure 4. BscF induction of inflammatory cytokines is largely dependent upon TLR4 activation.** Wild type, TLR2, and TLR4 knockout murine bone marrow cells were differentiated in the presence of GM-CSF and stimulated with 1 µg/ml of BscF, 1 µg/ml of LPS, or medium for 24 h. Release of (A) TNF-$\alpha$, (B) IL-6, and (C) IL-12p40 was measured in the supernatant by ELISA after 24 h stimulation. Data are presented as mean ± standard error of triplicate wells. Data are representative of three experiments. ** Indicates *p* is between 0.01 and 0.001; *** Indicates *p* is between 0.001 and 0.0001.

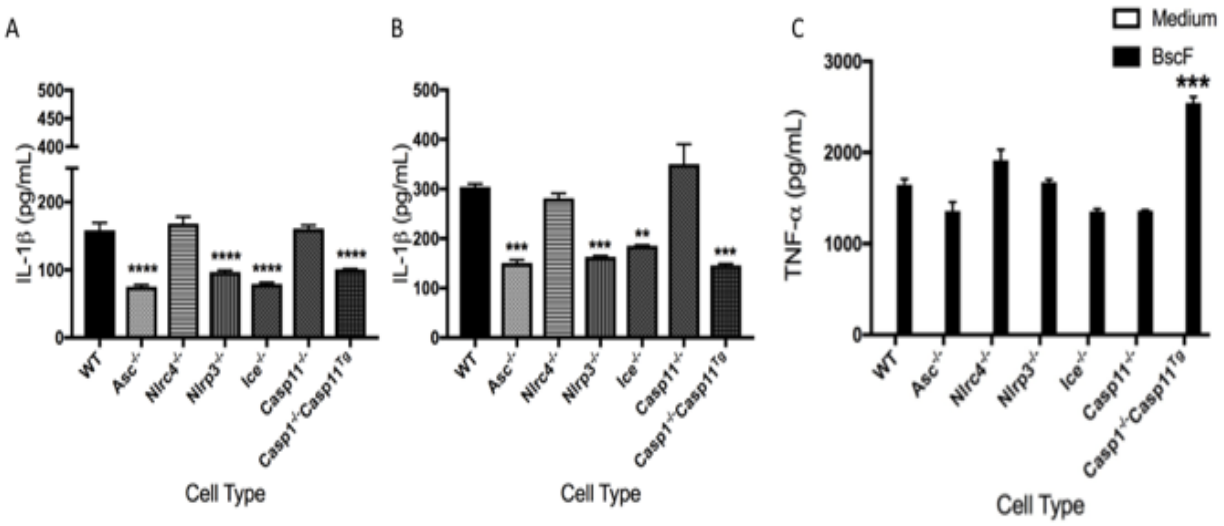


**Figure 5. BscF stimulates IL-1$\beta$ release in an Nlrp3 caspase-1 dependent mechanism**. Inflammasome component KO (A) BMDMs and (B) DCs were treated with 10 µg/mL of BscF and incubated for 24 hours at 37 ℃ with 5% $CO_2$. Following incubation, cell culture supernatant was collected and levels of IL-1$\beta$ and (C) TNF-$\alpha$ were measured by ELISA. Data are presented as mean $\pm$ SE of triplicate wells. ** Indicates *p* is between 0.05 and 0.001. *** Indicates *p* is between 0.001 and 0.0001. **** Indicates *p* is < 0.0001.

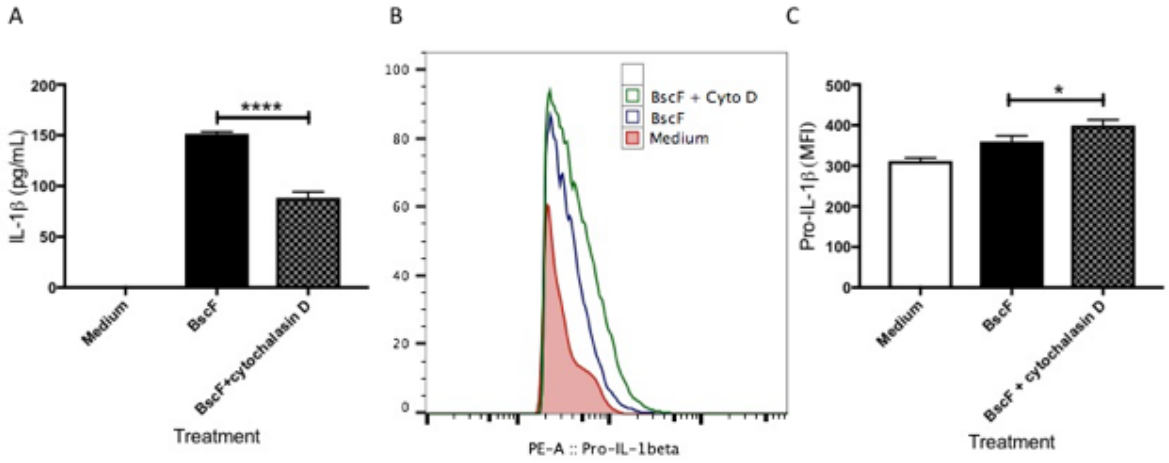


**Figure 6. Pre-treatment with cytochalasin D disrupts IL-1$\beta$ release and results in an increased accumulation of cytosolic pro-IL-1$\beta$**. DCs were treated with 0.25 µg/mL of cytochalasin D for 1 hour at 37 ℃ prior to stimulation with 10 µg/mL of BscF for 24 hours at 37 ℃ with 5% $CO_2$. Following incubation, cell culture supernatant was collected and level of (A) IL-1$\beta$ was measured by ELISA. Cells were collected, permeabilized, stained with anti-pro-IL-1$\beta$, and analyzed by flow cytometry. (B) pro-IL-1$\beta$ and (C) mean fluorescence intensity were measured. Data are presented as mean $\pm$ SE of triplicate wells. * Indicates *p* is between 0.05 and 0.01; **** Indicates *p* is < 0.0001.

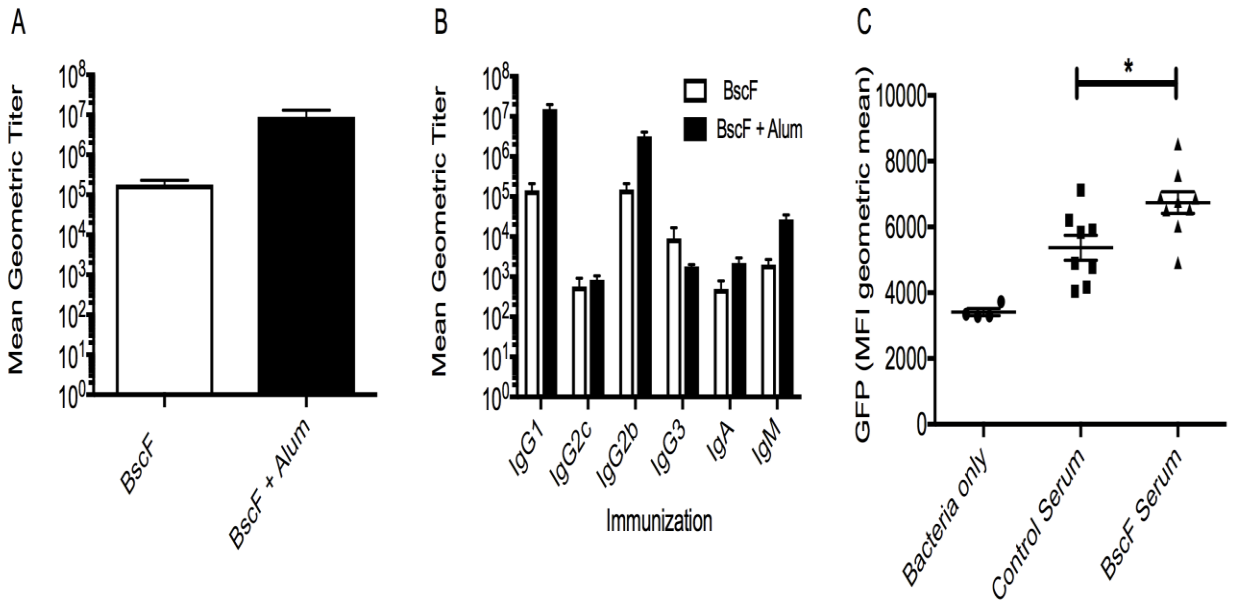


**Figure 7. BscF immunization induces a robust humoral response and *B. pertussis* opsonizing antibodies.** Mice were immunized with 40 µg/ml of BscF diluted in PBS or absorbed in aluminum hydroxide gel at day 0 followed by boost immunizations at 2 and 4 weeks with 20 µg/ml of BscF. Serum samples (n = 4-5) were collected at 6 weeks and (A) BscF-specific IgG titer and (B) IgG isotype titer were determined by ELISA. Pre-immunization serum samples were collected as control. GFP-expressing *B. pertussis* were opsonized with BscF immunized serum or naïve serum as control incubated with murine Raw 264.7 cells. Non-opsonized bacteria were used to determine background GFP mean fluorescence intensity. (C) GFP geometric mean fluorescence intensity was measured in $CD11b^+$ macrophages. Each dot represents a different serum sample. Antibody data presented as mean ± standard error. Antibody data are representative of two experiments. * Indicates *p* is between 0.05 and 0.01.

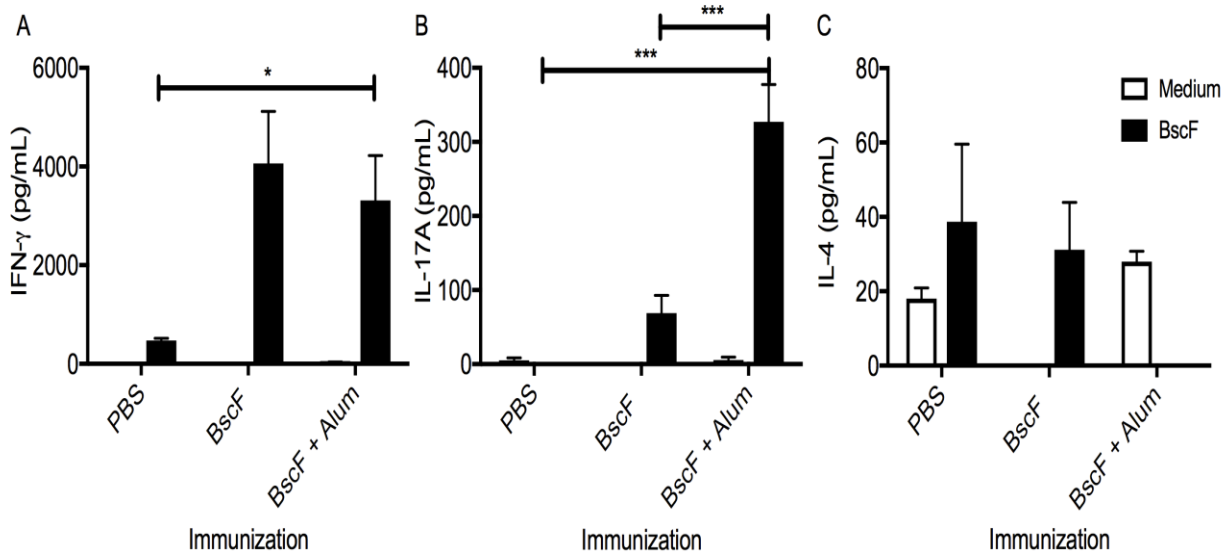


**Figure 8. BscF enhances activation of Th1 and Th17 cells, but not Th2 cells.** Spleen cells from BscF immunized mice were processed to a single cell suspension and stimulated with 1 µg/ml of BscF (black bars) or medium (open bars) for 3 days. Supernatants were tested for (A) IFN-$\gamma$, (B) IL-17A, and (C) IL-4 by ELISA. Data presented as mean ± standard error; n = 4 mice/group. Data are representative of at least two experiments. * Indicates *p* is between 0.05 and 0.01; *** Indicates *p* is between 0.001 and 0.0001.

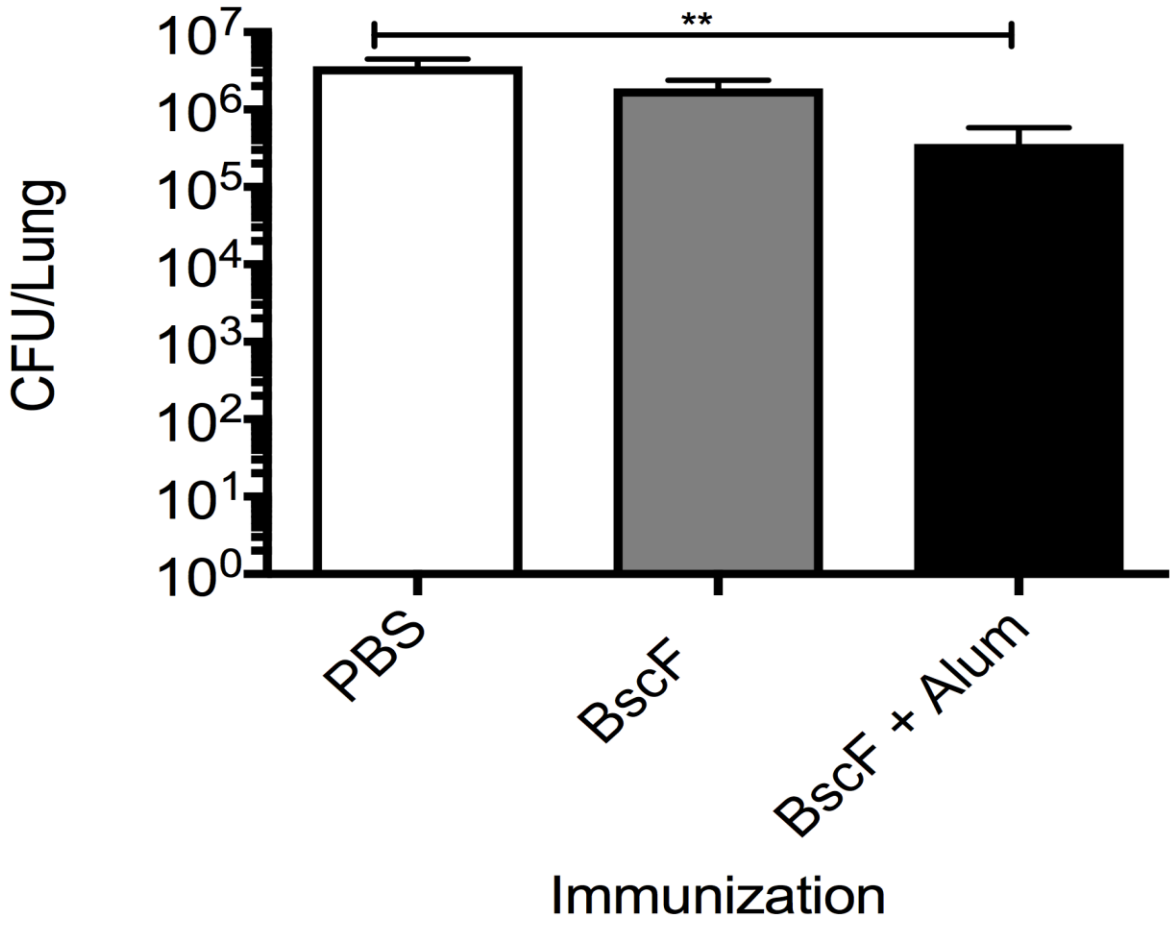


**Figure 9. BscF vaccination acts as a protective antigen against *B. pertussis*.** Mice were immunized with 40 µg/ml of BscF diluted in PBS or absorbed in aluminum hydroxide gel at day 0 followed by booster immunizations at 2 and 4 weeks with 20 µg/ml of BscF. Two weeks after the last immunization, mice were challenged with an intranasal inoculum of $2x10^6$ CFU. (A) CFU counts were performed on lung homogenates 7 days post infection. Data presented as mean ± standard error; n = 4-5 mice/group. Data are representative of two experiments. ** Indicates *p* is between 0.01 and 0.001.

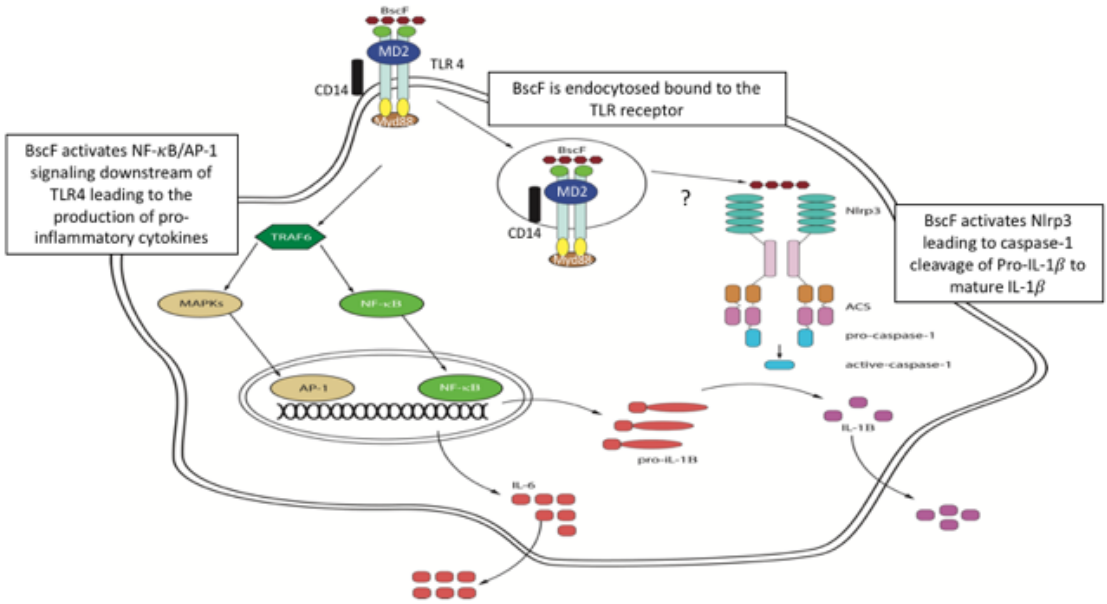


**Figure 10. Proposed model of T3S system needle protein-induced IL-1$\beta$ secretion**. BscF stimulates TLR4 receptor complex leading to activation of NF-$\kappa$B and expression of pro-inflammatory cytokines, including pro-IL-1$\beta$. We believe the BscF-TLR complex is internalized via actin rearrangement leading to endocytosis of BscF. BscF then can activate Nlrp3 and caspase-1 cleaving pro-IL-1$\beta$ to IL-1$\beta$, which is then secreted into the extracellular space.

## CHAPTER III

## PURIFIED TYPE III SECRETION SYSTEM NEEDLE PROTEINS INDUCE CLATHRIN-DEPENDENT NF-$\kappa$B/AP-1 SIGNALING FROM ENDOSOMAL COMPARTMENTS

### Introduction

The type III secretion (T3S) system is a protein secretion system that is conserved among many gram-negative bacteria used to deliver virulence factors into eukaryotic host cells to exploit the host by manipulating intracellular signaling (164,165,166,79). A key component of the T3S system is a needle-like structure that extends from the surface of the bacteria. This hollow structure is formed by the polymerization of single proteins: YscF in *Yersinia* spp., PrgI and SsaG (*Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2, respectively), PscF in *Pseudomonas* spp., MxiH in *Shigella flexneri*, and BscF in *Bordetella* spp. (166,167,168,169,170,171,172,173,174,175,176). Given the cellular localization of the T3S system needle complex, this structure has the potential to interact with the innate immune system.

During host-pathogen interactions, the innate immune system is the first to respond to invading pathogens by recognizing conserved pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are a family of receptors that become activated by microbial products to modulate innate and adaptive immune responses (177). The cellular localization of these receptors either at the plasma membrane or

within intracellular endosomal compartments allows immune surveillance of both extracellular and intracellular pathogens (178). Endocytosis of plasma membrane TLRs has been shown to amplify ligand-induced TLR signaling from endosomal compartments as well as activate distinct intracellular signaling pathways (179,180,181). One of the key players in TLR activation, endocytosis, and ligand specificity is CD14. CD14 is a GPI-linked or soluble protein expressed on many TLR4 expressing cells and was first identified as a pattern recognition receptor (PRR) that binds directly to lipopolysaccharide (LPS) (182). CD14 has also been implicated in TLR4 endocytosis upon LPS stimulation (183). In addition to TLR4, CD14 has been shown to associate with TLR2 to enhance ligand specificity (184,185,186) and endosomal-dependent NF-$\kappa$B signaling (187). The first TLR signaling event leading to early NF-$\kappa$B activation occurs at the plasma membrane through the adaptor proteins MyD88/TIRAP leading to the up regulation of inflammatory genes such as TNF-$\alpha$. A second signaling event occurs from the endosomal compartment through the adaptor proteins TRAM/TRIF leading to late NF-$\kappa$B signaling and type I interferon (IFN) production (188,189,190,191,192).

Recently, T3S system needle proteins have been identified as TLR2 and TLR4 ligands that induce NF-$\kappa$B/AP-1 activation and secretion of inflammatory cytokines in a MyD88-dependent manner (130). In addition, we identified that the N terminus of T3S system needle proteins is important for modulating TLR activation (131). Given the importance of endosomal TLR signaling we hypothesized that T3S system needle proteins may also exploit endosomal NF-$\kappa$B signaling to augment inflammatory cytokine release. In this study, we examined the mechanism leading to T3S system needle

protein-induced NF-$\kappa$B/AP-1 activation downstream of TLR2 and TLR4 in human HEK cells and monocytes. T3S system needle proteins: BscF, *B. pertussis* specific T3S system needle protein, and YscF, *Y. pestis* specific T3S system needle protein, were found to activate NF-$\kappa$B/AP-1 signaling from endosomal compartments mediated by a clathrin/dynamin dependent endocytosis as clathrin-dependent endocytosis inhibition significantly reduced NF-$\kappa$B/AP-1 signaling and inflammatory cytokine release. In addition, NF-$\kappa$B/AP-1 activation by T3S system needle proteins downstream of both TLR2 and TLR4 is mediated by CD14.

## Materials and Methods

### Bacterial strains and growth conditions

*Escherichia coli* Novablue (EMDMIllipore, Billerica MA), BL21 (DE3) star (Invitrogen, Carlsbad, CA), and TOP10 (Invitrogen) were stored at -80°C in 25% glycerol (vol/vol). *E. coli* strains were grown at 37°C in LB broth (BD, Franklin Lakes, NJ) or on tryptose blood agar base (TBA, BD) plates, with kanamycin (50 μg/ml) as needed.

### Expression and purification of His-tagged recombinant proteins

Template DNA for amplification was generated by using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Oligonucleotide primers (Eurofins MWG Operon, Inc. Huntsville, AL) were used to amplify BscF DNA from *B. pertussis* Tohama I strain: BscF forward (5'-CAC CAT GGC CAT TAA CCT GGG AGG-3') and BscF reverse (5'-TCA ACT CGC CTT CTG TAT GAC GCC C-3'). Oligonucleotide primers (Eurofins MWG Operon, Inc. Huntsville, AL) were used to amplify YscF DNA from *Y. pestis*: YscF forward (5'- CGG GAT CCG ATG AGT AAC TTC TCT GGA TTT 3') and YscF

reverse (5'-CCG CTC GAG TGG GAA CTT CTG TAG GAT GCC-3'). PCR was performed using Pfu Ultra polymerase (Agilent Technologies, Santa Clara, CA). The BscF amplified DNA was cloned in frame with a N-terminal His-tag into pET200 by using a Champion TOPO expression kit (Invitrogen). The YscF amplified DNA was cloned in frame with a C-terminal His-tag into pET24b (Novagen, Madison WI). Plasmids for protein expression were purified from *E. coli* TOP10 with a Qiaprep Miniprep kit (Qiagen). Purified plasmid DNA was then transformed into chemically competent *E. coli* BL21 (DE3) Star (Invitrogen). Plasmid constructs were verified by sequencing (Eurofins MWG Operon, Inc.).

Protein purification was performed as previously described (130,131). Briefly, *E. coli* BL21 (DE3) Star (Invitrogen) was grown overnight at 37°C in a shaking water bath in non-inducing medium (50X M, 1 M $MgSO_4$, 40% glucose, 5% aspartic acid (163)) supplemented with antibiotic. Bacteria were then inoculated into auto-inducing medium (50X M, 1 M $MgSO_4$, 50X 5052, NZ-amine S, yeast extract, distilled water (163)) supplemented with antibiotic and grown to an $A_{620}$ of 0.6 to 0.8. Cells were harvested by centrifugation at 4,000 x *g* for 10 min at 4°C and resuspended with wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10% glycerol (wt/vol)). The bacterial suspension was then French pressed at 20,000 lb/in$^2$ twice to lyse cells. The lysate was centrifuged at 10,000 x *g* for 20 min at 4°C. The supernatant was collected and diluted with 1,000 ml of wash buffer before application to a pre-equilibrated TALON metal affinity resin (Clontech, Mountain View, CA) column. The lysate was applied to the column twice followed by washing the column with 15 bed volumes of wash buffer. Bound protein was eluted in

elution buffer (50 mM $NaH_2PO_4$, 200 mM NaCl,150 mM imidazole, and 20% glycerol (wt/vol)). Purified protein was concentrated by centrifugation (Amicon Ultra centrifugal filters, Millipore, Billerica, MA), and dialyzed against phosphate-buffered saline (PBS) plus 10% glycerol (wt/vol) in Slide-A-Lyzer dialysis cassettes (Pierce, Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Bradford protein assay (Pierce, Thermo Fisher Scientific), and purified protein was stored at -80°C for future use.

**Human HEK and THP-1 cell stimulation**

HEK-Blue TLR2 and HEK-Blue TLR4 cells were suspended in DMEM and seeded at $5x10^4$ and $2.5x10^4$ cells/well, respectively in 96 well plates. T3S system needle proteins were added to a final concentration of 1 µg/mL. 100 ng/ml of Pam3CSK4 (InvivoGen) and 1 ng/ml of LPS (*E. coli* K12; InvivoGen) were used as positive controls for HEK-Blue TLR2 and HEK-Blue TLR4 cells, respectively. Cells were stimulated at 37℃ in 5% $CO_2$ for 24h. For inhibition experiments, cells were pre-treated with Chlorpromazine (CPZ), Chloroquine (CHQ), Dynasore (Dyn), or blocking antibodies (10 $\mu$g/ml; InvivoGen) for 1 hour at 37℃ in 5% $CO_2$ prior to stimulation. The plates were then centrifuged at 1,200 rpm for 5 min at 4℃ and the supernatant was added to detection media and incubated 37℃ in 5% $CO_2$ for 2h. Quantification of SEAP, the secreted embryonic alkaline phosphatase reporter gene under the control of NF-κB and AP-1 in the HEK-Blue cells was used as a surrogate to measure TLR activation and was measured by reading the absorbance at 630 nm. THP-1 cells were suspended in RPMI and seeded at $1x10^6$

cells/well in 24 well plates and stimulated as indicated above. TNF-$\alpha$ was measured in the cell culture supernatant at 24 hours by ELISA.

**mRNA silencing**

HEK-Blue TLR2 and HEK-Blue TLR4 cells were suspended in complete DMEM and seeded at $5x10^5$ cells/well in 6 well plates or $1x10^5$ cells/well in 12 well plates 1 day prior to transfection. The following day, the cells were washed with PBS and treated with 1 µM Accell siRNA against heavy chain clathrin 17 designed by supplier (GE Healthcare, Dharmacon™ Accell™ siRNA) or with 1 µM of non-targeting siRNA. After 24 h, complete DMEM was supplemented to the wells and transfection continued for a total of 72 h. The cells were re-seeded at $5x10^4$ cells/well in 96 well plates and stimulated at 37℃ in 5% $CO_2$ for 24h. The plates were then centrifuged at 1,200 rpm for 5 min at 4℃ and the supernatant was added to detection media and incubated 37℃ in 5% $CO_2$ for 2h. Quantification of SEAP was measured by reading the absorbance at 630 nm.

**Inhibitor cytotoxicity**

HEK-Blue TLR2 and HEK-Blue TLR4 cells were treated with either Chlorpromazine (CPZ), Chloroquine (CHQ), Dynasore (Dyn) as indicated above and cell toxicity was measured by lactate dehydrogenase (LDH) release as indicated using the manufacturer's instructions (ThermoFisher Scientific).

**Statistical analysis**

Data were assembled into graphs using GraphPad Prism, version 5.0f (GraphPad Software, La Jolla, CA). Data were analyzed using one-way analysis of variance (ANOVA)

followed by Bonferroni's multiple comparison test. Differences were considered statistically significant when $p<0.05$.

## Results

### T3S system needle proteins induce NF-$\kappa$B/AP-1 downstream of TLR2 and TLR4 by clathrin-dependent endocytosis

Recent data demonstrate the role of endosomal signaling downstream of TLR2 and TLR4 to augment plasma membrane TLR signaling (179,180,181,187). Clathrin-mediated signaling events, in addition to the GTPase dynamin, have shown to be crucial for localizing the TLR complex to the early endosome (180,187,193). Given the localization of TLRs within the endosomal compartments, and how endosomal signaling can augment NF-$\kappa$B activation and subsequent inflammatory cytokine production (187), we determined the role that endosomal TLR signaling plays in T3S system needle protein stimulated cells. To determine the role of TLR internalization in the activation of NF-$\kappa$B/AP-1 we utilized HEK-Blue TLR2 that express TLR2 and CD14, and HEK-Blue TLR4 cells which express TLR4, MD2, and CD14. These cells contain the secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of NF-κB and AP-1 in the HEK-Blue cells. HEK cells were treated with Chlorpromazine (CPZ), a clathrin-mediated endocytosis inhibitor, Chloroquine (CHQ), endosomal maturation inhibitor, and Dynasore (Dyn), the GTPase dynamin inhibitor, prior to T3S system needle protein stimulation. As shown in Figure 11, CPZ, CHQ, and Dyn significantly reduced NF-$\kappa$B/AP-1 activation induced by both BscF and YscF in both HEK TLR2 as well as HEK TLR4 cells. These data suggest that TLR2 and TLR4 internalization is important to augment T3S

system needle protein-induced NF-$\kappa$B/AP-1 signaling. In addition, TLR internalization is mediated by clathrin and dynamin dependent mechanisms.

**Clathrin-mediated endocytosis mediates T3S system needle protein induced TNF-$\alpha$ downstream of TLR2 and TLR4**

Because HEK cells are engineered cells that assess NF-$\kappa$B/AP-1 signaling by SEAP release, we wanted to examine the role of TLR endosomal signaling in a more realistic innate cell context. To that end, we treated human THP-1 cells with the CPZ, CHQ, and Dyn prior to T3S system needle protein stimulation and measured release of TNF-$\alpha$ in the cell culture supernatant. As indicated in Figure 12, CPZ, CHQ, and Dyn significantly reduced TNF-$\alpha$ release from T3S system needle protein stimulated THP-1 cells. These data demonstrate that clathrin-mediated endocytosis augments NF-$\kappa$B/AP-1 signaling and TNF-$\alpha$ expression downstream of TLR activation by T3S system needle proteins YscF and BscF.

We further confirmed the role of clathrin in mediating NF-$\kappa$B/AP-1 signaling downstream of TLR2 and TLR4 in the HEK cells. Interestingly, gene silencing of the clathrin heavy chain in HEK TLR2 cells significantly reduced BscF induced NF-$\kappa$B/AP-1 signaling, while the reduction of NF-$\kappa$B/AP-1 signaling in YscF stimulated cells did not reach significance (Fig 13A). Gene silencing of the clathrin heavy chain in HEK TLR4 cells did not significantly reduce NF-$\kappa$B/AP-1 signaling induced by BscF or YscF (Fig 13B). Brandt et al. demonstrated that targeting the clathrin heavy chain by gene silencing in HEK-BLUE TLR4 cells did not significantly reduce NF-$\kappa$B activation induced by LPS (187), and that the clathrin heavy chain was only partially inhibited by gene silencing. Our results suggest that T3S system needle proteins activate NF-$\kappa$B/AP-1 signaling

downstream of TLR2 and TLR4, which is augmented, in part, by clathrin-mediated endocytosis of the receptor complex.

**CD14 facilitates T3S system needle protein activation of NF-$\kappa$B/AP-1**

T3S system needle proteins from a number of gram-negative bacteria have been identified as TLR2 and TLR4 ligands that induce NF-$\kappa$B/AP-1 signaling and secretion of inflammatory cytokines in a MyD88-dependent manner (130,131). CD14 is a protein expressed on innate cells that has been shown to enhance ligand binding, increase ligand specificity, and facilitate endocytosis of TLR receptors (182,183,184,185,186,187). Using HEK-Blue TLR2 that express TLR2 and CD14, and HEK-Blue TLR4 that express TLR4, CD14, and MD2, we determined the contribution of CD14 to T3S system needle protein induction of NF-$\kappa$B/AP-1 signaling. CD14 blocking antibody significantly reduced NF-$\kappa$B/AP-1 signaling induced by YscF and BscF in HEK-Blue TLR2 cells, while isotype control antibody had no affect (Fig 14A). For HEK-Blue TLR4 cells, blocking both CD14 and MD2 significantly decreased NF-$\kappa$B/AP-1 signaling induced by YscF and BscF (Fig 14B). Isotype control antibody did not decrease signaling (Fig 4B). Neither CD14 or MD2 antibody alone significantly reduced NF-$\kappa$B/AP-1 signaling induced by YscF and BscF in HEK-Blue TLR4 cells (data not shown). These results suggest that CD14 enhanced T3S system needle protein induced NF-$\kappa$B/AP-1 signaling in HEK-Blue cells.

**LDH release from HEK-Blue cells treated with various pharmacological inhibitors**

To ensure the reduction in NF-$\kappa$B/AP-1 signaling observed by pharmacological inhibition was not due to direct cell toxicity, HEK-Blue TLR2 and HEK-Blue TLR4 cells were pre-treated with either CPZ, CHQ, or Dyn, at the highest and lowest concentrations

and LDH was measured following treatment. To our surprise, CPZ significantly reduced cell viability in HEK-Blue TLR2 cells at both the highest and lowest concentrations (Fig 15A). Lowest CPZ treatment did not reduce cell viability in the HEK-Blue TLR4 cells; however, the highest CPZ concentration contributed to cell toxicity (Fig 15B). Neither CHQ nor Dyn reduced cell viability at either the low or high concentrations (Fig 15). Despite the cytotoxic affect observed by CPZ, our results are in line with previously published data (187). Brandt et al., did not report any cytotoxic affects in human monocytes induced by CPZ at the same concentrations used in the current study (187). In addition, CPZ did not produce cytotoxic affects at the lowest concentration in HEK-Blue TLR4 cells (Fig 15B), but still significantly reduced NF-$\kappa$B/AP-1 signaling (Fig 11A). These results suggest that the reduced NF-$\kappa$B/AP-1 signaling observed with pharmacological inhibition cannot solely be contributed to direct cytotoxic affects of the inhibitors.

## Discussion

The innate immune system is the first line of defense against invading pathogens. TLRs are a group of innate receptors that are distinguished by ligand specificity and cellular localization (178). The internalization of plasma membrane TLRs has been shown to be a mechanism to enhance plasma membrane signaling or initiate distinct signaling cascades originating from the endosomal compartments, despite initially thinking that this process was a way to attenuate TLR signaling (180,181,190). We have shown that T3S system needle proteins from many different gram-negative bacteria act as TLR2 and TLR4 ligands, induce My-D88 dependent NF-$\kappa$B/AP-1 signaling,

and promote inflammatory cytokine release (130,131); however, the contribution of endosomal NF-$\kappa$B/AP-1 signaling has not been addressed. In the current study, we demonstrate that T3S system needle proteins, YscF and BscF, activate NF-$\kappa$B/AP-1 signaling from endosomal compartments. Our data indicate that clathrin-mediated endocytosis mediates NF-$\kappa$B/AP-1 activation, and that CD14 controls, in part, TLR activation.

Both TLR2 and TLR4 have been shown to be present within endosomal compartments (179,190,194,195). In the case of TLR4, early My-D88/TIRAP-dependent plasma membrane signaling leads to NF-$\kappa$B activation with a second endosomal-specific signaling event through TRAM/TRIF leading to late NF-$\kappa$B activation and IFN production (180,188,189,190,191,192). Like TLR4 signaling, endosomal-dependent signaling has been described by TLR2 as well (186,187,189). While plasma membrane signaling is a component in the generation of inflammatory cytokine production, data suggest that the contribution of endosomal signaling is primarily responsible for inflammatory cytokine production (180,196,197). Using our unique TLR ligands previously described to bind TLR2 and TLR4 (130,131), we demonstrate that these ligands also exploit clathrin/dynamin-mediated endosomal NF-$\kappa$B/AP-1 signaling to induce inflammatory cytokine release (Figs 11 and 12). Pharmacological inhibition of clathrin-mediated endocytosis with CPZ, endosomal maturation with CHQ, and dynamin with Dyn indicate endocytosis of T3S system needle proteins are necessary to augment NF-$\kappa$B/AP-1 signaling. These data are consistent with reports demonstrating the requirement of internalization of TLR ligands to induce NF-$\kappa$B-controlled inflammatory cytokine release

and ligand recognition (187,198,199), and demonstrate that endosomal localization and TLR signaling augments T3S system needle protein-induced NF-$\kappa$B/AP-1 signaling and inflammatory cytokine release from human THP-1 cells.

In the current study, the pharmacological inhibitor CPZ significantly decreased HEK-Blue TLR2 and HEK-Blue TLR4 cell viability at the highest concentration used (Fig 15). In addition, the lowest CPZ concentration also reduced cell viability in HEK-Blue TLR2 cells. This is surprising as it was reported by Brandt et al., (187) that no cell cytotoxicity in human monocytes was reported in their study using the same CPZ concentrations used in our study. No other cytotoxic affects were observed with any of the other inhibitors (Fig 15). Despite the direct cytotoxic effects of CPZ, specifically with the HEK-Blue TLR2 cells, CPZ reduced NF-$\kappa$B/AP-1 signaling in the HEK-Blue TLR4 cells in the absence of direct cytotoxic affects. Additionally, siRNA targeting the clathrin heavy chain in HEK-Blue TLR2 cells resulted in a significant inhibition of NF-$\kappa$B/AP-1 signaling induced by BscF (Fig 13A). Although YscF activation was reduced, it did not reach statistically significant levels (Fig 13A). No reduction in T3S system stimulated NF-$\kappa$B/AP-1 signaling in HEK-Blue TLR4 cells treated with clathrin heavy chain siRNA was observed (Fig 13B). These results are consistent with Brandt et al., (187), and could also be attributed to only partial reduction in clathrin heavy chain expression by siRNA.

CD14 has been shown to be an important co-receptor for TLR4 binding of LPS (182), TLR4 endocytosis (183), and for TLR2 by enhancing ligand specificity (184,185,186) and endosomal NF-$\kappa$B signaling (187). CD14's role in T3S system needle protein TLR activation remains unclear. Here we show that blocking CD14 on HEK-Blue

TLR2 cells significantly reduced NF-$\kappa$B/AP-1 activation by T3S system needle proteins (Fig 14A). Blocking both CD14 and MD2 in HEK-Blue TLR4 cells inhibited NF-$\kappa$B/AP-1 signaling induced by T3S system needle proteins (Fig 14B). HEK-Blue TLR2 cells only express CD14, while HEK-Blue TLR4 cells express MD2 in addition to CD14 so both blocking antibodies were required to block activation in the HEK-Blue TLR4 cells. These data suggest CD14 as an important co-receptor in TLR2 and TLR4 activation by T3S system needle proteins. CD14 has been shown to act as a co-receptor for both TLR7 and TLR9 (40), in addition to working in combination with TLR2 and TLR4 (201,202). At this point it is unclear if CD14 is augmenting T3S system needle protein NF-$\kappa$B/AP-1 by facilitating receptor internalization. It is possible that CD14 might be enhancing TLR binding by T3S system needle proteins.

In conclusion, we demonstrate that T3S system needle proteins activate NF-$\kappa$B/AP-1 and induce TNF-$\alpha$ release mediated by clathrin/dynamin-regulated receptor endocytosis and CD14 (Fig 16). Given the immune stimulating properties of T3S system needle proteins, they have potential as vaccine adjuvants, and further understanding their signaling mechanisms will serve as the groundwork for future vaccine development.

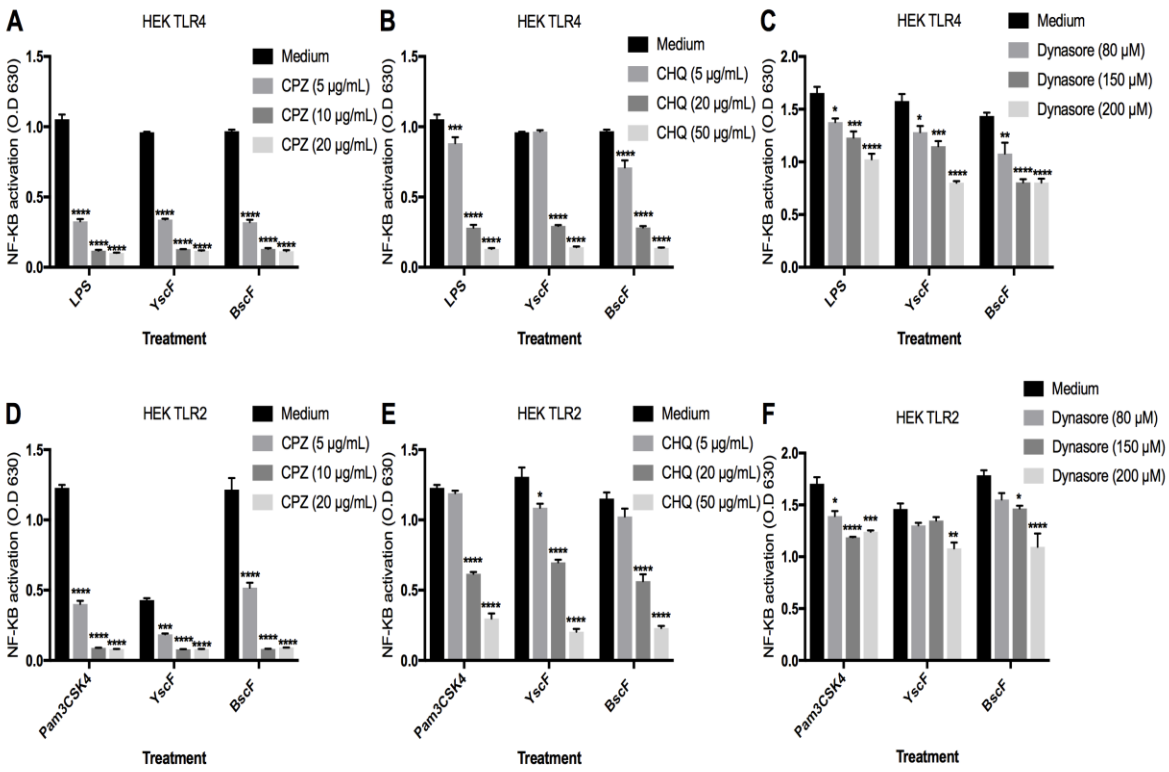


**Figure 11. Endocytosis inhibitors reduce NF-$\kappa$B/AP-1 signaling induced by T3S system needle proteins in HEK-Blue TLR2 and HEK-Blue TLR4 cells**. HEK-Blue TLR2 and HEK-Blue TLR4 cells were treated with CPZ (A,D), CHQ (B,E), and Dyn (C,F) at concentrations indicated in the figure for 1 hour prior to T3S system needle protein stimulation. Cells were stimulated with 1 $\mu$g/ml of T3S system needle protein, 100 ng/ml of Pam3CSK4, or 1 ng/ml LPS for 24 hours and NF-$\kappa$B/AP-1 activation was measured by SEAP production read at 630nm. Data are presented as mean $\pm$ SE of triplicate wells, and are representative of at least 3 independent experiments. * Indicates *p* is between 0.05 and 0.01; ** Indicates *p* is between 0.01 and 0.001; *** Indicates *p* is between 0.001 and 0.0001; **** Indicates $p < 0.0001$.

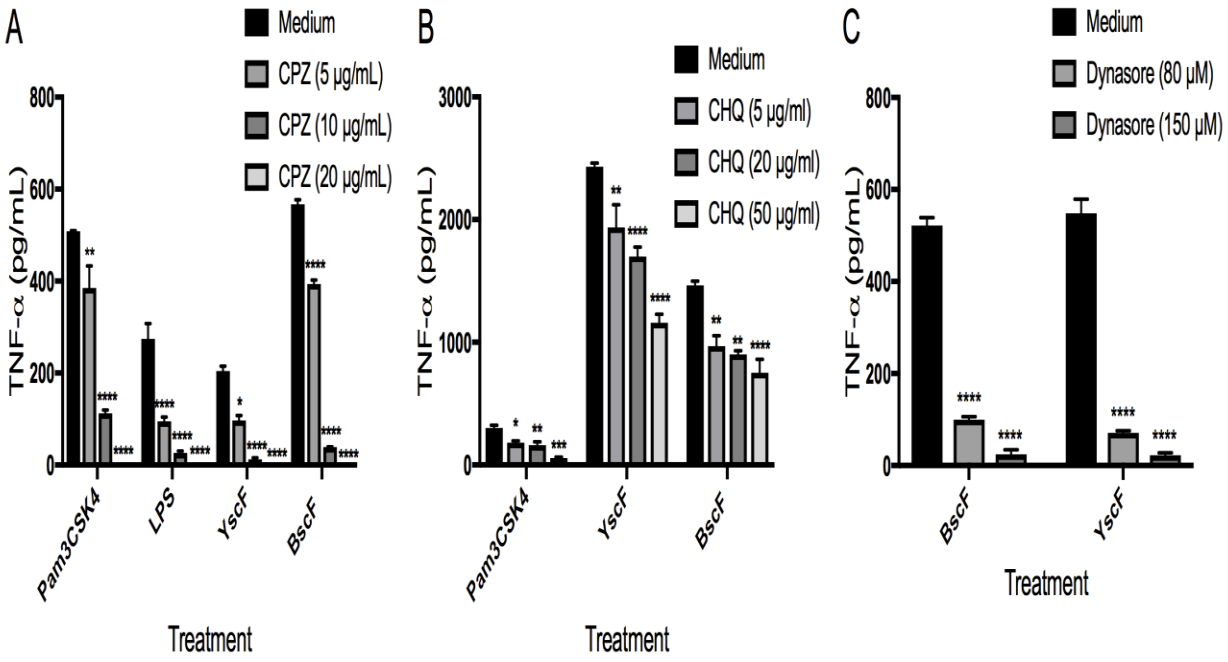


**Figure 12. Endocytosis inhibitors reduce TNF-$\alpha$ production in T3S system needle protein human THP-1 cells**. Human THP-1 cells were treated with (A) CPZ, (B) CHQ, or (C) Dyn for 1 hour prior to T3S system needle protein stimulation. Cells were stimulated with 1 $\mu$g/ml of T3S system needle protein, 100 ng/ml of Pam3CSK4, or 1 ng/ml of LPS for 24 hours. Release of TNF-$\alpha$ was measured in the cell culture supernatant by ELISA. Data are presented as mean $\pm$ SE of triplicate wells and are representative of at least 3 independent experiments. * Indicates *p* is between 0.05 and 0.01; ** Indicates *p* is between 0.01 and 0.001; *** Indicates *p* is between 0.001 and 0.0001; **** Indicates *p* < 0.0001.

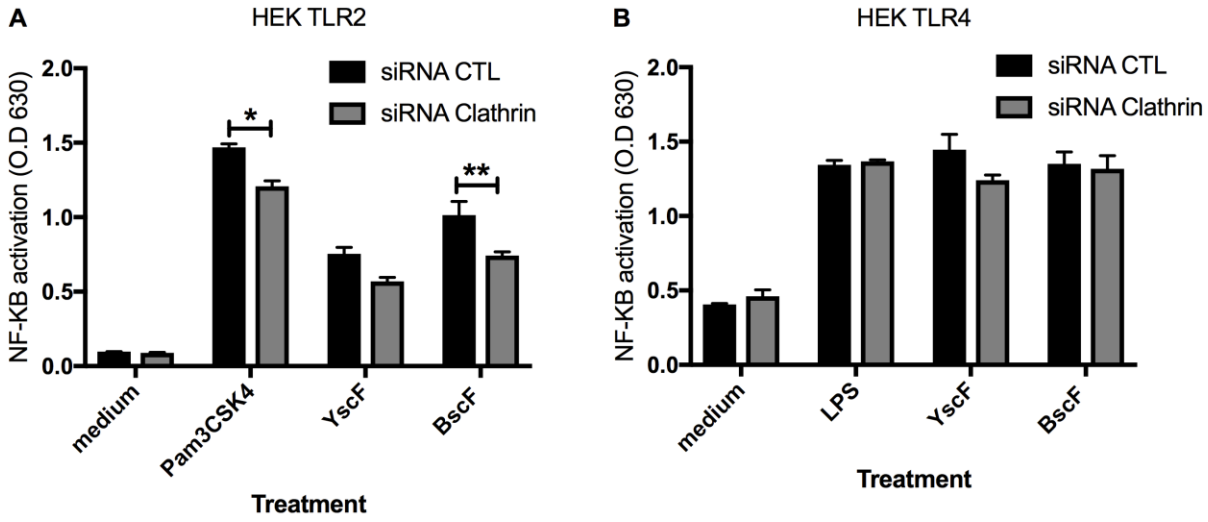


**Figure 13. siRNA gene knockdown of heavy chain clathrin reduced NF-$\kappa$B/AP-1 signaling induced by T3S system needle proteins in HEK-Blue TLR2 cells**. (A) HEK-Blue TLR2 and (B) HEK-Blue TLR4 cells treated with 1 µM Accell siRNA against heavy chain clathrin 17 (grey bars) designed by supplier or with 1 µM of non-targeting siRNA (closed bars). After 24 h, complete DMEM was supplemented to the wells and transfection continued for a total of 72 h. Following transfection, cells were stimulated with 1 $\mu$g/ml of T3S system needle protein, 100 ng/ml Pam3CSK4, or LPS for 24 hours. NF-$\kappa$B/AP-1 activation was measured by quantification of SEAP by reading the absorbance at 630 nm. Data are presented as mean $\pm$ SE of triplicate wells and are representative of 2 independent experiments. * Indicates *p* is between 0.05 and 0.01; ** Indicates *p* is between 0.01 and 0.001.

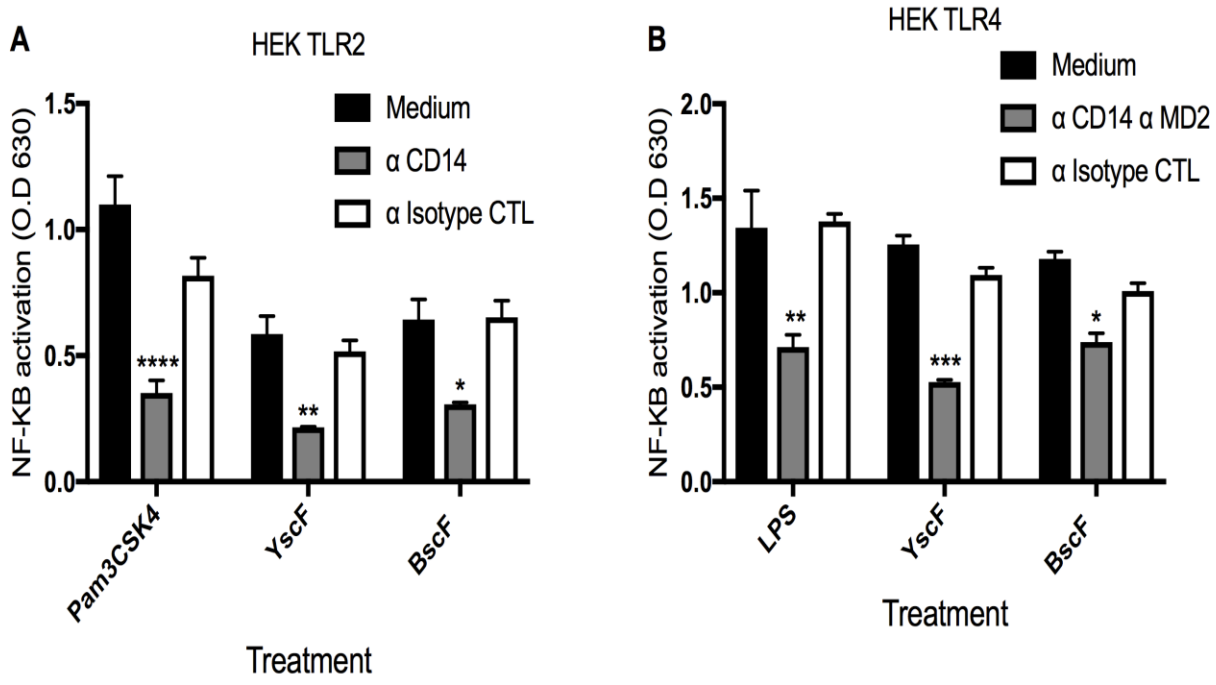


**Figure 14. CD14 mediates NF-$\kappa$B/AP-1 signaling induced by T3S system needle proteins in HEK-Blue TLR2 and HEK-Blue TLR4 cells.** (A) HEK-Blue TLR2 cells were treated with 10 $\mu$g/ml of anti-CD14 antibody (grey bar), 10 $\mu$g/ml of isotype control antibody (white bar), or no antibody (closed bar) prior to T3S system needle protein stimulation. (B) HEK-Blue TLR4 cells were treated with 10 $\mu$g/ml of both anti-CD14 and anti-MD-2 (grey bar), isotype control antibody (white bar), or no antibody (closed bar) prior to T3S system needle protein stimulation. NF-$\kappa$B/AP-1 activation was measured by quantification of SEAP by reading the absorbance at 630 nm. Data are presented as mean $\pm$ SE of triplicate wells and are representative of at least 3 independent experiments. * Indicates *p* is between 0.05 and 0.01; ** Indicates *p* is between 0.01 and 0.001; *** Indicates *p* is between 0.001 and 0.0001; **** Indicates $p < 0.0001$.

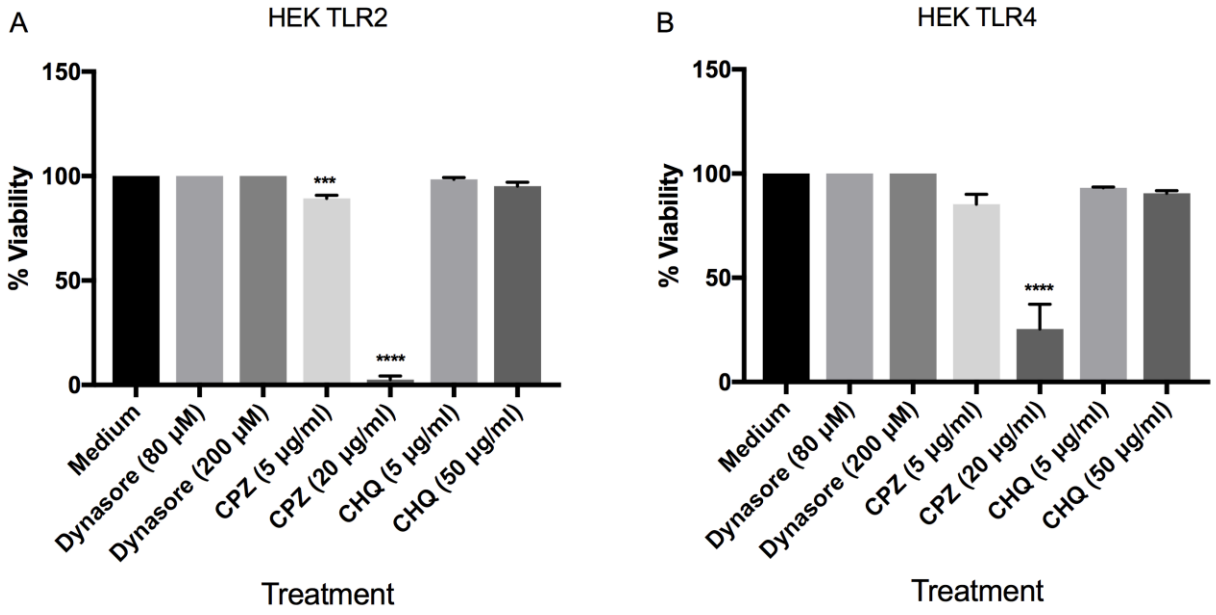


**Figure 15. Direct cell cytotoxicity of endocytosis inhibition in HEK-Blue TLR2 and HEK-Blue TLR4 cells.** (A) HEK-Blue TLR2 and (B) HEK-Blue TLR4 cells were treated with the highest and lowest concentration of each inhibitor and LDH release was measured as a cell cytotoxicity measure. % cell viability was calculated from non-treated cells. Data a presented as mean $\pm$ SE of triplicate wells and are representative of 2 independent experiments. *** Indicates *p* is between 0.001 and 0.0001; **** Indicates $p < 0.0001$.

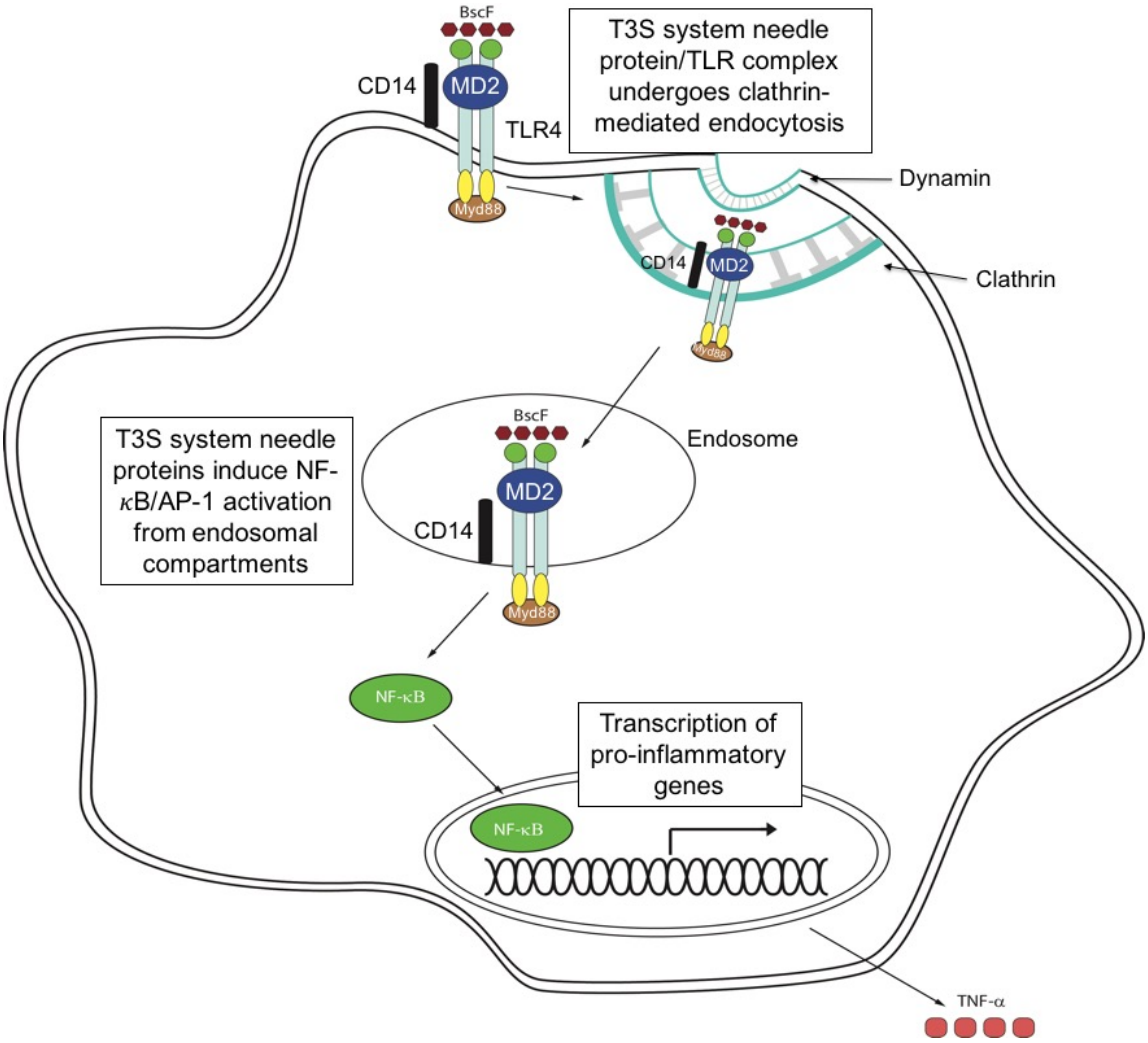


**Figure 16. Proposed model of T3S system needle protein endosomal activation of NF-*κ*B/AP-1 and pro-inflammatory release**. T3S needle proteins are recognized by TLR2 and TLR4 on the surface of innate cells and can be internalized by clathrin-mediated endocytosis. This internalization augments T3S needle protein-induced NF-*κ*B/AP-1 activation through endosomal signaling, resulting in inflammatory cytokine release.

## CHAPTER IV

## BSCF AS A VACCINE CANDIDATE FOR A NEXT GENERATION *BORDETELLA PERTUSSIS* ACELLULAR VACCINE

### Introduction

*Bordetella pertussis* is a gram-negative bacterium and the causative agent of the toxin-mediated disease whooping cough (pertussis). Despite widespread vaccination, pertussis kills roughly 200,000 infants worldwide, mainly in developing countries. Pertussis is characterized by airway inflammation and severe coughing episodes, followed by the characteristic "whooping" sound. Pertussis cases are most commonly found in infants and young children, however adolescents and adults can also be infected. *B. pertussis* attaches to ciliated epithelial cells in the respiratory tract (51,203). Although traditionally thought of as an extracellular pathogen, *B. pertussis* has been shown to invade and reside within epithelial cells and alveolar macrophages (23,24). With the advent of a whole cell pertussis (wP) vaccine in the 1940/1950s, cases of pertussis were dramatically reduced. Effectiveness of the wP vaccine has been largely attributed to its many antigens and PAMPs that bind and activate innate PRRs. The wP vaccine proved to be too reactogenic and was replaced with a less reactogenic, subunit acellular pertussis (aP) vaccine in the 1990s. The aP vaccine does not contain classical PAMPs. The introduction of the aP vaccine was accompanied by a resurgence of pertussis cases in both developed and developing countries (88). Given the contribution

of the many PAMPs to the effectiveness of the wP vaccine, we propose that the addition of PRR stimulating agents could enhance the effectiveness of the current aP vaccine.

There are a number of contributing factors that have lead to a resurgence of pertussis cases attributed to deficiencies in the aP vaccine. First, many circulating isolates of *B. pertussis* have been shown to be deficient in the protective antigens included in the aP vaccine; namely pertussis toxin (PT), pertactin (Prn) and fimbriae (Fim2 and Fim3) (103,134,135,76,136). In addition, a *B. pertussis* strain lacking Prn and PT was recently isolated from an unvaccinated infant in the United States (136). Due to the lack of these key antigens, it is possible that the aP vaccine is not providing robust immune responses against many currently circulating pertussis strains. Second, many studies have demonstrated that the aP vaccine does not elicit effective immunological memory (137,138,139,140). This lack of long-term immunity is particularly concerning because small infants that are most susceptible to severe symptoms of pertussis rely on herd immunity. This incomplete immunity results in adults spreading pertussis to infants, despite the adults receiving the full immunization schedule.

Finally, the current aP vaccine does not elicit strong immune responses that are necessary for long-term protection (86,113). The aP vaccine has been shown to prevent clinical pertussis symptoms, but the aP vaccine may not prevent bacterial colonization or transmission (113). Requirements for the correct type of immune responses needed to be effective against *B. pertussis* are evident in natural infections of *B. pertussis*. Athymic *nu/nu* mice, which lack all T cells, are unable to clear a *B. pertussis* infection

(84,85)and adoptive transfer of total splenic T cells into athymic mice was able to prevent chronic infection in *nu/nu* mice. Transfer of only *B. pertussis* specific $CD8^+$ T cells was not able to reverse chronic infection, indicating a critical role for $CD4^+$ T cells in controlling *B. pertussis* infections (85). $CD4^+$ T cells collected from the draining lymph nodes of *B. pertussis* infected mice produce high levels of interferon gamma (IFNy), a prototypical T helper cell (Th) Th1-type cytokine, with little interleukin-4 (IL-4), a prototypical Th2-type cytokine (85). In addition, IFNy mRNA levels in the lungs increased sharply during the first week of infection (85), suggesting a protective role for IFNy and $CD4^+$ Th1 cells. The contribution for Th17 cells was demonstrated by persistent lung colonization with *B. pertussis* in IL-$17^{-/-}$ mice (86). The lack of IL-17 and persistent infection was correlated with a decrease in neutrophil recruitment into the lungs (86,204,123). Both Th1 and Th17 cells have shown to be important in humans as well. wP vaccines have been shown to induce predominately mixed Th1/Th17 type $CD4^+$ T cell responses in children, similar to natural infection (114,144,205) In terms of humoral immunity, B cells appear to play an auxiliary role, potentially by activating the adaptive immune response (206,207). These studies highlight the importance of both Th1 and Th17 cells in protective immunity induced by a natural *B. pertussis* infection.

In contrast to a natural infection with *B. pertussis* or the wP vaccine, the aP vaccine has been shown to have distinct immune stimulating properties, likely contributing to the current resurgence of pertussis cases. In humans, aP vaccination has been shown to induce a robust Th2 response, with minimal Th1/Th17 $CD4^+$ T cell responses (114,142,117). Mouse studies also identify a strong Th2 and a Th17 response

generated by the aP vaccine. aP vaccination is associated with $CD4^+$ T cells that produce IL-4, interleukin-5 (IL-5), and interleukin-17 (IL-17), but little IFNy (86,123). The protective immunity generated by the aP vaccine was shown to be mediated through Th17 cells, while the Th2 cells appear to be unnecessary (86). In addition, the ability of the aP vaccine to provide protection for long periods of time has been called into question. aP vaccination promoted differentiation of *B. pertussis* specific terminally differentiated T cells ($T_{TD}$) over central memory T cells ($T_{CM}$) (208). In addition, two years after vaccination, peripheral blood mononuclear cells (PBMCs) from children that received the aP vaccine produced lower levels of *B. pertussis* specific IL-17 compared to PBMCs from wP vaccinated children (117). It is clear that the aP vaccine elicits sub-optimal immunity, and with the resurgence of pertussis cases, the need for a next-generation pertussis vaccine is apparent.

As mentioned above, the numerous PAMPs associated with the wP vaccine significantly enhanced its effectiveness. These endogenous PAMPs activate the innate immune system through PRRs – specifically Toll-like receptors (TLRs), promote pro-inflammatory cytokine release, and direct pertussis-specific adaptive immunity (118). Because PAMPs appear to be an important aspect of the wP vaccine, it has been suggested that the addition of TLR agonists to the aP vaccine could re-direct the immune response generated by the aP vaccine to a more wP-like immune response. A TLR2 agonist of *B. pertussis* when combined with the components of the aP vaccine enhanced protection from an aerosol *B. pertussis* challenge, induced robust IgG2a antibodies, and enhanced IL-17 and IFNy production from antigen stimulated

splenocytes *ex vivo* compared with the aP vaccine in alum (120). A TLR4 agonist, monophosphoryl lipid A (MPL), when mixed with the aP vaccine increased protection against *B. pertussis* challenge when compared with the aP vaccine in alum adjuvant, while suppressing Th2 responses (121). A separate TLR agonist, LpxL1 (122), a genetically engineered LPS from *Neisseria meningitidis*, enhanced antigen specific IFNy and IL-17 $CD4^+$ T cells and increased the number of specific memory $CD4^+$ $T_{CM}$ cells when added to the aP vaccine (123). Finally, CpG oligonucleotides from bacterial DNA that activate TLR9 have been shown to induce antigen-specific IgG2a titers (124) and Th1 and Th17 cells (125) when added to the aP vaccine (86). These studies show the feasibility and effectiveness of incorporating novel TLR agonists into the aP vaccine to enhance pertussis specific immunity that will not only promote the proper adaptive immune responses, but could also augment the long-term efficacy of the aP vaccine.

We have recently identified that needle proteins from bacterial type III secretion (T3S) systems are novel TLR agonists that activate TLR2 and TLR4, promote inflammatory cytokine release, and can be modified to modulate TLR signaling (130,131). In addition, we have also characterized the innate stimulating properties of BscF, a purified T3S system needle protein from *B. pertussis* (unpublished results). In the current study, we investigated the contribution of BscF when added to a laboratory prepared aP vaccine. We demonstrate that BscF's ability to engage TLRs has the potential to enhance aP induced vaccine immunity to generate long lasting protective immunity against *B. pertussis*.

## Materials and Methods

### Bacterial strains and growth conditions

*E. coli* Novablue (EMDMIllipore, Billerica MA), BL21 (DE3) star (Invitrogen, Carlsbad, CA), TOP10 (Invitrogen), and *Bordetella pertussis* (Tohama I, ATCC BAA-589 and 12743, ATCC 5375[3865]) were stored at -80°C in 25% glycerol (vol/vol). *E. coli* strains were grown at 37°C in LB broth (BD, Franklin Lakes, NJ) or on tryptose blood agar base (TBA, BD) plates, with kanamycin (50 μg/ml) as needed. *B. pertussis* was maintained as previously described (162). Briefly, *B. pertussis* was grown at 37°C on Bordet-Gengou (BG) solid medium (Remel™ Thermo Fisher Scientific, Lenexa, KS) supplemented with glycerol and 15% sterile sheep's blood (Lampire Biological Labs, Pipersville, PA). *B. pertussis* liquid cultures were grown in Stainer-Scholte broth supplemented with heptakis (2,6-di-*O*-methyl-ß-cyclodextrin; Sigma-Aldrich, St. Louis, MO) and Stainer-Scholte supplements at 37°C. In the case of the GFP-expressing *pertussis*, both solid and broth medium were supplemented with kanamycin (50 μg/ml) and gentamicin (30 μg/ml).

### Expression and purification of His-tagged recombinant proteins

Template DNA for amplification was generated by using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Oligonucleotide primers (Eurofins MWG Operon, Inc. Huntsville, AL) were used to amplify BscF DNA from *B. pertussis* Tohama I strain: BscF forward (5'-CAC CAT GGC CAT TAA CCT GGG AGG-3') and BscF reverse (5'-TCA ACT CGC CTT CTG TAT GAC GCC C-3'). PCR was performed using Pfu

Ultra polymerase (Agilent Technologies, Santa Clara, CA). The amplified DNA was cloned in frame with a N-terminal His-tag into pET200 by using a Champion TOPO expression kit (Invitrogen). Plasmid for protein expression was purified from *E. coli* TOP10 with a Qiaprep Miniprep kit (Qiagen). Purified plasmid DNA was then transformed into chemically competent *E. coli* BL21 (DE3) Star (Invitrogen). Plasmid construct was verified by sequencing (Eurofins MWG Operon, Inc.).

Protein purification was performed as previously described (130,131). Briefly, *E. coli* BL21 (DE3) Star (Invitrogen) was grown overnight at 37°C in a shaking water bath in non-inducing medium (50X M, 1 M $MgSO_4$, 40% glucose, 5% aspartic acid (163)) supplemented with antibiotic. Bacteria were then inoculated into auto-inducing medium (50X M, 1 M $MgSO_4$, 50X 5052, NZ-amine S, yeast extract, distilled water (163)) supplemented with antibiotic and grown to an $A_{620}$ of 0.6 to 0.8. Cells were harvested by centrifugation at 4,000 x *g* for 10 min at 4°C and resuspended with wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10% glycerol (wt/vol)). The bacterial suspension was then French pressed at 20,000 lb/in$^2$ twice to lyse cells. The lysate was centrifuged at 10,000 x *g* for 20 min at 4°C. The supernatant was collected and diluted with 1,000 ml of wash buffer before application to a pre-equilibrated TALON metal affinity resin (Clontech, Mountain View, CA) column. The lysate was applied to the column twice followed by washing the column with 15 bed volumes of wash buffer. Bound protein was eluted in elution buffer (50 mM $NaH_2PO_4$, 200 mM NaCl,150 mM imidazole, and 20% glycerol (wt/vol)). Purified protein was concentrated by centrifugation (Amicon Ultra centrifugal filters, Millipore, Billerica, MA), and dialyzed against phosphate-buffered saline (PBS)

plus 10% glycerol (wt/vol) in Slide-A-lyzer dialysis cassettes (Pierce, Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Bradford protein assay (Pierce, Thermo Fisher Scientific), and purified protein was stored at -80°C for future use.

**Bone marrow derived cell isolation and differentiation**

Bone marrow cells were collected from femurs of WT naïve mice. Femurs were aseptically removed from each hind leg, briefly soaked in 70% ethanol, and placed in fresh RPMI medium (10% heat inactivated FBS, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 50 µg/ml Pen-Strep). Both ends of the femur were cut and the bone was flushed with 10 ml of RPMI and the cells were collected in a 50 ml conical tube. The cell suspension was centrifuged at 400 x *g* for 10 min at 4°C. The cells were resuspended in RPMI supplemented with 40 ng/ml granulocyte-macrophage colony-stimulating factor (GMCSF; PeproTech, Rocky Hill, NJ), seeded at a density of 4 X $10^6$ in 20 ml of medium in a 150 x 20 mm round culture dish, and incubated at 37°C with 5% $CO_2$. On day 3, the cells were supplemented with 20 ml of fresh RPMI + 40 ng/ml GMCSF and incubated for an additional 3 days. At day 6, the non-adherent cells were removed (BMDCs), and were used for subsequent analysis.

**Innate cytokine analysis and maturation assessment of BMDCs**

BMDCs were seeded in triplicate at 1 X $10^6$ cells/ml into 24-well plates and stimulated with 1 µg/ml of needle protein for 24 h at 37°C with 5% $CO_2$. PBS, 1 µg/ml LPS (E. coli K12; InvivoGen), and/or 1 µg/ml $Pam_3CSK_4$ (InvivoGen) were used as negative and positive controls. Following stimulation, cells were centrifuged at 400 x *g*

for 5 min at 4°C and the cellular supernatant was removed and stored at -20°C for future analysis. Numerous human and mouse innate cytokines were measured by DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) or mouse inflammation panel cytometric bead analysis (CBA) kit (BioLegend, San Diego, CA). CBA samples were collected by flow cytometer (LSR II, Becton Dickinson, San Jose, CA), and analyzed by software provided by the manufacturer (LEGENDplex v7.0). For BMDC maturation analysis, the stimulated cells were collected and surface stained for antibodies specific for MHCII, CD80, and CD86. Surface expression and activation markers were analyzed by flow cytometry (LSR II, Becton Dickinson, San Jose, CA).

**Mouse immunization and intranasal *B. pertussis* challenge**

6-8 week old C57BL/6 male mice were immunized intra peritoneally with 100 µL of a laboratory prepared aP vaccine composed of 0.5 µg mutant PT (genetically inactivated by site-directed mutagenesis (R9K) and (E129A) in the S1 subunit; List Biological Laboratories, Campbell, CA), 1 µg FHA (List Biological Laboratories, Campbell, CA) mixed with PBS (aP + PBS) or 40 µg purified BscF (aP + BscF). Mice were boosted at 4 weeks with the same components. Mice that received PBS injections served as controls. Two weeks after the last immunization, mice were intra nasally challenged with 6 X $10^6$ CFU of *B. pertussis* 12743 in a 25 µl inoculum. 7 d post infection, lungs were aseptically removed, and homogenized (Bullet blender, Next Advance, Averill Park, NY) in 1 ml of sterile PBS. Lung homogenate was centrifuged at 130 x *g* for 1 min at 4°C, serial dilutions were plated on 15% blood BG plates, and incubated at 37°C for 4-5 d. Lung bacterial

burden was determined by counting CFUs. All animal experiments were approved by IACUC at the University of North Dakota.

**ELISA assay of antibody levels in mouse serum**

ELISA plates (Costar EIA/RIA, Corning) were coated with 100 µl of 1 µg/ml mPT or FHA diluted in PBS and incubated overnight at 4°C. Plates were washed with wash buffer (1X PBS, 0.05% Tween-20), and blocked with blocking buffer (1X PBS, 1% BSA, 0.05% Tween-20) and incubated at room temperature for 1 h. Plates were again washed and incubated with diluted mouse serum for 1 h at room temperature. Following incubation, plates were washed, blocked for 10 min at room temperature with blocking buffer, and incubated with rabbit anti-mouse IgG biotinylated (Invitrogen) antibody diluted 1:10,000 in blocking buffer for 1 h at room temperature. Both wash and blocking steps were repeated as indicated above, and plates were incubated with streptavidin-HRP (Invitrogen) diluted 1:2,000 in blocking buffer for 1 h at room temperature. For measuring IgG isotypes, isotype specific goat anti-mouse IgG1, and IgG2c, (Sigma-Aldrich) diluted 1:1,000 in blocking buffer was incubated following diluted serum for 30 min at room temperature. Plates were washed and blocked as indicated above, and bound IgG was detected with biotinylated rabbit anti-goat IgG (Sigma-Aldrich) for 30 min at room temperature. Plates were again washed and blocked, and incubated with streptavidin-HRP (Invitrogen) diluted 1:2,000 in blocking buffer for 30 min at room temperature. Plates were washed with wash buffer and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 10 min at room temperature. The reaction was stopped by adding 50 µl of 1 M $H_2SO_4$. Optical densities (OD) were measured at 450

nm with a microplate reader (Synergy HT, BioTek) and were analyzed with KC4 v3.3 software (BioTek). IgG was quantified by reading absorbance at 450 nm, correcting for the background (day 0 serum).

**T cell cytokine production**

At 2 weeks post last immunization, spleens were collected from mice receiving either PBS, aP+PBS, or aP+BscF, and processed to a single cell suspension. Following red blood cell lysis, splenocytes were suspended in RPMI (10% heat inactivated FBS and 50 µg/ml of Pen-Strep) and seeded at 2 X $10^6$ cells/ml into 24 well plates. Splenocytes were stimulated with 1 µg/ml purified mPT, FHA, BscF, or medium alone as negative control for 72 h at 37°C with 5% $CO_2$. Plates were centrifuged at 400 x *g* for 5 min at 4°C and cellular supernatant was removed. IFN-y and L-17A production was determined by DuoSet ELISA kits (R&D Systems).

**Characterization of T cell response and T cell memory induced by aP vaccination**

At 2 weeks post last immunization, spleens, inguinal lymph nodes, and blood were collected. Splenocytes were prepared to single cell suspension as indicated above. Lymph nodes were homogenized and prepared to a single cell suspension in complete RPMI medium. 100 µl of blood from each mouse was used for staining and subsequent analysis with the remaining blood being processed for serum and stored at -80°C. 1-2x$10^6$ splenocytes and lymphocytes as well as 100 µl of blood were stained with anti-mouse CD3, CD4, CD8, CD44, and CD62L for 30 minutes at room temperature. Blood samples were incubated with red blood cell lysis buffer for 10 minutes at room temperature. The cells were washed 2 times and suspended in flow cytometry staining

buffer (2% FBS 1X PBS). Data were collected by flow cytometer (LSR II, Becton Dickinson, San Jose, CA) and data were analyzed by FlowJo (FlowJo, LLC, Ashland, OR). The following gating scheme was used to identify the populations of $CD4^+$ and $CD8^+$ T cells: The first gate was on the cell population using SSC-A (cell complexity) vs FSC-A (cell size). From there, singlets were identified by FSC-W (cell width), and the population of $CD3^+$ cells was identified. Within the $CD3^+$ population, a quadrant gate of CD4 and CD8 was used to identify $CD4^+$ and $CD8^+$ cells. To identify naïve, memory ($T_{cm}$) and effector ($T_{em}$) T cell subsets, cells and singlets were identified as indicated above. Within the singlets population, a quadrant gate was used to identify either $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. Within those populations a combination of CD62L and CD44 was used to identify naïve T cells ($CD62L^+CD44^{low}$), $T_{cm}$ ($CD62L^{high}CD44^{int\text{-}high}$), and $T_{em}$ ($CD62L^{neg}CD44^{high}$). Refer to Figure 20 for a demonstration of the gating scheme.

## Statistical analysis

Data were assembled into graphs using GraphPad Prism, version 5.0f (GraphPad Software, La Jolla, CA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered statistically significant when $p<0.05$.

## Results

### BscF promotes DC maturation and inflammatory cytokine release

Since we have demonstrated that a number of T3S system needle proteins are potent TLR agonists and induce inflammatory cytokine release from innate cells, we

examined the ability of BscF to activate mouse DCs *in vitro*. BscF stimulation for 24 hours promoted bone marrow derived DC maturation as measured by increased surface expression of MHC class II, and the co-stimulatory molecules CD80, and CD86 (Fig 17A-C). BscF stimulation also produced robust IL-6, TNF-$\alpha$, IL-1$\beta$, and IL-12p40 release, accompanied by a modest increase in IL-23 production (Fig 17D-F). These data indicate that BscF is a TLR agonist and activates murine DC maturation and cytokine release *in vitro*.

**BscF is immunogenic *in vivo* and act as an adjuvant when added to a laboratory prepared aP vaccine**

Having shown that BscF promotes inflammatory cytokine release by murine DCs *in vitro*, we assessed BscF's *in vivo* adjuvant capability when added to a laboratory prepared aP vaccine. 6-8-week-old C57BL/6 male mice were immunized intra peritoneally with 100 µL of a laboratory prepared aP vaccine composed of 0.5 µg mPT and 1 µg FHA diluted in PBS (aP + PBS), or supplemented 40 µg purified BscF (aP + BscF). Mice were boosted at 4 weeks with the same components. At 6 weeks post first vaccination, antibody characterization was performed for both antigen specific IgG as well as isotype antibody analysis. The addition of BscF (aP + BscF) to the aP vaccine significantly increases FHA specific total IgG responses (Fig 18A). BscF did not significantly enhance mPT specific total IgG responses (Fig 18D). FHA specific IgG1 as well as IgG2c isotypes were significantly increased in the aP + BscF group when compared to the aP + PBS (Fig 18B-C). mPT specific IgG1 and IgG2c isotypes were not increased by the addition of BscF to the laboratory aP vaccine (Fig 18E-F). These data

demonstrate that BscF can enhance the aP vaccine specific antibody immune response generated by a laboratory prepared aP vaccine.

**BscF indirectly enhances IFN-$\gamma$ and IL-17 production by stimulated splenocytes *in vitro***

We have shown that BscF promotes inflammatory cytokine release by murine DCs, including IL-6, IL-12, and IL-23. These cytokines are associated with the expansion of Th1 and Th17 cells. To further characterize the immune modulating properties of BscF when included in our laboratory prepared aP vaccine, splenocytes from mice immunized with either aP + PBS, aP + BscF, or PBS were processed to a single cell suspension and stimulated with each antigen, including BscF *in vitro*. The addition of BscF to the aP vaccine significantly increased IFN-$\gamma$ from FHA stimulated splenocytes, when compared to the aP + PBS (Fig 19A). Interestingly, IL-17 release was more robust in the aP + PBS group when stimulated with FHA (Fig 19B). aP + BscF splenocytes stimulated with mPT as well as BscF produced significantly more IFN-$\gamma$ when compared to aP + PBS splenocytes (Fig 19A). IL-17 release did not differ between groups when stimulated with mPT; however, BscF stimulated aP + BscF splenocytes produced robust IL-17 release whereas no detectable levels were reported in the aP + PBS group (Fig 19B). These data suggest that BscF's immune stimulating properties indirectly enhanced our aP vaccine immune responses *in vivo*.

**BscF modulates memory response generated by the aP vaccine**

We next assessed the ability of BscF to influence the long-term immunity generated by our laboratory aP vaccine. $CD4^+$ and $CD8^+$ T cells within the blood (data not shown), spleen, and inguinal lymph nodes were investigated *ex vivo* based on CD62L

and CD44 expression within a population of $CD3^+CD4^+$ or $CD3^+CD8^+$ subsets following vaccination. Within those populations a combination of CD62L and CD44 was used to identify naïve T cells ($CD62L^+CD44^{low}$), $T_{cm}$ ($CD62L^{high}CD44^{int\text{-}high}$), and $T_{em}$ ($CD62L^{neg}CD44^{high}$) as indicated in Figure 20. Total $CD4^+$ and $CD8^+$ T cells were increased in the lymph nodes in aP + BscF mice compared to aP + PBS mice (Fig 20A,E). Interestingly, naïve $CD8^+$ T cells, but not $CD4^+$ T cells within the lymph nodes were increased in the BscF group (Fig 20B,F). $T_{em}$ cells were not increased in either compartment by the addition of BscF (Fig 20C,G). aP + BscF significantly enhanced both $CD4^+$ and $CD8^+$ $T_{cm}$ cells within the lymph nodes when compared to the aP + PBS group (Fig 20D,H). These results indicated that the addition of BscF to the aP vaccine induced $CD4^+$ and $CD8^+$ T cells, but also enhanced a central memory T cell phenotype, potentially indicating improved long term immunity generated by the aP vaccine with BscF.

**The addition of BscF to our laboratory aP vaccine promotes protective immunity against a sub lethal *B. pertussis* challenge**

Mice were immunized with either aP + PBS, aP + BscF, or PBS as indicated above. At 6 weeks post vaccination mice were challenged intranasally with live *B. pertussis* and lungs were harvested 7 days post infection, homogenized in 1 mL of sterile saline, and plated to enumerate CFU in the lungs. PBS control mice were well colonized with *B. pertussis* at 7 days post infection (Fig 21). Immunization with aP + PBS provided modest protection when compared to non-immunized mice (Fig 21). In contrast, mice immunized with aP + BscF demonstrated enhanced bacterial clearance when compared to both non-immunized mice as well as aP + PBS mice (Fig 21). These data demonstrate

the adjuvant properties of BscF when included in our laboratory aP vaccine by increased bacterial clearance in the aP + BscF mice.

## Discussion

In the current study, we characterized a novel TLR ligand from the T3S system needle complex of *B. pertussis* that activates the innate immune system to enhance protective immunity generated by our laboratory prepared aP vaccine. This protein, BscF, has been shown to activate TLR2 and TLR4, induce intracellular NF-$\kappa$B/AP-1 signaling, and promote inflammatory cytokine release from innate cells *in vitro* [current study and unpublished results]. The ability of BscF to engage multiple TLRs is in line with other purified T3S system needle proteins that we have previously characterized (130,131). BscF demonstrated potent immune modulating activities *in vitro*, driving murine dendritic cell (DC) maturation and inflammatory cytokine production. The addition of BscF to the laboratory prepared aP vaccine enhanced vaccine-specific IgG responses, indirectly induced Th1 and Th17 responses in *ex vivo* stimulated splenocytes, and provided enhanced protection against *B. pertussis* challenge.

The importance of generating cellular immunity, specifically Th1 and Th17 cells has been highlighted in both natural and vaccine-induced immunity (86,84,85,114,141,143). One of the inadequacies of the current aP vaccines is the lack of innate immune stimulating properties necessary to effectively induce protective cellular immunity. The wP vaccine was considered a strong Th1 producing vaccine due to its numerous endogenous pathogen associated molecular patterns (PAMPs) that engaged numerous pattern recognition receptors (PRRs), significantly enhancing its effectiveness

at generating protective immunity (118). We demonstrated that BscF has strong innate immune stimulating properties and matures murine DCs as measured by inflammatory cytokine release IL-12, IL-6, IL-23, and IL-1$\beta$, and surface expression of prototypical maturation markers. Expansion of Th1 and Th17 cells is influenced by the presence of these innate cytokines (209). Due to the immune modulating properties of BscF, we suggest that the addition of BscF to the aP vaccine may promote Th1 and Th17 responses and drive protective immunity.

Current literature has exploited a host of PRR stimulating agents in combination with current aP vaccines to drive protective Th1 and Th17 responses that are generally lacking with aP vaccination. Unlike natural *B. pertussis* infection or vaccine induced immunity generated by the wP vaccine, the current aP vaccine in alum adjuvant preferentially induces Th2-type responses (86,123,114,142,117). These stimulating agents include TLR2 agonists (120), TLR4 agonists (123,121,122), and TLR9 agonists (86,124,125). Interestingly, many of these agonists have proven effective at skewing the aP vaccine-induced immunity to a more effective wP-like immune response by increasing IgG2a antibody production, expanding Th1 and Th17 cellular immunity, and providing enhanced protection from *B. pertussis* infections in mice. In the current study, we report enhanced pertussis specific antibody titers, including the Th1 indicating IgG2c isotype, when our novel T3S system needle protein, BscF, was included in our laboratory prepared aP vaccine. Additionally, BscF indirectly enhanced Th1 and Th17 cytokine production from *ex vivo* stimulated splenocytes.

Another limitation to the current aP vaccines absorbed in alum adjuvant is the lack of long-lasting immunological memory (137,138,139,140). Waning immunity, in addition to other factors, may be contributing to the rise in whooping cough cases despite relatively high vaccination rates. Brummelman et al., demonstrated that the addition of a TLR4 ligand to the aP vaccine resulted in an increased pertussis-specific $T_{cm}$ phenotype, suggesting enhanced long-term efficacy of the vaccine (123). The generation of $T_{cm}$ cells is a good predictor of long-term immunity (210,211,212,213). Here we report that the addition of BscF to the aP vaccine increased $CD4^+$ and $CD8^+$ T cells within the inguinal lymph nodes when compared to the aP vaccine in PBS. In addition, Total $CD4^+$ $T_{cm}$ and $CD8^+$ were increased in the presence of BscF. These data provide evidence of BscF's ability to modulate vaccine induced immunity. It is worth noting that we did not use any methods necessary to assess pertussis-specific memory T cells. This study differs from Brummelman et al., who utilized MHC class II tetramer analysis to identify pertussis-specific $CD4^+$ T cells (123).

Although aP vaccine has been able to prevent clinical pertussis symptoms, it did not reduce bacterial colonization of transmission in baboons (113). Here we demonstrated the benefit of the immune modulating capacity of BscF to enhance bacterial clearance of the aP vaccine. This benefit is likely the result of BscF promoting protective cellular immunity through its innate stimulating properties. Taken together, our results demonstrate that BscF, a purified T3S system needle protein from *B. pertussis* has potential to improve aP vaccine-induced immunity by indirectly promoting protective cellular immunity through its TLR stimulating properties. BscF has been

shown to promote inflammatory cytokine release from human cells (unpublished data), suggesting the immune modulating capacity of BcsF may extend to humans as well. In addition, mice immunized with BscF prior to *B. pertussis* challenge had modest reductions in lung bacterial burdens compared to non-immunized mice (unpublished data). Given the extracellular localization of BscF on the bacterial surface, we believe BscF has the ability to act not only as an adjuvant but also as a modest protective antigen, thus providing potentially unique advantages over other PRR stimulating agents. The need for improved vaccines exists, and BscF may hold potential for inclusion in a next-generation *B. pertussis* vaccine.

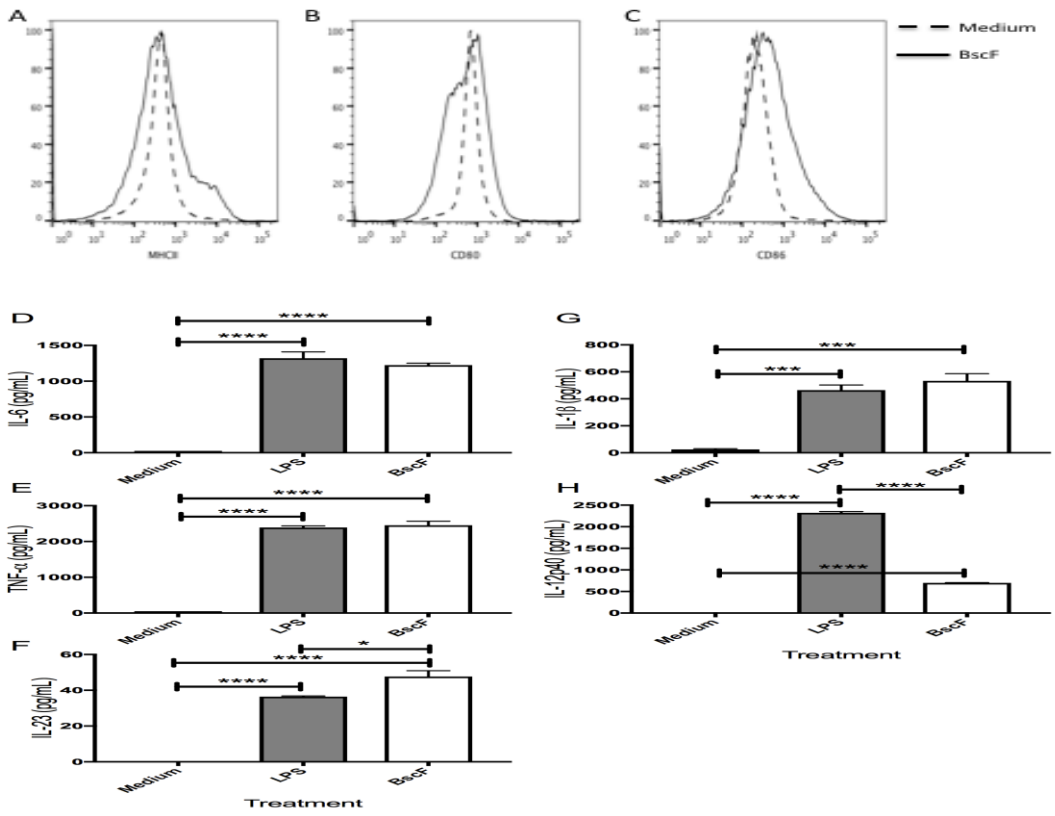


**Figure 17. BscF promotes murine DC maturation and inflammatory cytokine release**. Murine DCs were stimulated with 1 $\mu$g/ml of BscF (solid line) or medium (dashed line) for 24 hours. Following stimulation surface expression of (A) MHC class II, (B) CD80, and (C) CD86 was determined by flow cytometry. Cell culture supernatant was collected following 24 hour stimulation with 1 $\mu$g/ml BscF (open bars), 1 $\mu$g/ml LPS (positive control; grey bars), or medium (negative control; closed bars), and levels of (D) IL-6, (E) TNF-$\alpha$, (F) IL-23, (G) IL-1$\beta$, and (H) IL-12p40 were measured by ELISA. Data are presented as mean $\pm$ SE of triplicate wells and are representative of 2-3 independent experiments. * Indicates *p* is between 0.05 and 0.01. *** Indicates *p* is between 0.001 and 0.0001. **** Indicates $p < 0.0001$.

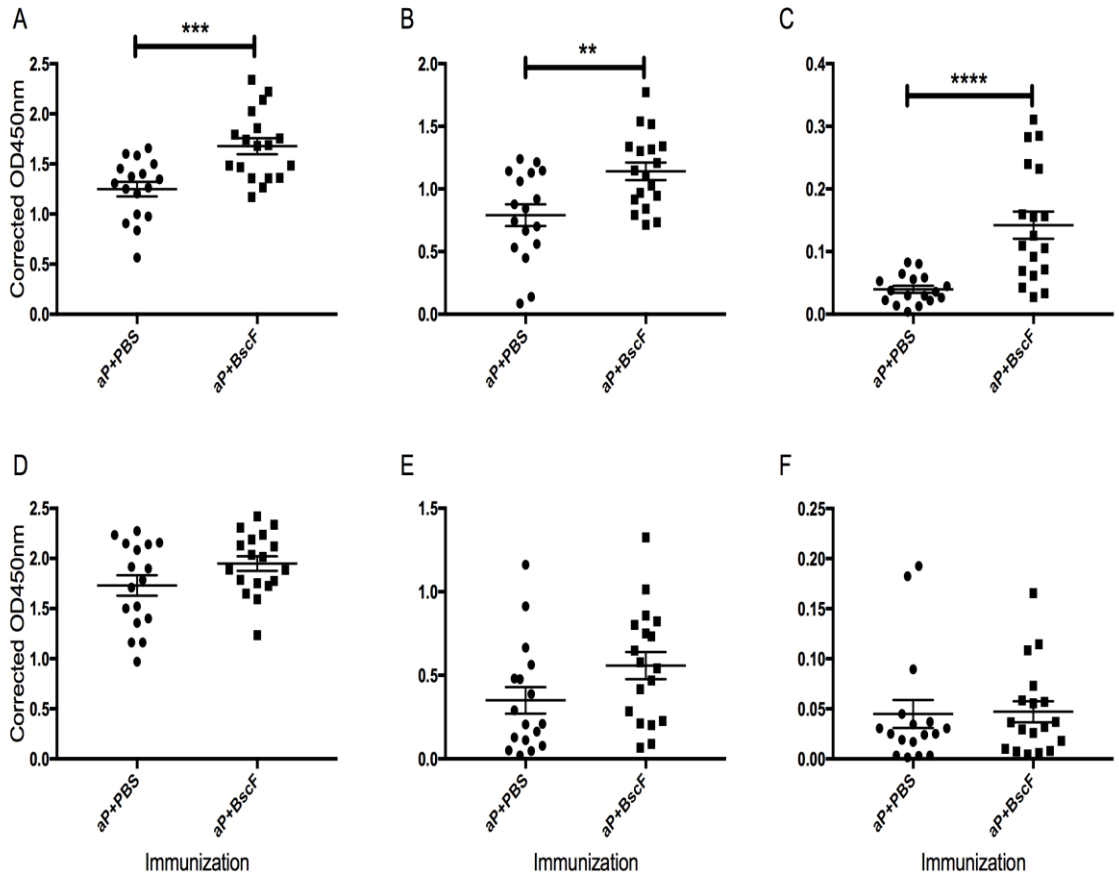


**Figure 18**. **BscF acts as an adjuvant to enhance aP vaccine specific antibody responses**. Mice were immunized with aP + PBS or aP + BscF twice (0 and 4 weeks), and serum was collected at 6 weeks. (A) Total FHA specific IgG or antibody isotype (B) IgG1 and (C) IgG2c were measured in serum samples diluted 1:100. Serum mPT specific (D) total IgG, (E) IgG1, and (F) IgG2c were determined in serum samples diluted 1:100. Data are presented as mean ± SE of OD450nm absorbance readings corrected by non-immunized serum, and are compiled from 4 independent experiments. aP + PBS n = 17 and aP + BscF n = 18. ** Indicates *p* is between 0.01 and 0.001. *** Indicates *p* is between 0.001 and 0.0001. **** Indicates $p < 0.0001$.

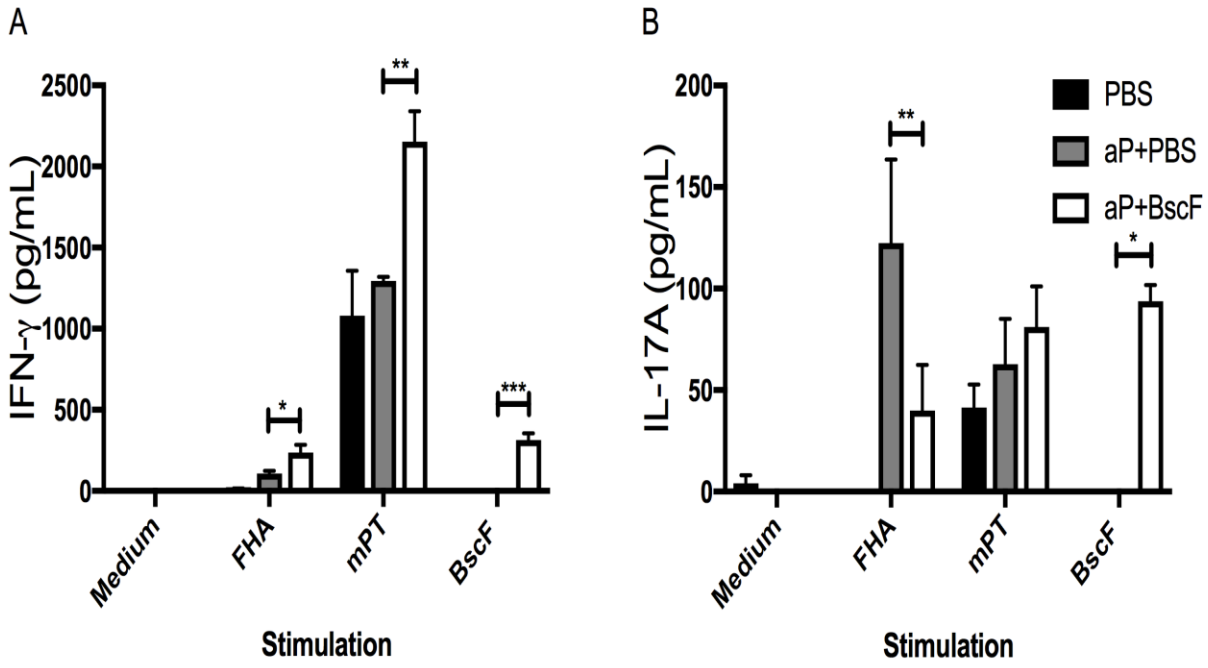


**Figure 19. BscF indirectly enhances IFN-$\gamma$ and IL-17 production from *ex vivo* stimulated splenocytes**. Mice were immunized with aP + PBS (grey bars) or aP + BscF (open bars) twice (0 and 4 weeks). PBS immunized mice (closed bars) served a naïve control. At 6 weeks, spleens were harvested and stimulated ex vivo with 1 $\mu$g/ml of either FHA, mPT, or BscF, and release of (A) IFN-$\gamma$ and (B) IL-17 was measured in the cell culture supernatant following 72 hour stimulation by ELISA. Data presented as mean $\pm$ SE of 4 mice per group, and are representative of 2 independent experiments. * Indicates *p* is between 0.05 and 0.01. ** Indicates *p* is between 0.01 and 0.001. *** Indicates *p* is between 0.001 and 0.0001.

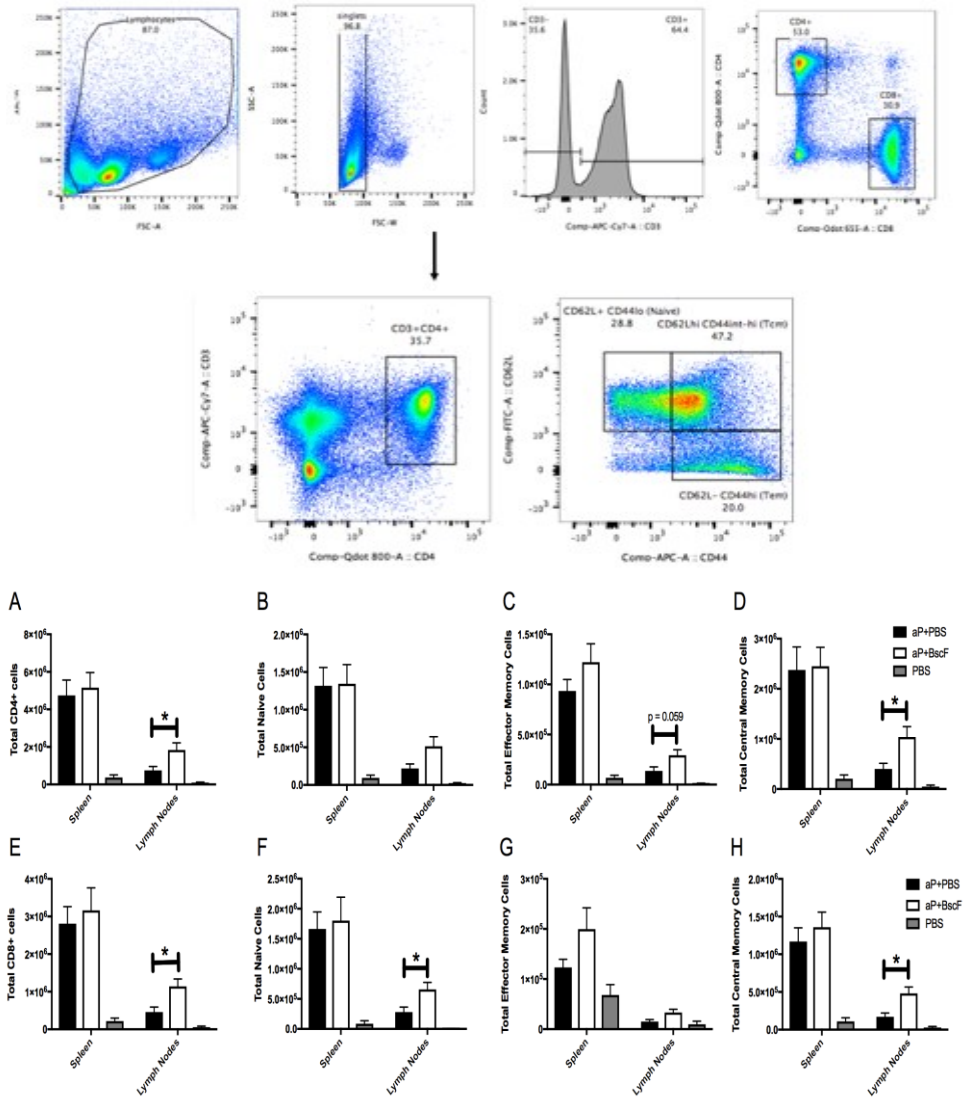


**Figure 20. The addition of BscF to the laboratory aP vaccine enhanced a central memory T cell phenotype**. Mice were immunized with aP + PBS (closed bars) or aP + BscF (open bars) twice (0 and 4 weeks). PBS immunized mice (grey bars) served a naïve control. The following gating scheme was used to identify the populations of $CD4^+$ and $CD8^+$ T cells: The first gate was on the cell population using SSC-A vs FSC-A. From there, singlets were identified by FSC-W, and the population of $CD3^+$ cells was identified. Within the $CD3^+$ population, a quadrant gate of CD4 and CD8 was used to identify (A) $CD4^+$ and (E) $CD8^+$ cells. To identify naïve, memory ($T_{cm}$) and effector ($T_{em}$) T cell subsets, cells and singlets were identified as indicated above. Within the singlets population, a quadrant gate was used to identify either $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. Within those populations a combination of CD62L and CD44 was used to identify (B and F) naïve T cells ($CD62L^+CD44^{low}$), (C and G) $T_{em}$ ($CD62L^{neg}CD44^{high}$) and (D and H) $T_{cm}$ ($CD62L^{high}CD44^{int\text{-}high}$). Data presented as mean $\pm$SE of 4 mice per group, and are representative of 2 independent experiments. * Indicates *p* is between 0.05 and 0.01.

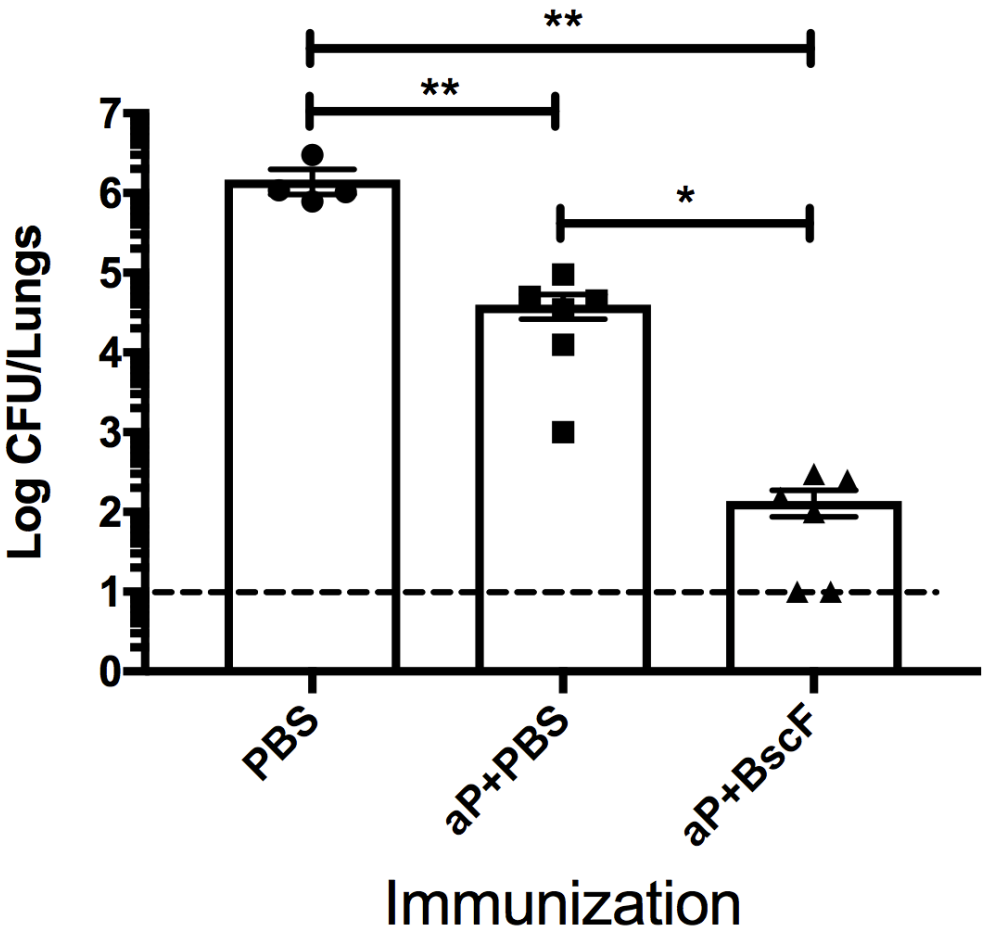


**Figure 21. The addition of BscF to the laboratory aP vaccine enhanced protective immunity against a sub lethal *B. pertussis* challenge**. Mice were immunized with aP + PBS (squares) or aP + BscF (triangles) twice (0 and 4 weeks). PBS immunized mice (circles) served a naïve control. At 6 weeks, mice were challenged with an intranasal inoculum of live *B. pertussis*. Lung homogenate CFU counts were recorded at 7 days post infection. Data are presented as mean $\pm$ SE of 4-6 mice per group, and are representative of 2 independent experiments. * Indicates *p* is between 0.05 and 0.01. ** Indicates *p* is between 0.01 and 0.001.

# CHAPTER V

# DISCUSSION

*Bordetella pertussis*, the causative agent of whooping cough, produces significant morbidity and mortality worldwide. Because there are no known non-human *B. pertussis* reservoirs, *B. pertussis* is truly a vaccine preventable disease. Current aP vaccines have fallen short over the last 20-30 years or so of their use, partly contributing to the reemergence of *B. pertussis* incidences in a number of developed and developing countries. *B. pertussis* research has been disadvantaged mostly due to the lack of suitable animal models; however, much of the work in these animal models on understanding *B. pertussis* pathogenesis and the critical role both the innate and adaptive arms of the immune system play in vaccine development have been validated in a newly developed baboon model that exhibits clinical symptoms more similar to humans. Moreover, human trials examining both the wP and aP vaccine safety and immunogenicity have also been instrumental in moving the field forward. From both animal and human studies, it is clear that a next-generation aP vaccine is necessary. In the studies included here, we characterize the immune stimulating properties of a *B. pertussis* specific protein called BscF, and assess its immune modulating capabilities when added to an aP vaccine.

With the replacement of the wP vaccine with aP vaccines in the 1990's cases of *B. pertussis* have been on the rise in many countries. Waning and ineffective immunity generated by the aP vaccine have been, in part, the cause of *B. pertussis* reemergence. While there have been a number of unique approaches in the effort to develop better vaccines, a large amount of work has been focused on the use of innate immune agonists (specifically TLR agonists) to skew aP vaccine induced immunity from the prototypical Th2 responses to protective Th1 and Th17 responses (86,120,121,122,123,124,125). These studies have reported success in animal models and provide evidence of the feasibility of adding other TLR ligands to a next-generation aP vaccine. Recent work from our lab focusing on purified T3S system needle proteins has identified a number of novel TLR agonists from a number of Gram-negative bacteria (130,31). These proteins are of interest for a number of reasons. First, T3S system needle proteins have been shown to be TLR2 and TLR4 agonists. Second, these proteins activate MyD88 dependent NF-$\kappa$B/AP-1 signaling downstream of TLR activation, and promote inflammatory cytokine release (130,131). Third, the strength of innate activation and inflammatory cytokine release can be modulated by modifying the N-terminus of the protein (131), allowing great flexibility if using these proteins and their immune stimulating properties in newly developed vaccines.

In the current studies, we demonstrated that BscF, a purified T3S system needle protein from *B. pertussis* acts as a strong TLR2 and TLR4 ligand. BscF activated NF-$\kappa$B/AP-1 signaling and promoted inflammatory cytokine release from both mouse and human cells *in vitro*. It is noteworthy that human cells respond similarly to BscF

stimulation when compared to mouse cells, indicating the possibility of the translational application of BscF in human aP vaccines. While we have extensively characterized the innate stimulating properties of T3S system needle proteins (130,131), to date, the contribution of endosomal TLR NF-$\kappa$B/AP-1 signaling has not been addressed. Additionally, clues to the mechanism of how T3S system needle proteins activate TLRs warrants further investigation. Clathrin-mediated endocytosis of plasma membrane TLRs (specifically TLR2 and TLR4) has been shown to amplify ligand-induced TLR signaling from endosomal compartments as well as activate distinct intracellular signaling pathways (179,180,181,187). One of the key players in TLR2 and TLR4 activation, endocytosis, and ligand specificity is CD14 (183,184,185,186,187). Our data indicated that clathrin-mediated endocytosis mediated NF-$\kappa$B/AP-1 activation, and that CD14 controls, in part, TLR2 and TLR4 activation. This is the first report demonstrating endocytosis of the TLR augments NF-$\kappa$B/AP-1 signaling by T3S system needle proteins. In addition, these data highlight the critical role of CD14 during T3S system needle protein activation of TLR2 and TLR4. Understanding the signaling mechanisms leading to TLR2 and TLR4 activation by T3S system needle proteins will facilitate their use in vaccine development.

In addition to TLR activation, we demonstrated that BscF activates the NLRP3 inflammasome and requires internalization to process pro-IL-1$\beta$ into mature IL-1$\beta$. It is thought that the wP vaccine was effective at driving $CD4^+$ Th1 and Th17 cellular immunity primarily through its immune stimulating properties. wP containing PAMPs activate the innate immune system and promote inflammatory cytokine release and DC

maturation to induce Th1/Th17 cellular immunity (118). In addition, *B. pertussis* specific Th17 cellular protective immunity and effective bacterial clearance were shown to be dependent on stimulation of the NLRP3 inflammasome by adenylate cyclase toxin, and subsequent IL-1$\beta$ release (60). BscF stimulation of mouse DCs resulted in robust IL-12, IL-6, IL-23, and IL-1$\beta$ release. These cytokines have been shown to be effective at polarizing naïve T cells to Th1 and Th17 subsets during antigen presentation.

*Ex vivo* BscF stimulated splenocytes from mice vaccinated with BscF produced robust IFN-$\gamma$ and IL-17 release, indicating a strong Th1 and Th17 adaptive immune response in mice immunized with BscF. Mouse and human studies have clearly demonstrated the critical role for $CD4^+$ Th1 and Th17 cells in protective immunity elicited by natural infection or vaccination (84,85,86,123,204,114,144,205). In addition, BscF immunization resulted in a modest, yet significant, reduction of bacterial burden in the lungs 7 days post challenge. While BscF vaccinated mice were still highly colonized with *B. pertussis*, these data indicate that BscF may also act as a protective antigen in addition to its immune modulating capabilities in a next-generation aP vaccine. We believe that the extracellular localization of BscF makes it an ideal therapeutic target. Serum from BscF immunized mice was able to significantly enhance *B. pertussis* opsonization and phagocytosis by mouse cells *in vitro*. It has been demonstrated that T3S system needle and translocon proteins act as protective antigens, presumably through the generation of a robust antibody response (145,132,146,147,148,149). Further work is needed to identify the protective role of BscF specific antibodies during a live *B. pertussis* mouse infection.

Although we saw modest protection in mice vaccinated with BscF alone, we do not envision BscF as a stand-alone vaccine for *B. pertussis*. We next addressed the ability of BscF to skew the immune response elicited by aP vaccination to a protective Th1/17 response by adding BscF to our laboratory prepared aP vaccine containing mPT and FHA. Both mPT and FHA are included in any licensed aP vaccine, so our results may provide insight for how BscF would work in future aP vaccines. aP vaccination has shown to primarily induce Th2 type responses in both animal models and humans (84,86,113,114,115). Our results demonstrate that the addition of BscF to the aP vaccine induced significantly higher FHA specific antibody titers, and indirectly induced IFN-$\gamma$ and IL-17 from *ex vivo* stimulated splenocytes when compared to the aP vaccine in PBS. In addition, bacterial clearance was enhanced by the addition of BscF to the aP vaccine 7 days post infection in mice compared to the aP vaccine alone. Our results are consistent with other studies demonstrating the effectiveness of skewing aP induced immunity to a protective Th1 and Th17 response by adding TLR ligands to aP vaccines (86,120,121,122,123,124,125). One advantage of BscF compared to the other TLR ligands is that BscF is *B. pertussis* specific, and could therefore be used as a protective antigen as well as an adjuvant in next-generation aP vaccines. In addition, given the prevalence of new circulating *B. pertussis* strains that have genetic modifications of many common antigens included in the aP vaccines (103,104,105,77,108,109,110), the use of new antigens that will likely not be lost by the bacteria would be advantageous. *B. pertussis* mutants of the T3S system demonstrated reduced colonization and induced

exacerbated inflammation during infection (83), suggesting that the loss of the T3S system due to vaccine pressure is highly unlikely.

This work has provided the foundation for future studies to further investigate the immune mechanisms mediating BscF's role during *pertussis* infections. For example, characterizing the role that BscF specific antibodies play during *B. pertussis* infection through passive transfer of BscF immune serum. Second, utilizing MHC class II tetramer staining to facilitate our understanding of BscF and pertussis specific $CD4^+$ T cell responses during immunization. Third, investigating if BscF is able to provide any cross protection to other *Bordetella* species (i.e. *B. bronchiseptica* and *B. parapertussis*).

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