ROLE OF *BRUCELLA* TOLL/INTERLEUKIN-1 RECEPTOR (TIR) DOMAIN CONTAINING PROTEIN (Δ*TCP*B) DELETION MUTANT IN PROTECTIVE IMMUNITY AGAINST BRUCELLOSIS

A Dissertation

by

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ABSTRACT

Brucellosis is an important zoonotic disease affecting about 500,000 people annually. The development of safer and more efficacious *Brucella* Live attenuated vaccines addresses safety concerns that include the identification of reproducible and reliable surrogates of protection and mechanisms to bolster longevity. *Brucella* encodes a toll interleukin receptor domain containing protein (*Tcp*B/Btp-1). These proteins subvert host innate immunity by abrogating NF-κB mediated cytokine production, by binding to and/or causing the degradation of signaling molecules TIRAP (MAL) and MyD88. *Tcp*B has also been shown to directly reduce the CTL killing activity of infected host cells.

In the current study, we investigated the effect of deleting *tcp*B from *Brucella* on *invitro* and *invivo* immune responses. We also evaluated an in vitro murine *Brucella* growth inhibition co-culture assay to determine the capacity of immune splenocytes from mice exposed to the *tcp*B mutant or wild type to control the growth of *Brucella melitensis* in murine bone marrow derived macrophages. A *tcpB* knockout constructed by gene replacement in the *Brucella abortus* S19 genetic background was used to vaccinate C57BL/6 mice assessed for development of $CD4^+$ memory T cells. Mice vaccinated with the mutant displayed an elevated Th1 response, compared to the parental S19 and non-vaccinated controls, as manifested by multiple factors. These include; elevated IFN-γ early post vaccination, and a significant elevation of memory $CD4^+CD44^+CD62L^+$ within $CD4^+$T cell population in splenocytes derived from mice vaccinated with the mutant. S19 and S19Δ*tcp*B strains induced a significant increase in the IgG_{2a} levels post

vaccination. Consistent with a shift to a Th1 response, S19ΔtcpB induced a higher response later in vaccination. Splenocytes obtained from mice vaccinated with the S19Δ*tcp*B mutant exhibited significantly higher levels of killing activity compared to cells derived from S19 vaccinated mice and PBS controls. Consistent with enhanced immune protection, fewer bacteria were recovered from the spleens of mice vaccinated with the S19Δ*tcp*B mutant, which had reduced inflammatory lesions consistent with reduced bacterial burden. These results provide strong evidence that *tcp*B deletion improves immunogenicity, longevity and protective efficacy of S19 and that *ex vivo* co-cultivation may be employed to predict potential vaccine efficacy.

DEDICATION

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1.1 Background

1.1.1 Brucellosis

Brucellosis is an important zoonotic disease caused by facultative intracellular bacteria of the genus, *Brucella*. *Brucella spp* belong to family Brucellaceae in the α2 subdivision of the phylum Proteobacteria. These are small, non-motile, non-spore forming, aerobic or microaerophilic, Gram-negative coccobacilli [1]. Ten species of *Brucella* have been identified based on their preferred hosts; these are *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. ovis* (sheep), *B. canis* (dogs), *B. suis* (pigs), *B. neotomae* (desert woodrat), *B. pinnipedialis* (seal), *B. ceti* (dolphin, porpoise, whale), *B. microti* (common vole) and *B. inopinata* (unknown). All members of the genus are closely related genetically and immunologically [2]. Of these, *B. abortus, B. melitensis, B. suis, and B. canis* are pathogenic to humans [3]. *Brucella* strains may exist as either smooth or rough variants depending on the expression of O-polysaccharides (OPS), which is a major component of the bacterial outer membrane lipopolysaccharide (LPS) [4]. The smooth LPS (S-LPS) expresses the OPS, which is responsible for host antibody responses. The

absence of OPS on R-LPS determines a rough phenotype, which is associated with lost of invasion, clearance from host tissues and virulence [5,6].

Brucella infections are characterized by preference for cells of the reticulo-endothelial system and reproductive organs. The organism colonizes and persists in tissue macrophages, dendritic cells and is carried to many parts of the body. Infection of the placental trophoblasts causes necrosis of the fetal cotyledons, suppurative placentitis and abortion in the female. Abortion occurs in the fifth month of gestation and the fetus may be autolysed. Infection in bulls is manifested by seminal vescicultis and orchitis that may cause sterility [7,8]. In addition to brucellosis severely impairing the reproductive potential of a herd by impacting negatively on productivity; the disease has a severe public health importance, causing an acute and chronic disease in humans [9]. Control of the disease in humans is through animal vaccination or culling, as no vaccine is licensed for use in humans.

1.1.2 Impacts of brucellosis on human health

Although human brucellosis has almost been eradicated in many developed countries, it remains a major neglected zoonosis of low-income nations [10]. The disease negatively impacts agriculture, and is a major public health hazard affecting about additional 500,000 people annually [3,11]. The disease manifests in humans in an acute or chronic debilitating course; the most common signs include undulating fever, headaches, chills, depression, weakness, arthralgia, myalgia and weight loss [12]. The disease may also present as a reproductive form manifesting as orchitis/epididymitis in men and

spontaneous abortion in pregnant women [13,14]. The disease can take a chronic course accompanied by complications like endocarditis, arthritis and osteomyelitis [15,16]. Treatment of brucellosis in humans involves a prolonged antibiotic course, the optimal treatment regimen being a doxycycline-aminoglycoside-rifampicin combination, with the aminoglycoside administered for the first seven to 14 days and doxycycline-rifampicin combination continued for six to eight weeks [17]. Transmission of *B. abortus*, *B. melitensis, B. suis and B. canis* between animals occurs by contact with discharges from the placenta, fetus and the reproductive tract from infected animals. Transmission from animals to humans may occur as an occupational hazard, involving people that work with farm animals or come into contact with environments contaminated by animal products. These may include farmers, animal attendants, stockmen, shepherds, sheep shearers, goat herders, pig keepers, veterinarians and inseminators. Infection may occur by inhalation, conjunctival contamination, accidental ingestion, skin contamination through abrasions, or accidental self-inoculation with live vaccines in laboratory workers [18]. Clinical signs in humans are often incorrectly interpreted by medical personnel, and as a result, human brucellosis is severely underreported or ignored [19].

1.1.3 Impacts of brucellosis on agriculture

Livestock rearing is the principal economic activity supporting livelihoods of more than 50% of the population in sub Saharan Africa [20]. Many diseases that affect both livestock and humans like brucellosis are poorly controlled due to lack of financial

resources [21]. Brucellosis has been recognized as one of the most important and widespread zoonosis in sub Saharan Africa [21]. The disease not only affects human health, but also their socio economic livelihoods through the health of their livestock [10,22]. The negative impact on the livestock sector is primarily due to abortion or stillbirths, weakened or sickened calves, decreased milk production and infertility [10]. The latter is a result of prolonged inter-calving intervals due to stillbirths and abortions, and increased periods between lactations and the costs involved in control. Economic losses in small ruminants manifest as suboptimal breeding resulting from infertility in rams and reduced milk production [21]. In developed countries, mandatory testing and depopulation is the method of choice in disease control and management of infected herds. Infected animals are eliminated, and owners are paid a federal indemnity. In addition to the loss of valuable income, this culling slows the genetic progress to improving the quality of the herd by reducing the genetic pool and reduces export trade of any affected state [23].

1.1.4 Significance of the work performed

The negative impacts of endemic brucellosis cut across agriculture, public health and social development sectors. It is therefore important to control the disease in order to improve the socio-economic livelihood of affected communities. Management of the disease relies heavily on vaccination in the animal population, and the best course of action is removal of the infected animals from the herd. However, currently available vaccines are unsafe for use in pregnant animals, and may induce abortion. Their use can

therefore exacerbate the threat to human health in the absence of a licensed vaccine for use in humans and a prolonged and often unsuccessful therapeutic management [24]. Live attenuated vaccines (LAV) against brucellosis present the best method of control due to the elevated, long lived immunity they engender [25]. Generation of *Brucella* live attenuated vaccines can be naturally obtained by spontaneous or targeted deletions of virulence genes from wild type strains [26]. Several vaccines have been generated and have demonstrated successful use [25-27]. However, these vaccines have several side effects including residual virulence, abortion in pregnant animals, and ability to cause disease in humans [25]. Use of *B abortus* S19 (also referred to as S19) vaccine in field conditions has been associated with abortions when administered to pregnant animals at rates between 1 and 2.5% [28]. In experimental conditions, attenuated *B. abortus* S19 is transmitted to the fetus and often causes higher abortion rates [29,30]. Several attempts have been made to develop safer *Brucella* LAV, by identifying and deleting virulence genes and providing improved delivery systems with varying results [31]. In contrast to those studies, we have hypothesized that the presence of immuno-subversive factors may be explored in the quest for safe and efficacious *Brucella* live attenuated vaccines. Recent studies have revealed that *Brucella* genomes bear genes encoding factors that subvert toll like receptor (TLR) and host innate immune responses. These are proteins that mimic the toll interleukin 1 receptor, including *Brucella* toll interleukin-1 receptor domain containing proteins (*Tcp*B/Btp1 and Btp2) [32,33]. The presence and activity of these factors may compromise the efficacy and safety of LAV. Deletion of genes

encoding these factors from live attenuated vaccines offers a novel strategy for improving their safety and/or efficacy.

1.1.5 Innovation associated with the project

*Tcp*B/Btp1 and Btp2 proteins expressed by *Brucella* have significant subversive effects on the host immune resistance to *Brucella* infection [33]. *Tcp*B binds to components of the TLR signaling pathway, Myeloid differentiation factor 88 (MyD88) and Toll interleikin-1 receptor adaptor protein (TIRAP), causing TIRAP degradation and subverting innate immunity and enhancing pathogen establishment and survival [34,35]. Subsequent studies have shown that mutants defective in *Tcp*B expression up-regulate host innate immune mechanisms during *Brucella* infection by stimulating enhanced pro inflammatory cytokine secretion [32]. In addition, *Tcp*B also reportedly subverts T cell activity and contributes to the establishment of chronic brucellosis [36]. The net effect of expression of TIR containing proteins like *Tcp*B during *Brucella* infection is host immune subversion and enhancement of pathogen survival. The flip side of this coin is that the absence of *Tcp*B expression is expected to result in an enhanced innate immune response potentially improving vaccine efficacy. The goal of this project was to design safer and more efficacious *Brucella* live attenuated vaccines by introducing a *tcpB* mutation to *Brucella* LAV (S19) and to use this novel vaccine to identify correlates of immune protection against brucellosis. The specific aims of the research included construction of a *tcpB* deletion mutant of *Brucella abortus S19* and evaluation of the resulting innate and adaptive immunity and protective efficacy in a murine model of

brucellosis. The central hypothesis of the objective was that *tcpB* deletion mutant would confer improved immunogenicity and protective efficacy to the currently available vaccine strain *B. abortus S19*.

1.2 Problems unique to intracellular pathogens

Intracellular bacteria reside within macrophages, dendritic cells or other cells of the reticulo-endothelial system, a niche that provides unique physicochemical conditions that impact both the survival of the pathogen and the host. Intracellular bacteria are therefore faced with a task of developing mechanisms that render them inaccessible to the antimicrobial effector mechanisms of the host while providing nutrients necessary for survival and proliferation. Intracellular pathogens have therefore evolved mechanisms enabling them reside within the hostile environment of macrophages or other cells of the reticulo-endothelial system [37]. Upon infection by bacteria, macrophages deploy a wide range of killing mechanisms, these may include, secretion of proinflammatory cytokines, activation of intracellular pathogen killing and activation of Th1 cell responses [38]. Bacteria have therefore developed mechanisms of inhibiting or modifying these mechanisms in their favor.

1.3 Microbial mechanisms of host immune evasion

1.3.1 Lysosomal degradation

Following phagocytosis, engulfed bacteria are located in phagosomes, which subsequently fuse with lysosomes, acidify and mature into phagolysosomes.

Phagolysomes contain proteases, antimicrobial peptides, and lysozyme that mediate the killing of the engulfed bacteria. Intracellular bacteria have developed various ways of avoiding phagosomal maturation and fusion with the lysosome [39]. These mechanisms may range from enclosure in a vacuole resistant to lysosomal fusion, or expression of factors that neutralize the lysosomal effectors [40]. *Brucella* is enclosed in a *Brucella* containing vacuole (BCV) that is resistant to lysosomal degradation [41].

1.3.2 Nutrient access

Since location in lysosome resistant compartments presents a problem of access to nutrients, bacteria may need the lysosomal processes to acquire nutrients [42]. Cells use the autophagic process by enclosing dead organelles in autophagosomes and fuse them with lysosomes for degradation. Autophagy may play a dual role in host defense by encapsulating and degrading microbes, but it may also facilitate delivery of nutrients to enclosed pathogens [43]. *Brucella* has been documented to induce the unfolded protein response, which may activate autophagic processes that enhance its survival. The unfolded protein response mediated autophagy been proposed to be a mechanism of nutrient acquisition by *Brucella* and is responsible for enhancing its survival [44]

1.3.3 The oxidative burst

The oxidative burst is a release of reactive oxygen species [superoxide radical (O^{-2}) and hydrogen peroxide (H_2O_2)] by macrophages following contact with microorganisms. Macrophages use NADPH oxidase to reduce O_2 to O^{-2} and then to H_2O_2 [45].

Macrophages also use the interaction of myeloperoxidase with H_2O_2 and Cl^- to generate hypochlorite, which is toxic to most bacteria. *Brucellae* are protected from the respiratory burst of host macrophages by expression of superoxide dismutase (SOD) encoded by *sodC*. These enzymes catalyze the dismutation of superoxide (O_2^-) to hydrogen peroxide ($H_2O_{2),\ which}$ can then be further detoxified through the action of catalases and peroxidases[46].

1.3.4 Macrophage response to cytokine activation

Macrophages respond to bacterial infection by activating signaling cascades leading to expression of pro-inflammatory cytokines such as IL-12, TNF-α, and IFN-γ [47]. These cytokines either activate intramacrophage killing of the bacteria or enhance the adaptive immune response by up regulating T cell activity. They may also induce production of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-4 to down-regulate host defense mechanisms [48]. *Brucella* may also trigger down regulation of signaling pathways that trigger programmed cell death

When macrophages fail to control the pathogen, they may use programmed cell death as a way of pathogen elimination [43]. This may occur by either apoptosis, pyroptosis or necrosis [49]. Apoptotic cells are then engulfed by neighboring phagocytes, causing pathogen degradation and presentation of peptides to CD8+T cells I the context of MHC class 1 [50]. Necrosis and pyroptosis can trigger rapid inflammation, due to secretion of pro-inflammatory cytokines via extracellular release of cytoplasmic contents [51].

Intracellular *Brucella* can also use programmed cell death or cytotoxicity as a strategy for egress and possible cell-to-cell spread [52,53].

1.4 Host response to *Brucella* infection

1.4.1 Innate immunity

1.4.1.1 NF-κB mediated TLR pathway

Early in infection, the TLRs expressed by the host cell recognize pathogen-associated molecular patterns (PAMPs) from bacterial cells [54]. This triggers protective innate immune responses, including expression of proinflammatory cytokines and co stimulatory molecules [55]. TLR signaling therefore significantly affects the host innate and adaptive immune responses [56]. However, *Brucella* spp lack structures that commonly activate classic TLR signaling and a strong innate immune response; including, possession of non-classic LPS, capsules, fimbriae and pili, and a flagellum that escapes recognition by TLR5 [57]. Under classic conditions, potent TLR engagement stimulates interaction with specific, TIR adaptors, MyD88, MyD88-adapter-like (MAL)/TIRAP, TIR domain containing adaptor inducing IFN-β (TRIF) or TRIF-related adapter molecule (TRAM). However, *Brucella* infection does not induce strong innate immune responses due in part to the poor agonist activity of *Brucella* PAMPs and to *Tcp*B-dependent abrogation of TLR signaling [58]. *Tcp*B binds to TIRAP and myD88, causing degradation of TIRAP and abrogation of the MyD88 mediated signaling pathway. The net action of *TcpB* is abrogation of NF-κB translocation and inhibition of expression of proinflammatory cytokines. The cytokines produced by APCs direct the

differentiation of $CD4^+$ T cells to either Th1 or Th2 cells. Activation of TLR signaling pathways therefore significantly affects the type of $CD4^+$ T cell differentiation [59]. *Brucella* infection restricts the host's innate immune response by expressing LPS with reduced agonist activity to enhance efficient invasion and establishment in addition to limiting the priming of an effective adaptive immune response. It has, however, been found that toll like receptors play a significant role in of *Brucella* infections. Experimental evidence has shown that TLR 2, 4 and 9 play important roles in aiding clearance, susceptibility, and dissemination of *Brucella* in the host [60]. Experimental evidence has shown that TLR 2,4, 9 and MyD88 KO mice have enhanced susceptibility to *Brucella,* and that this enhanced susceptibility has been attributed to reduced expression of pro-inflammatory cytokines, reduced uptake and dissemination [60-62]. Due to the MyD88 subversive effect of *tcp*B, it is expected that *tcp*B bearing wild type *B. abortus* will significantly impact the infectivity and dissemination of *Brucella*. MyD88 has, however, been shown to be more important than TLR2, 4 and 9 in clearance of *Brucella* infections [63-65]. The subversive effect of *tcp*B to MyD88 is therefore expected to have a negative impact on the host response to *Brucella*.

1.4.1.2 Mitogen activated kinase (MAPK) mediated TLR pathway

TLR activation by *Brucella* and subsequent production proinflammatory cytokines also involves activation of the mitogen-activating protein kinase-signaling pathway (MAPK) [66]. The MAPK signaling cascade has been described in bacterial pathogenesis as demonstrated by the induction or inhibition of ERK1/2 and p38 MAPKs during

Mycobacterium spp [67]. The differences in inflammation associated with smooth and rough *Brucella* have been explained by differential activation of MAPK. Smooth *Brucella* have been shown to mildly activate the MAPK while activation is greatly enhanced by the rough bacteria, this has been associated with the presence and absence of the LPS O chain that leads to up regulation of TLR signaling[68].

1.4.2 Host adaptive immune mechanisms in brucellosis

Following phagocytosis by antigen presenting cells (APCs), *Brucellae* are enclosed in a *Brucella* containing vacuole (BCV) which releases antigens that become associated with MHC class II which traffic to the cell membrane where they are presented to $CD4^+$T cells. The normal outcome is activation of T cells that includes both enhanced cytotoxic activity and MyD88 dependent secretion of a variety of chemokines and cytokines including; IL-12, IL-1, IL-6, TNF-α and IFN-γ to initiate an innate immune response [65]. IL-12 initiates clonal expansion of Th1 cells, and stimulates production of IFN-γ and TNF-α from T cells and NK cells while reducing IL-4 expression. IL-12 also enhances the cytotoxic activity of NK cells and $CD8^+$ T lymphocytes demonstrated experimentally by increasing innate and adaptive immunity in *Brucella*-susceptible mice treated with IL-12 [69,70]. IFN-γ is a critical cytokine for host control of *Brucella* infection [71]. IFN-γ activates the cytotoxic action of $CD8^+$ T cells on *Brucella* infected cells, by activating macrophages and by inducing class II major histocompatibility complex (MHC) molecule expression on antigen presenting cells [72]. TNF-α, another a pro-inflammatory cytokine produced during *Brucella* infection drives the secretion of

IL-12 and IFN-γ, directing an acquired secondary response of the Th1 phenotype by $CD4^+$ and $CD8^+$ lymphocytes [73]. Th1 cells play an important role in the control of *Brucella* infection. Although immunity to *Brucella spp*. requires both $CD4^+$ T cells and $CD8^+$ CTLs [74], there are conflicting reports as to which plays a more important role. Several studies describe the importance of $CD8^+$ cells based on the absence of β_2 macroglobulin significantly exacerbating infection [75-77]. β_2 macroglobulin is an essential component of MHC class I, and is present on all nucleated cells. Lack of β_2 macroglobulin therefore compromises antigen presentation to CD8+ T cells via MHC class I [78]. Furthermore, in vivo depletion has also revealed that $CD8^+$ T cells are the primary responders to DNA vaccines encoding outer membrane proteins and reduction in number results in higher bacterial loads in *B. abortus* infected BALB/c mice [79,80]. *Brucella*-specific $CD8^+$T cells play a dual role in control of *Brucella* infection; they initiate lysis of *Brucella*-infected macrophages and also secrete low levels of IFN-γ that enhances macrophage killing of intracellular bacteria [74]. It has, however, been shown that IFN-γ–producing $CD8^+$ T cells alone fail to protect mice during primary infection [81]. $CD4^+$ T cells have been shown to display a variety of helper functions necessary for an efficient adaptive immunity. $CD4^+$ T cells produce IFN-γ that promotes intra-macrophage killing of *Brucella*-infected cells. $CD4^+$ CTLs are also capable of cytolytic action against *Brucella*-infected APC [82]. This suggests that both $CD4^+$ and $CD8^+$ T cells may a play a synergistic role in the anti-Brucella activity [74]. Deletion of *tcp*B from live attenuated vaccines may result in increased expression of $CD4^+$ and $CD8^+$ T

cells by virtue of the increased expression of pro inflammatory cytokines that are induced early in infection.

1.5 *Brucella*-specific resistance to host immune mechanisms

1.5.1 Resistance to innate immunity

Brucella infections are characterised by subversion of innate immune rsponses that leads to persistence in tissues and failure of the host to clear the infection. This is suspected to derive from the cell surface lipopolysaccharide composed of a relatively non-toxic lipid A structure composed of unusually long chain fatty acids and an O-antigen homopolymer of N-formyl perosamine. The absence of O-antigen results in "rough" variants which induce elevated inflammatory responses accountable for the highly attenuated virulence of these organisms [83,84]. In the absence of documented changes in the lipid A in rough organisms, this difference is attributable to the loss of O-antigen sidechains alone. Following phagocytosis, the organism relies upon the type IV secretion system (T4SS) to survive by secreting effector proteins to alter the intracellular environment and intracellular trafficking of *Brucella* containing vacuoles to provide a replicative niche [85]. One of these effectors, VceC, activates a proinflammatory response via the unfolded protein response (UPR) via the endoplasmic reticulum (ER). These two factors appear to control contradictory results, but it is important to recognize that these activities operate at different stages of infection. The contribution of TIR-containing proteins like *Tcp*B/Btp-1 and Btp-2 have only recently been revealed [32,33]. Encoded within the bacterial genome, *Tcp*B and Btp-2 encode proteins which are

molecular mimics of host proteins that interfere with host cell signaling pathways by interacting with pathway components and altering host cell functions. For example, *Tcp*B prevents TIRAP-mediated recruitment of MyD88 to the plasma membrane (PM) resulting in a failure of TLR4/2 signaling [86]. The *Brucella* TIR proteins also have phosphatidyl inositol (PtdIns) binding sites similar to host cell factors that contribute to PM localization to enhance TIR:TIR interactions. Whether *Tcp*B can reduce intracellular PIP2 levels in an effort to regulate cell function has not been addressed. Strains lacking *Tcp*B would be expected to exhibit an enhanced proinflammatory response due to a reduction in the inhibitory affect of TIRAP binding with MyD88 which enhances TLR4/2 signaling and the UPR and an associated improvement in the adaptive immune response.

1.5.2 *Brucella* lipopolysaccharide (LPS)

Brucella possesses a non-classical LPS with weak agonistic activity to TLR as compared with the potent endotoxin produced by *Escherichia coli* [87]. This difference is attributable to the presence of long chain fatty acids conjugated to the lipid A portion of the *Brucella* LPS. *Brucella* lipid A possesses a di-aminoglucose backbone with long acyl groups (C28), which are linked to the core by amide bonds. The reduced potency of *Brucella* LPS is thought to represent an evolutionary adaptation that avoids activation of the early host innate immune response and leads to early establishment of the infection and acquisition of a BCV [88,89].

1.5.3 The two-component system (BvrR/BvrS)

This is an adaptive mechanism used by *Brucella* to regulate the expression of specific genes in response to changes in environmental conditions. The two components include an environmental sensor (BvrS), a histidine kinase found on the cytoplasmic membrane that transmits a signal to the second component, a cytoplasmatic response regulator (BvrR) that mediates changes in gene expression [90]. Environmental stimuli are detected by BvrS that undergoes autophosphorylation on a histidine residue. The phosphorylation generates a series of reactions that activate transcription factors inducing expression of specific host genes [91]. *Brucella* uses this system for the expression of genes involved in the maintenance of cell envelope integrity including multiple members of the Omp3 family of proteins, Omp25, Omp22 or Omp3b [92]. This system also regulates genes that modify the fatty acid composition of the lipid A moiety of the LPS. This system also controls the expression of the *vir*B type 4-secretion system (T4SS) via BvrR binding at the *vir*B promoter and activating the transcription of VjbR a master regulator of expression of *Brucella* virulence factors [90].

1.5.4 The *Brucella* type 4 secretion system (T4SS)

The *Brucella* type IV secretion system (T4SS) is a bacterial organelle comprised of 12 proteins exposed on the surface of the bacterial cell envelope. Its main function is to translocate effector proteins from *Brucella* into infected host cells. The *virB* operon encoding the *Brucella* T4SS consists of 12 open reading frames *virB1* to *virB12 (loci*

BMEI10025-BMEI10035) controlled by a single promoter just upstream of *virB1*[93,94]. The T4SS is expressed in response to acidification and/or partial lysosomal fusion with the BCV. The acidified BCV acquires the late endosome markers LAMP-1, CD63 and Rab7 [95], then progressively looses these markers and associates with the ER exit sites as determined by accumulation of the ER markers calreticulin, Rab2 and GAPDH. Acquisition of these markers is associated with conversion of the BCV into a replicative BCV (rBCV). Expression of the T4SS is required for maturation of the BCV following sustained interaction and fusion with the endoplasmic reticulum [96]. VjbR, a LuxR family transcriptional regulator, promotes expression from the *vir*B operon by directly binding to a region of the *virB* promoter containing an 18 bp palindromic motif [93]. Also among the genes activated by VjbR are two novel T4SS effectors, VceA and VceC [97]. Five additional T4SS effectors that may play a role in persistence of *Brucella* have been described; these are BAB1_0678 (BspA), BAB1_0712 (BspB), BAB1_0847 (BspC), BAB1_1671 (BspE) and BAB1_1948 (BspF) [85].

1.5.5 Cyclic beta-1, 2 glucans (CβG)

CβG is a virulence factor expressed by *Brucella* that interrupts host cellular functions like causing perturbation of intracellular trafficking to benefit the pathogen [98]. CβG interacts with cholesterol and interferes with lipid rafts and intracellular trafficking to the pathogen's advantage. *Brucell*a CβG deficient mutant strains display a defect in intracellular trafficking in epithelial cells. This deficiency can be complemented by addition of purified CβG [98].

1.5.6 *Brucella* TIR domain containing proteins

Brucella encodes and expresses TIR domain containing proteins, which are implicated in enhancing virulence by subversion of host immune responses [33]. These proteins suppress TLR signaling by subverting NF-κB activity, reducing the host inflammatory response and enhancing infection of the "stealth" pathogen [99]. Three dimensional crystallography studies have shown that TIR containing proteins are composed of uniform structures of five stranded parallel β sheets and five α helices connected by surface exposed loops [100]. A BB loop connects the B sheet to the B helix and has been shown to be important for signal transduction and microtubule-binding site [101,102]. Their function stems from the shared sequence homology they have with mammalian TIR domains [103]. The greatest similarity between *Brucella* and mammalian TIR proteins is found in domains designated box 1, box 2, and box 3 [58]. The features in these areas are highly conserved in both the mammalian and the bacterial TIR domains. The consensus protein sequences for box 1 is (YDAFISYS), box 2 (LKLCLXXRDXXPGXSI), while box 3 has a conserved W surrounded by basic residues. It has been proposed that boxes 1 and 2 are involved in the binding to TIRAP and MyD88, whereas box 3 is primarily involved in localizing proteins to microtubules [34,86,104]. *Tcp*B binds to both MyD88 and TIRAP, but the binding to MyD88 occurs via death domains (DD) and is stronger than TIRAP binding [34]. MyD88 forms a central adaptor in the TLR family signaling pathways by linking TLR family members to IL-1R-associated kinase (IRAK) family kinases [105]. The C-terminal TIR (Toll IL-1R)

domain interacts with other TIR domain-containing proteins; the N-terminal death domain (DD) associates with the IRAK family members [106]. Activation of IRAK kinases leads to activation of nuclear factor-kappa B (NF-κB), mitogen-activated protein kinases (MAPK), and activator protein and subsequently expression of pro inflammatory cytokines. Binding of MyD88 DDs to IRAK DD forms a structure, termed the Myddosome, comprising of a ring of six MyD88 DDs that serve as a seed for assembly of a ring of four IRAK4 DDs and ultimately four IRAK2 DDs in a helix. The Myddosome forms a scaffold for the assembly of downstream signaling molecules [105]. Binding of *Brucella Tcp*B to MyD88 prevents assembly of the activated Myddosome complex, thus preventing downstream signaling [107]. The result is abrogation of translocation of NF-kB into the nucleus and reduced transcription of genes expressing proinflammatory cytokines [108] . The binding of *Tcp*B to the DD of MyD88 or binding to TIRAP and subsequent TIRAP degradation causes subversion of host cell signaling that aid in the establishment of infection [86]. *Brucella* Δ*tcpB* mutants may be expected to stimulate an enhanced innate response and provide improved immune protection.

1.5.7 *Tcp*B: Phosphoinositide (PIPs) binding and immune subversion

Phosphoinositides, the phosphorylated derivatives of phosphatidylinositol (PI), regulate many critical cell processes involving membrane signaling, trafficking, and reorganization [109]. Sequence analysis confirms that TIRAP shares phosphoinositide-binding domains similar to those identified in the *Brucella Tcp*B protein [86]. This

binding enhances *Tcp*B co-localization with the plasma membrane and components of the cytoskeleton [110]. Phosphoinositides participate centrally in several cell signal transduction activities in addition to structural and developmental functions [111,112]. By virtue of PIP binding, *Tcp*B is expected to participate in structural and signaling properties of cells to facilitate *Brucella* infection and dissemination [86]. Phosphoinositides play an important role in the organization and rearrangements of the actin cytoskeleton and in phagosomal maturation during classical phagocytosis. Bacteria utilize phosphoinositides to recruit effectors required to facilitate invasion and enhance maturation of intracellular compartments that promote bacterial survival [113]. These observations prompted studies to confirm that *Tcp*B targets the TLR-TIRAP-mediated signaling pathway to subvert NF-κB translocation by binding to and promoting ubiquitination of TIRAP [58,86]

1.5.8 TcpB effect on unfolded protein response (UPR) and innate immunity

B. melitensis infection triggers UPR in order to facilitate its survival after infection by activating all three branches of UPR signaling in macrophages; IRE1, PERK and ATF6 [44]. *Brucella* mediated activation of UPR has been shown to be associated with TcpB [44]. *Tcp*B has a strong MyD88 binding affinity that is predicted to disrupt UPR mediated expression of proinflammatory cytokines [44]. *Brucella* therefore uses TcpB to inhibit XBP-1 splicing, a critical branch of UPR, to subvert cytokine production [114]. Thus, expression of *Tcp*B may suppress cytokine gene expression via MyD88 binding and subsequent TIRAP degradation, leaving two of the three arms of the UPR active.

1.5.9 *Brucella* Δ*tcp*B mutants in the mouse model

Due to its mechanism of abrogation of NF-κB translocation, *Brucella* TIR domain containing proteins reduce pro inflammatory cytokine expression. In studies performed with *B. abortus* TIR mutants, deletion of BtpA and BtpB resulted in significant increase in secretion of TNF-α in BMDCs [32,115]. Deletion of BtpB was also shown to upregulate the proinflammatory cytokine IL-12 in BMDCs [32]. Analysis of cytokine expression in infected IRF-$1^{-/-}$ mice infected has also shown upregulation of TNF-α in animals infected with *Tcp*B-deficient *Brucella* compared with the wild type [86]. Studies comparing survival of *Brucella* Δ*tcpB* mutants and wild type bacteria in a mouse model have shown that Δ*tcp*B mutants exhibit an early attenuated phenotype in mice that is resolved at later time points [86]. Mice infected with the Δ*btpB* mutant also survive longer than those infected with the wild type *B. abortus* [32]. Histological examination of spleens obtained from wild type BALB/c mice revealed a significantly higher number of granulomas in mice infected with Δ*btpB* and Δ*btp*AΔ*btp*B [32]. Granuloma formation may reflect the host's ability to develop a protective immune response. Increased granuloma formation in infection by Δ*btpB* and Δ*btp*AΔ*btp*B mutants therefore suggests that *tcp*B deletion increases the host's ability to develop a protective immune response in LAV. Mice infected or vaccinated with *tcp*B LAV mutants are therefore expected to show elevated immune response, making the case for *tcp*B deletion in LAV as an attractive vaccine development strategy.

1.6 *Brucella* vaccines

Studies have established that the ability of a *Brucella* vaccine strain to multiply in the host before clearance was a condition necessary to trigger protective immunity, making the case for live attenuated vaccines [116]. A degree of persistence in host tissues without causing pathology may be a favorable factor in designing LAV [25]. The best available vaccine against *Brucella* (*B. abortus* S19 and *B. melitensis* Rev 1) are live, attenuated strains that persist in tissues for some time [117]. *Brucella* virulence genes are related to induction of virulence, persistence may, therefore, be partly associated with the side effects observed [118]. *Brucella* LAV may cause abortion in pregnant animals and interference with serological diagnosis, and virulence for humans [7]. Efforts to improve LAV have focused on creating deletion mutants with reduced persistence and virulence [26,116,119,120]. However, this strategy has also resulted in reduced efficacy [25]. To address the problem created by reduced efficacy, improved delivery methods and additional immune enhancing mutations may be introduced [25]. Multiple experimental live attenuated vaccines with varying efficacy have been created for use against brucellosis in animals. These LAV against brucellosis have been designed by identification and deletion of genes encoding virulence factors [31,121,122]. Deletion of these genes confers upon the vaccines increased safety. Attempts have also been made to improve delivery platforms of LAV in order to improve their efficacy [123]. Attempts have also been made at designing subunit vaccines by expressing immunogenic peptides from *Brucella* and optimizing the expression systems [124]. An example of a promising LAV has been made by deletion of the, vjbR gene that encodes

the quorum sensing regulator. The resulting mutant made from the strains *B. melitensis* 16M is rapidly cleared from mice manifested as a drastic reduction in splenic CFU burden by 28 days post-infection [31]. This deletion confers protection against *B. melitensis* similar to Rev-1 vaccine [26]. The efficacy of this strain has been further enhanced by microencapsulation of a related mutant *B. melitensis* vjbR::Tn5, in alginate microspheres [31]. Despite the availability of many experimental *Brucella* LAV, very few have been licensed for use due to the caveat of residual virulence and/or reduced efficacy [27]. To further improve the efficacy of Brucella LAV, strategies that result in elevated memory cells may be adopted. Elevated T cell immunological memory is a critical component of vaccine efficacy [125]. The lack of protection, particularly longevity of protection, afforded by many vaccines may be attributed to poor induction of memory cell differentiation. Nowhere is this more evident than in the development of bacterial subunit vaccines. The failure to identify protective immunogens capable of inducing demonstrable immunological memory has compelled the continued use of live, attenuated vaccine candidates with the capacity to deliver antigens for proper immune stimulation without prolonged persistence or systemic spread that could resulting in disease. Yet, the use of live, attenuated organisms may also be compromised by the expression of factors specifically designed to restrict host immunity. Among these gene products, the *Tcp*B protein, a novel effector for immune evasion [115]. *Tcp*B accomplishes this action by abbrogating NF-kB mediated cytokine expression [86] and inhibiting $CD8^+$ T cell effector function as well as memory cell differentiation. It has been established that *Tcp*B contributes to T cell memory exhaustion and persistance of

infection in brucellosis [36]. The mechanism by which *Tcp*B disrupts memory cell differentiation has been proposed as a disruption of mTOR activation signaling pathway [36]. Based on these factors, we proposed that a vaccine deficient in *Tcp*B expression should stimulate enhanced differentiation of memory T cells and confer improved immunogenicity relative to the parental strain. Because $CD4^+$ T cells have been identified as major effectors in *Brucella* infection, a successful *Brucella* vaccine is expected to elicit a robust $CD4^+$ T cell memory differentiation [126]. Minimal attempts have been made to characterize the longevity and associated ability of *Brucella* vaccines to enhance T cell memory differentiation. Determining the presence and quality of long-lived memory T cells *ex vivo* may be expected to simplify the processes of vaccine candidate identification.

In this study, we hypothesized that presence of immune subversive factors play a crucial role in compromising the safety and efficacy of *Brucella* LAV. We therefore proposed to create LAV deficient of immune subversive factor, *tcp*B, a novel strategy that will improve their efficacy and safety.

CHAPTER II

EFFECT OF *TCP*B DELETION ON IMMUNITY IN BRUCELLA INFECTED MACROPHAGES

2.1. Introduction: Immune response to *Brucella* infection in macrophages

Macrophages are normally activated during infection via pattern recognition by cell surface proteins including the toll like receptors to stimulate an innate immune response including expression of proinflammatory cytokines. The cytokines produced during the early stages of host immune response are essential in defining the type of innate and subsequent adaptive host immune response to be developed; proinflammatory cytokines expressed during infection elicit a Th1 immune response [127,128]. *Brucella* infection fails to fully activate these pathways based on the weak agonist properties of cell surface components like non canonical LPS and mechanisms designed to restrict downstream signaling such as the activity of *Tcp*B [58,88]. In this study, we evaluated the effect of *tcpB* deletion on the host's innate immune responses in macrophages infected with *B. abortus* S19. We constructed a *tcp*B deletion in the wild type strain *B melitensis 16M* to create 16MΔ*tcpB*, to determine the effects of *tcp*B deletion on a fully virulent organism with fully active immune-suppressive activity. We infected murine macrophages and studied the resulting replication and immuno-modulatory effects resulting from *tcpB* deletion, including the bacterial burden in murine RAW264.7 macrophages over time and up-regulated expression of proinflammatory cytokines (IL12, TNF and IL6). The results are consistent with *Tcp*B-mediated suppression of innate immunity and the

potential to rationally improve *Brucella* LAVs via removal of similar immune-suppressive functions. We hypothesized that deletion of *tcp*B will improve the host immune response of *Brucella* LAV (*B. abortus* S19), and presumably other LAVs as well. To test this hypothesis, we constructed a Δ*tcpB* deletion mutant in the *Brucella abortus* S19 vaccine strain to create a derivative vaccine S19Δ*tcpB*.

2.2 Rationale for the experimentation outlined

Identification and deletion of genes encoding immune subversion factors is a novel strategy that can be employed to improve vaccine efficacy. Microbial Toll/interleukin 1 receptor proteins are a family of virulence factors that modulate the toll receptor signaling cascades [33]. Following *Brucella* infection, *Tcp*B subverts the host's innate response by blocking development of immunological memory and altering development of an adaptive response. *Tcp*B (and/or BtpB) has been shown to abrogate MyD88 mediated NF-κB mediated cytokine gene expression representing novel effectors of immune evasion. *Tcp*B deletion is expected to maximize high quality T cells that avoid excessive reduction of T cell subsets after the initial proliferation of early effector T cells and avoid functional exhaustion during the late phase in order to induce protective memory.

There is no doubt that innate immune subversion is a primary activity employed by intracellular pathogens to prevent clearance from the infected host. Among these mechanisms, TLR2, TLR4, MyD88 are essential for clearance, and by extension the TIR domain containing proteins reportedly play pivotal roles in preventing clearance by

impairing the host innate immune response [60]. We propose that removing the genes encoding TIR domain-containing proteins from *Brucella* will enhance innate immunity induced by vaccination. This is built on several observations indicating increased innate immunity in the absence of these proteins [32]. In line with these observations, we hypothesized that *tcp*B deletion in LAV S19 is associated with increased expression of proinflammatory cytokines and reduction of bacterial burdens in murine macrophages. We constructed the S19Δ*tcp*B and *B. melitensis* 16MΔ*tcp*B mutants and demonstrated reduced intra-bacterial burdens in infected macrophages. We also hypothesized that this reduction could be partly associated with cytokine expression induced by S19Δ*tcp*B and S19 in murine macrophages. Indeed, we demonstrated elevated cytokine expression in macrophages infected with the S19Δ*tcp*B.

2.3 Materials and methods

2.3.1 Bacterial strains, culture conditions and plasmids for cloning

E. coli strains Top10 DHQ (Invitrogen) were used as hosts for plasmid purification. *E. coli* cultures were grown on Luria-Bertani (LB, Difco Laboratories) plates overnight at 37°C with or without antibiotic supplementation. Antibiotics were obtained from Sigma-Aldrich® and used at the following concentrations, kanamycin (100 mg/l), carbenicillin (100 mg/l) or chloramphenicol (50 mg/l). Frozen stocks were prepared in LB broth supplemented with 50% (v/v) glycerol and stored at -80°C. Various plasmids were utilized for cloning and electroporation purposes: pKD4 FLP/FRT, Km^R, pEX18Ap*sacB* Ap^R (the kind gifts of P. Schweizer), pBBR.MCS::Cm^R (the kind gift of

M. Roop) and pET His6 Sumo::Kan^{R} (Invitrogen™). RAW 264.7 cells (ATCC® TIB-71™) were cultivated in Dulbeco's minimum essential media (DMEM) (Life Technologies) containing 4.5 g/l glucose, 10mM L-glutamine, 10mM sodium pyruvate and 10% (v/v) fetal bovine serum (FBS) (Life Technologies) in an atmosphere containing 5% (v/v) CO_2 with 100% relative humidity. Passaged cells ($n \leq 15$) were harvested at 90% confluence in T75 flasks, counted and used for infection.

2.3.2 Construction of *Brucella abortus* S19ΔtcpB deficient mutant (S19ΔtcpB)

The S19Δ*tcp*B mutant was constructed as previously described with minor modifications [119]. Sequences upstream of the *tcp*B gene BAbS19_I07080 were amplified from *Brucella abortus* S19 genomic DNA using the primer pair 5'-GGAATTCATTTCGATACGGTGCTGGAC-3' (upstream EcoRI site) and 5'-AGCGGCGCGCCATTTGGTCAAGCGTTACCGAC-3' (downstream AscI site). The sequence downstream was amplified using the primer pair 5'-AATGGCGCGCCGCTCCTCACAATACGGGTGT-3' (upstream AscI site) and 5'-GGAATTCGGCTGACGACTTTCTCCTTG-3' (downstream EcoRI site). These two products were ligated to each other via overlapping PCR placing the AscI site at the junction of the upstream and downstream fragments. The overlap product was ligated into pUC9ApR using the unique EcoRI site to create a construct from pEX18AP containing sites flanking *tcpB* separated by a unique Asc1 site. Finally, a kanamycin resistance gene amplified from the plasmid pKD4 (Promega™) was ligated into the plasmid at the unique AscI site. This new construct was then used for electroporation

into *B. abortus* S19 and *B. melitensis* 16M. Candidate deletion mutants were identified based on kanamycin resistant growth and carbenicillin sensitivity and verified by PCR and DNA sequencing, using primers that flank the tcpB locus 5'-GGAATTCATTTCGATACGGTGCTGGAC-3' and GGAATTCGGCTGACGACTTTCTCCTTG-3. The resulting product was then subjected to electrophoresis. The product was isolated and DNA extracted using a commercial DNA extraction Kit (Qiagen). The resulting DNA was subjected to a sequencing cycle on a Thermocyler (Biorad) and run cyc-seq program. (96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 4 min) X 25 cycles then read using a Beckman coulter CEQ-800 genetic analysis DNA system.

2.3.3 Evaluation of bacterial growth and expression of *Tcp*B

B. abortus strains S19 and S19Δ*tcp*B from frozen stock were grown on solid media [tryptic soy broth (TSB) prepared according to the manufacturer's instructions (BD Difco™) supplemented with 1.5% (w/v) Bacto-Agar (BD Difco™)]. Colonies from these plates were used to inoculate TSB. Cultures on solid media or in broth were incubated up to 72 hours at 37°C. For determination of the growth curve, liquid cultures were grown to a similar OD, determined using a Klettmeter, equal samples from this culture were then used to inoculate study cultures. Liquid cultures were agitated sufficiently to prevent bacteria from forming a pellet. At selected times portions of the culture were removed and bacterial density determined via serial dilution and plating on TSA plates. For nucleic acid isolation, portions of the culture were removed and bacteria

recovered following centrifugation at 5000xg for 5 minutes at 4°C. RNA was extracted and purified using a commercial kit (Qiagen®) according to the manufacturer's instructions. Bacterial cDNA was prepared using primers designed to amplify *tcp*B transcripts using RNA to DNA EcoDry™ kit from Clontech®. Briefly, between 1 and 5μg of total RNA was diluted to a final volume of 20μl. Fifty mg of EcoDry premix was added and the mixture was briefly centrifuged to settle the contents and bring them into close proximity, prior to incubation for 60 minutes at 42°C. The reaction was stopped by heating at 70°C for 10 minutes.

2.3.4 Macrophage infections and survival assay (gentamicin protection assay)

Murine macrophage-like RAW264.7 cells (passage number 4-15) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, and 1mM nonessential amino acids. 2.5×10^5 cells were seeded into each well of a 24-well plate and incubated as described above. The cell monolayers were infected at a multiplicity of infection (MOI) of 200 CFU per cell unless stated otherwise. To synchronize the infection, the plates were centrifuged at 250xg for 5 min at room temperature. Following a 20-min incubation at 37°C, the cells were washed three times in DMEM medium and incubated for 1 h at 37°C in fresh DMEM supplemented with 100 μg of gentamicin per ml to kill extracellular bacteria. Following a 1 hour incubation, the monolayers were washed with DMEM to remove gentamicin, followed by cellular lysis with 0.5 ml of 0.5% (v/v) Tween 20 in sterile water to determine bacterial uptake (time zero). The colony forming units (CFU) per well were obtained by plating serial

dilutions on TSA plates with or without supplementation with 100 μg of kanamycin per ml, as needed. The outcome of bacterial uptake or invasion was presented as the $\log_{10}$ of the number of bacteria recovered per ml of solution. To assess intracellular growth of the bacteria, the concentration of gentamicin was reduced to 20 μg/ml and the cultures incubated up to 72 hours. Infected cells were lysed at selected time points post infection (p.i), and bacterial recovery determined. All assays were performed in triplicate wells, and the results represent the average of at least three independent experiments.

2.3.5 Analysis of cytokine responses from infected macrophages *invitro*

Murine macrophage-like RAW264.7 cells were maintained and infected with *Brucella* as described in the previous section and supernatants were collected at selected times post-infection. Briefly, late log phase cultures of *Brucella* strains **S19** and S19Δ*tcp*B were used to infect the cells at MOIs of 100, and the supernatants harvested from triplicate wells and stored at -20^0C. Analysis of cytokine secretion was performed using ELISA kits from eBiosciences^{TM} using plates coated with capture antigen at 20μg/ml (e.g. anti mouse IL12/23 or IL6) in coating buffer (1×PBS) following incubation overnight at 4°C. The plates were washed (5X) with 0.05% (w/v) Tween 20 in PBS (PBS-T) and blocked with 1X assay diluent (eBiosciences) for one hour at room temperature to prevent non-specific binding. Supernatants from the infected and control cells were added and incubated for two hours at room temperature to allow binding. The wells were washed as described above and 100μl/well biotin-labeled secondary antibody was added at 1-2 μg/ml and incubation was continued for one hour. The wells were

washed, and AVIDIN conjugated HRP added at 0.5-1.0 μg/ml and incubated for a further one hour. TMB substrate was added and reactivity was characterized following 15 minutes incubation at room temperature after which 50 ul of stop solution was added. Reactivity was quantified by absorbance at OD_{450} using an ELISA plate reader (Multiscan™, Thermolab Systems). Viability of cells was also determined by Cytotox 96® non-radioactive cytotoxicity assay with 90% viability acceptable (Promega).

2.3.6 Cloning and expression of histidine-tagged *Tcp*B

S19 cultures were grown to log phase, at which point they were inactivated by incubation at 68°C for 24 hours. The cells were pelleted and genomic DNA extracted using Qiagen® DNA isolation kit as per the manufacturer's instructions. TcpB specific primers (ATG TCT AAA GAG AAA CAA GCC and GAT AAG GGA ATG CAG TTC TTT CGC) were used to amplify the *tcp*B open reading frame (ORF). The amplified product was cloned into the plasmid pET-SumoHis to generate the construct pETSumo-*Tcp*B. Cloning of the *tcpB* ORF was validated by DNA sequencing, and the plasmid was then used to transform *E. coli* BL21 (DE3) cells (Invitrogen™). The transformed BL21 cells were transferred and grown LB supplemented with antibiotic when appropriate and 1 mM glucose, and expression was induced at log phase by the addition of IPTG (Thermo Fisher Scientific) to a final concentration of 1mM. Following incubation for an additional 3 hours at 37°C, the bacteria were pelleted by centrifugation for 20 minutes at 5000xg and the pellets were resuspended and lysed in 2×SDS. The denatured proteins were separated by electrophoresis on 12.5% (w/v) SDS-PAGE gels. The gels were

stained with 0.25% (w/v) Coomassie blue R-250 for 2 - 4 hours until the gel was a uniform blue color (Sigma-Aldrich®) and then destained in water. Expression of TcpB was confirmed via Western blotting as described below.

2.3.7 Purification of his-tagged *Tcp*B

The expressed *Tcp*B-sumo protein was purified using native conditions by the ProBond™ purification system according to the manufacturer's instructions (Life Technologies™). Briefly, cells were harvested from a 50 ml culture prepared as described in the previous section via centrifugation at 3000xg for 10 minutes in a Sorvall RC 6 C centrifuge rotor. The pellet was re-suspended in 8 ml native binding buffer (50 mM $NaHPO_4$ and 0.5 M NaCl) supplemented with 1mg/ml of lysozyme and incubated on ice for 30 minutes. The cells were then lysed on ice via sonication (with a Cole Palmer Ultrasonic processor Model GEX130) using six 10-second bursts at high intensity with a 10-second cooling period between each burst. The lysate was centrifuged at 3000×g for 15 minutes to pellet the cellular debris. His-tagged *Tcp*B was purified from the lysate using nickel chelating resin (Invitrogen) according to the manufacturers instructions. Briefly, the lysate was gently agitated with a nickel chelating resin after which the resin was allowed to settle by gravity or centrifugation at 800×g for 1 minute. Native wash buffer was then used to remove unbound protein in a series of five washes. The resin was then poured into a resin-column (Invitrogen™) clamped in a vertical position and bound protein eluted using native elution buffer (50mM NaH_2PO_4, 0.5M NaCl and 250mM Imidazole). The eluted protein was subjected to SDS PAGE

electrophoresis and stained with coomassie blue (Sigma-Aldrich) and subjected to western blot analysis. The eluted protein was stored at -20°C until needed for use.

2.3.8 Western blots for detection of his-tagged protein

Following purification of the protein, it was subjected to SDS-PAGE gel electrophoresis after which the protein was transferred to a PVDF membrane (Millipore). The membrane was blocked using blocking buffer (5% skimmed milk in 1XPBS) overnight at 4°C. The membrane was then washed 3 times in wash buffer (0.5% Tween 1XPBS), 5 minutes each. The membrane was then incubated with a 1:1000 dilution of mouse anti histidine antibody (Sigma-Aldrich) in 1% blocking solution overnight at 4°C. The membrane was then washed three times, 5 minutes each. The membrane was incubated with a 1:5000 dilution of secondary antibody HRPO-conjugated rabbit anti mouse IgG (Sigma-Aldrich) in 5% blocking buffer in TBST at room temperature for 1 hour. The membrane was then washed three times. For signal development the membrane was incubated in 5 ml of HRP-chemiluminescent substrate (Denville Scientific) Excess reagent was removed and the membrane covered in transparent plastic wrap. The signal was acquired using darkroom development techniques for chemiluminesence.

2.3.9 Removal of endotoxins from expressed proteins

Endotoxins were removed from expressed proteins using the proteospin endotoxin removal kit (Norgen, Biotek, CA) according to the manufacturers instructions described as follows. Briefly, the expressed protein sample was diluted to a final volume of 450 µL

using sterile, deionized water. 20 µL of Binding Buffer was added to the protein and the pH of the resulting solution was adjusted to 3.5 by addition of 2N HCl. The endotoxin binding column was then activated by adding the provided activating solution. The protein sample was then transferred to the activated column and a 1% volume of endotoxin removal solution added. The column was mixed gently by inversion and allowed to stand for 5 minutes following which $1/10^{th}$ volume of isopropanol was added. Following centrifugation at 14,000xg for 1 minute, the protein was eluted into 9.3µl of neutralizer solution in an elution tube by centrifugation at 14,000xg for 1 minute. The absence of endotoxin was confirmed using Toxin-Sensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, Scotch Plains, NJ)

2.3.10 Determination of uptake of *Tcp*B by RAW264.7 cells

RAW 264.7 cells (1×10^5) in DMEM media were seeded into each well of 24 well plates and incubated for 24 hours at 37°C. The DMEM media was replaced with media supplemented with 50µg/ml of His tagged protein and the plates were then incubated at 37°C for 2, 5, 24, 48 and 72 hours. At the specified time points, culture media was aspirated, and the wells were rinsed 5 times with PBS. Adherent cells were harvested into the same solution by scraping and the cell suspension transferred to 1 ml tubes and centrifuged to collect the cells. Cells were lysed with 2×SDS-PAGE sample buffer and proteins resolved on SDS PAGE gel in the presence of His tagged-*Tcp*B via Western analysis using anti-His antibody conjugated with HRP as described above

2.3.11 Complementation of the *tcpB* mutation

To complement the *tcp*B defect, the His-tagged protein was purified using a nickel column system as described above (section 2.3.9). The concentration of the purified protein was determined using the Bradford assay [129]. *Tcp*B protein was added to RAW264.7 cells growth medium at a final concentration of 50 ug/ml and uptake of the protein was monitored as originally described by Radhakrishnan et al. [35]. The cells were washed 5 times with 1×PBS following which they were lysed with 2×SDS buffer. The samples were subjected to electrophoresis via SDS-PAGE and the separated proteins were transferred to PVDF membrane and probed using mouse anti-His antibody as described above. Cells were pre-incubated with the protein prior to infection with the *B. abortus* Δ*tcp*B mutant; the effect on replication was determined using the gentamicin protection assay described previously.

2.3.12 Determination of viability of cells (cytotoxicity assay)(Promega)

Supernatants were collected from experimental wells in the assays from the 24 wells plates and stored at -20^{0}C until ready for use. Lactate dehydrogenase (LDH) levels in the medium were measured using the cytotox 96 LDH kit (Promega) according to the manufacturer's instructions. Briefly, supernatants were equilibrated at room temperature and 100 ul added to wells in a 96 well microtiter plate. The provided cytotox 96 reagents was then prepared and added at 1:1 to the cell culture supernatants. The assay plate was incubated for 30 minutes at room temperature protected from light. 50ul of stop solution was then added to each well of the plate. The absorbance was read at 430nm. The

percentage cytotoxicity was determined as percentage experimental LDH release release/Maximum LDH release. Maximum release was determined by addition of LDH lysis reagent provided in the kit.

2.4 Statistical analysis

Bacterial burdens in cell culture were expressed as $\log_{10}$ CFU/ml +/- standard deviation (SD). Immune responses of infected cells were expressed in the form of cytokine concentration were obtained by plotting ODs against concentrations obtained from kit standards. Concentrations were then plotted against time post inoculation. Statistical comparisons between groups was done by use a two way ANOVA followed by a Student's *t* test to determine the statistical validity of observed differences between experimental data sets. P values ≤ 0.05 were considered to be significant.

2.5 Results

2.5.1 Construction of S19ΔtcpB and *B. melitensis* 16MΔtcpB deletion mutants

Primers were designed to amplify a 500bp region flanking the *tcpB* gene on the 5' end and a 300bp region flanking the *tcp*B gene on the 3' end. The two segments were then joined in an overlap extension PCR with adjoining AscI restriction site between the fragments. The resulting PCR product was ligated into the pEX18Ap plasmid. A kanamycin resistance cassette was amplified from a plasmid pKD4 and inserted into the AscI site. The resulting construct was electoporated into S19 and homologous recombination between the genome and the plasmid-encoded Δ*tcp*B (Km) results in the

replacement of *tcp*B gene. The mutants were identified by resistance to kanamycin and sensitivity to ampicillin, i.e., exclusion of co-integrates and designated as *Brucella abortus S19 Δtcp*B(Km) or S19*ΔtcpB*(Km) (see Fig. 1)

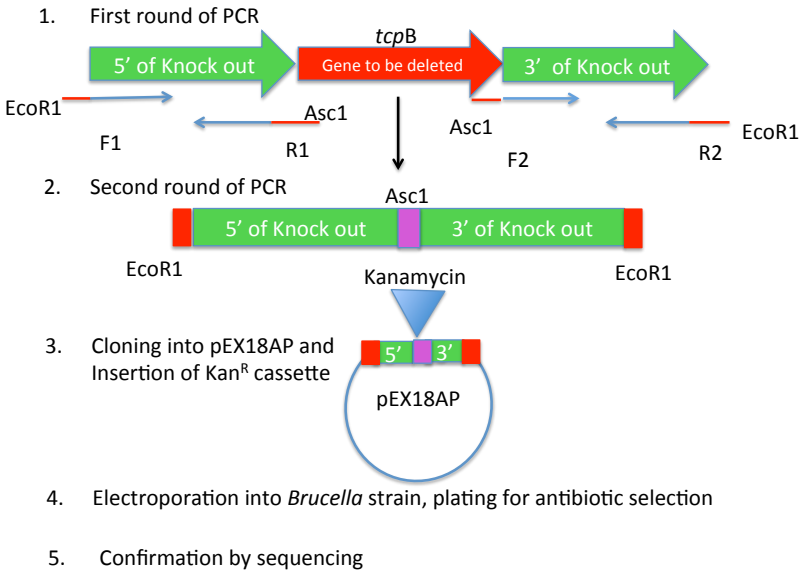


Fig. 1. Schematic representation of the method of construction of *tcp*B mutant strains. Two fragments flanking the *tcp*B gene were amplified in the first round of PCR using primers F1, R1 and F2, R2 with EcoR1 and Asc1 unique restriction sites as shown on the diagram. The fragments were then joined in the second round of overlap extension PCR, creating a unique Asc1 restriction site. The resulting product was cloned into the plasmid pEX18AP and a kanamycin resistance cassette was inserted between the two PCR fragments, and the resulting product used to electroporate *B. abortus S19* and *B. melitensis* 16M. Marked mutants *B. melitesis* Δ*tcpB*:Kan and *B. melitensis*Δ*tcp*B:Kan were formed by homologous recombination

2.5.2 Validation of the Δ*tcp*B deletion

Primers were designed to amplify a region encompassing the 700 bp region at the 5' end of the *tcpB* locus and a 500 bp region at the 3' end. PCR amplification of the template from the Δ*tcpB* mutant yielded a 2.8 kb fragment due to the insertion of a 1.5 kb kanamycin cassette in addition to the flanking fragments. Amplification of the DNA template from S19 yielded a smaller 1.8 kb fragment. The two fragments were then sequenced to confirm deletion of *tcpB* observed (see Fig. 2 & 3)

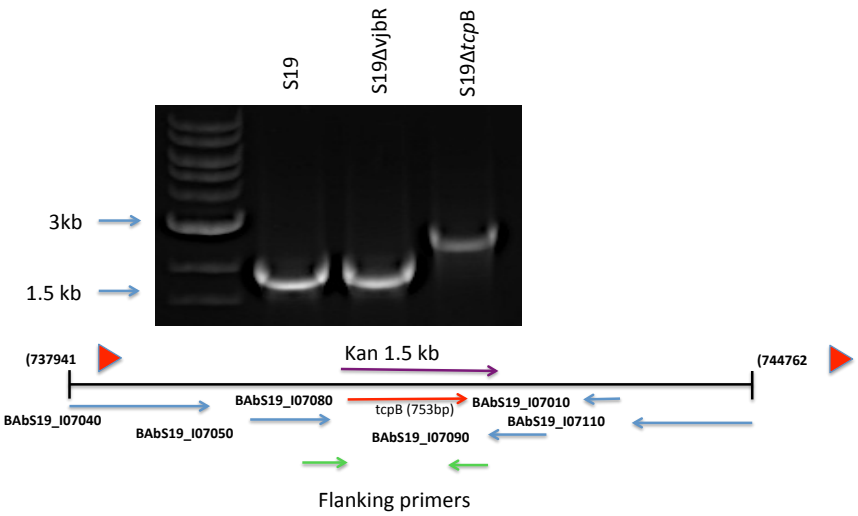
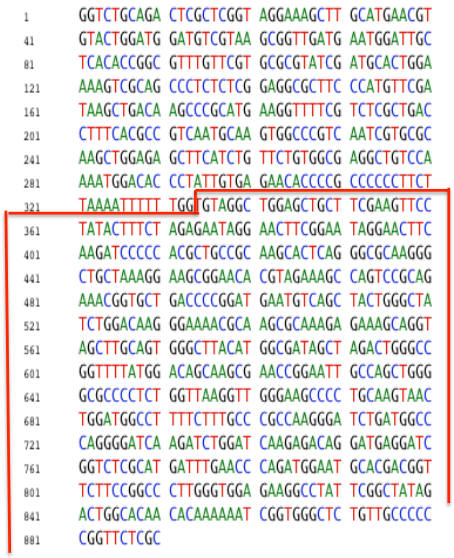
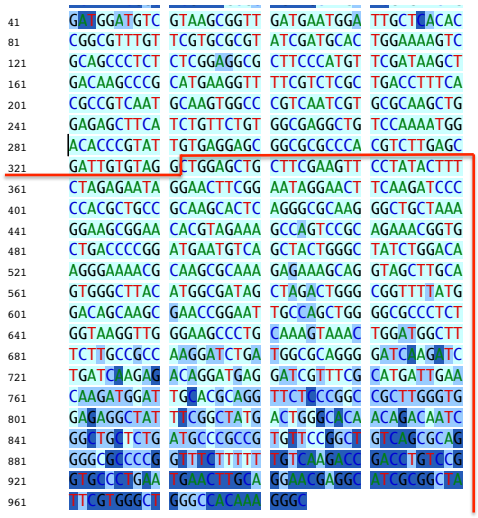


Fig. 2. PCR validation of S19*ΔtcpB* mutants. PCR products from genomic DNA extracted from *Brucella* strains S19, S19*ΔvjbR,* and S19*ΔtcpB* were amplified with primers flanking the *tcp*B gene. (S19Δ*vjb*R was created by deletion of the *vjb*R gene [119]). Lower panel, Genetic map of *B. abortus* S19, showing the location of the *tcp*B gene (S19_I07090) and the neighboring genes. To create the mutants, a kanamycin cassette (Kan 1.5 kb) displaced the *tcp*B (tcpB 753 bp) gene by homologous recombination as indicated in the diagram.



a)

Fig. 3 a). Sequences of 3'junction between *tcp*B mutants and kanamycin cassette in *B. abortus* S19. S19Δ*tcp*B genomic DNA showing the 3' junction of insertion of Kanamycin resistance cassette (pKD4). PCR products for S19ΔtcpB and 16MΔtcpB were cut from the gel and DNA extracted using a commercial kit (Qiagen). Specific primers were used to sequence the extracted DNA and data acquired using a Beckman coulter CEQ-800 genetic analysis DNA system.



b)

Fig. 3 b) Sequences of 3'junction between *tcp*B mutants and kanamycin cassette in *B. melitensis 16M*Δ*tcp*B. *B. melitensis 16M*Δ*tcp*B genomic DNA showing the 3' junction of insertion of Kanamycin resistance cassette (pKD4). PCR products for S19Δ*tcp*B and 16MΔ*tcp*B were cut from the gel and DNA extracted using a commercial kit (Qiagen). Specific primers were used to sequence the extracted DNA and data acquired using a Beckman coulter CEQ-800 genetic analysis DNA.

2.5.3 Growth of S19, S19ΔtcpB, *B. melitensis* 16M and 16MΔtcpB in TSB media

Replication of S19Δ*tcp*B and S19 was characterized as normal sigmoidal growth curves with defined lag, log and plateau phases indistinguishable from the parental strain. It was therefore concluded that deletion of *tcp*B had no effect on replication of the organism outside of the host under the conditions described (Fig. 4).

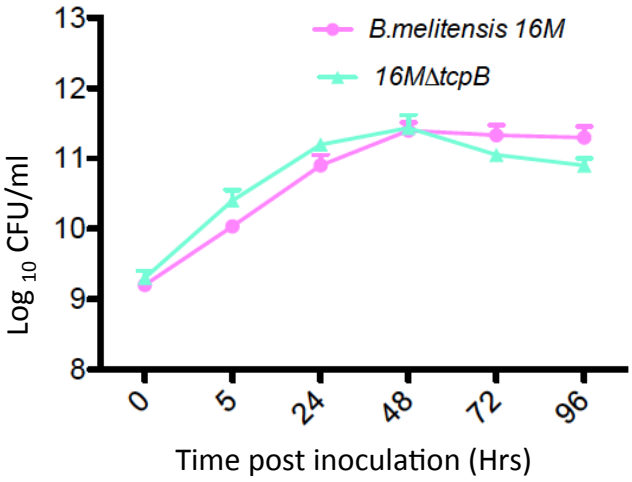


Fig. 4 a). Growth of *Brucella melitensis* (16M and 16MΔ*tcp*B) strains TSB media. *B. melitensis* 16M and *B. melitensis* 16MΔ*tcp*B in rich culture media (TSB). 5 ml of TSB media were inoculated with equal cultures of *B. abortus* S19 and S19Δ*tcp*B or *B. melitensis* 16M and *B melitensis* 16MΔ*tcp*B, and at specified intervals, samples were collected from the cultures and platted on TSA plates. The plates were incubated for 3 days at 37^0C, after which the colonies were counted and documented The cultures were grown until late plateau phase at which point culture viability was on the decline. Results shown are one of three independent experiments. Bacterial loads are expressed as Log $_{10}$CFU/ml +/-SD.

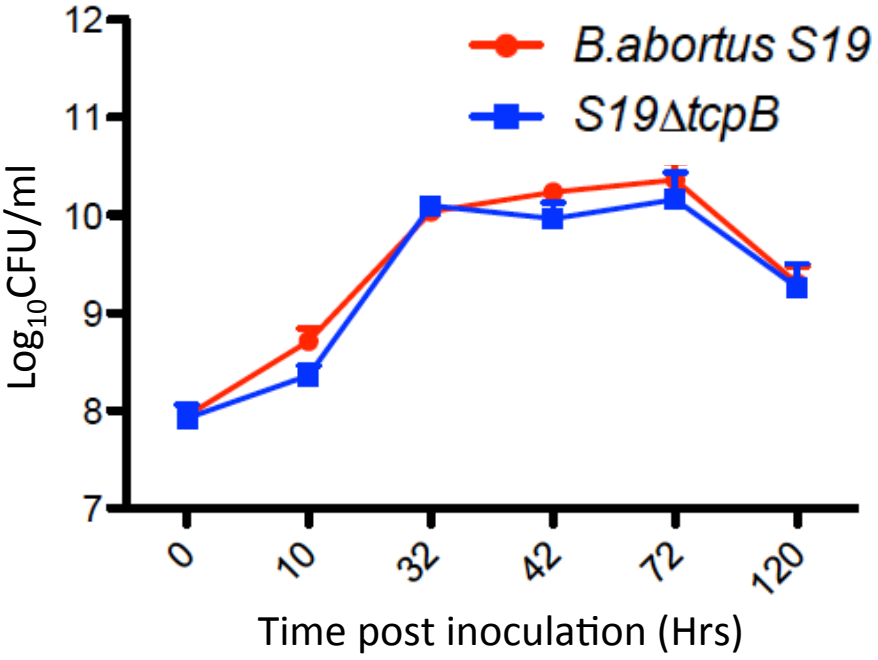


Figure 4 b) Growth of *Brucella abortus (*S19 and S19Δ*tcp*B) strains in TSB media. *B. abortus* S19 and S19Δ*tcp*B and in rich culture media (TSB). 5 ml of TSB media were inoculated with equal cultures of *B. abortus* S19 and S19Δ*tcp*B or *B. melitensis* 16M and *B melitensis* 16MΔ*tcp*B, and at specified intervals, samples were collected from the cultures and platted on TSA plates. The plates were incubated for 3 days at 37^0C, after which the colonies were counted and documented The cultures were grown until late plateau phase at which point culture viability was on the decline. Results shown are one of three independent experiments. Bacterial loads are expressed as Log $_{10}$CFU/ml +/-SD.

2.5.4 Cloning, expression and purification of his-tagged *Tcp*B

For complementation studies, tcpB was cloned and expressed from the plasmid pET SUMO (Champion™, Life Technologies®). SUMO-CAT was used an expression control. Primers were designed and used to amplify the full ORF of *tcpB* from S19

genomic DNA (see section 2.2.8). The PCR product was then cloned into pET SUMO to create pET SUMO-*tcpB*. The plasmid was then used to transform BL21 cells. The cells were grown in LB medium and induced by the addition of IPTG. The proteins were purified, expressed and analyzed as described materials and methods (Fig 5).

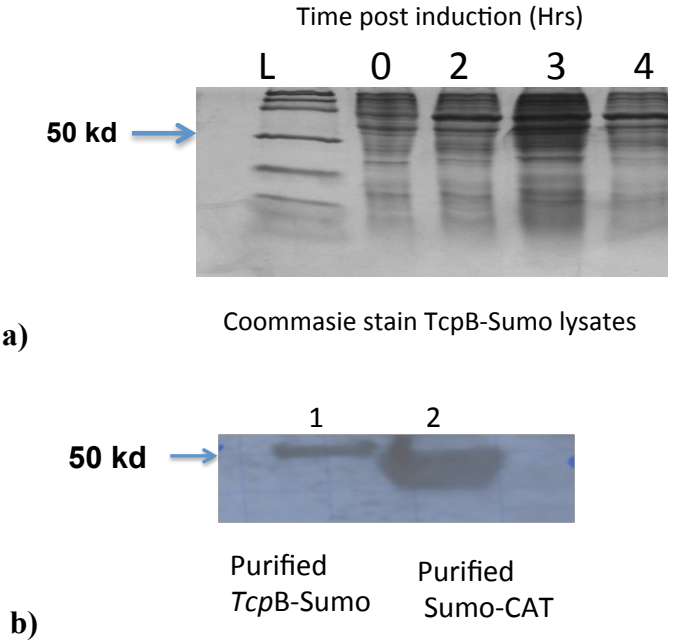
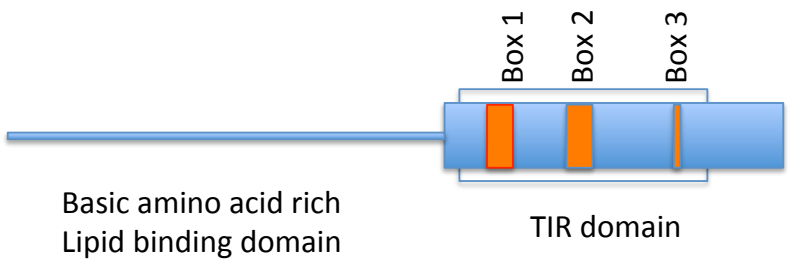


Fig. 5. Cloning and expression of *Tcp*B. a) SDS PAGE gel images of coomassie blue stain of TcpB at various times of induction. Lanes 1 (ladder) 0, 2, 3 and 4 induced lysates at 0, 2, 3 and 4 hours respectively. b) Western blots stain for TcpB-sumo (lane 1) and Sumo-CAT (lane 2).

2.5.5 Uptake of *Tcp*B by RAW 264.7 cells

The structure of TcpB depicts an N terminus with a basic amino acid rich lipid-binding domain and the C-terminus encodes a TIR domain (Fig. 6a) [35]. *Tcp*B harbors endogenous cationic motifs conferring cell permeable properties. The cationic amino acid residues bind to the cell surface trapping the protein, and the protein is then taken up by the cellular endocytic pathway (Fig 6b) [35]. In this study, we sought to analyze the internalization of His-tagged *Tcp*B by RAW264.7 cells. Murine macrophages were incubated with purified with His-tagged *Tcp*B at a final concentration of 50μg/ml and verified the translocation of *Tcp*B was verified by western blotting. Sumo-*Tcp*B could be located in the cell, while internalization was not observed in cells incubated with the Sumo-CAT control protein indicating that the cell permeable property is attributed to TcpB (Fig 7). Sumoylation is needed for proteins to enter stable complexes or functional states enhancing solubility and expression, but is dispensable after the initial establishment [130].



6a)

M S K E K Q A Q S K A H K A Q Q A I S S A K S L S T Q K S K M S E L E R A T R

D G A A I G K K R A D I A K K I A D K A K Q L S S Y Q A K Q F K A D E Q A V K

K V A Q E Q K R L S D E R T K H E A F I K Q S L S S M R T T A S A T M E A E E E

Y D F F I S H A S E D K E A F V Q D L V A A L R D L G A K I F **Y D A Y T L K V**
-------box1------- --------box 2-------
G D S L R R K I D Q G L A N S K F G I V V L S E H F F S K Q W P A R E L D G L

T A M E I G G Q T R I L P I **W H K V S Y D** E V R R F S P S L A D K V A L N T S L

K S V E E I A K E L H S L I

6 b)

Fig. 6. Schematic representation and protein sequence of *Tcp*B.
a) Schematic representation of the *Brucella* tcpB protein showing the position of the TIR domain, TIR boxes and the lipid binding domain. b) Protein sequence of *Tcp*B showing the location of basic amino acids (Lysine, arginine, histidine) (blue) and the TIR domains (red)

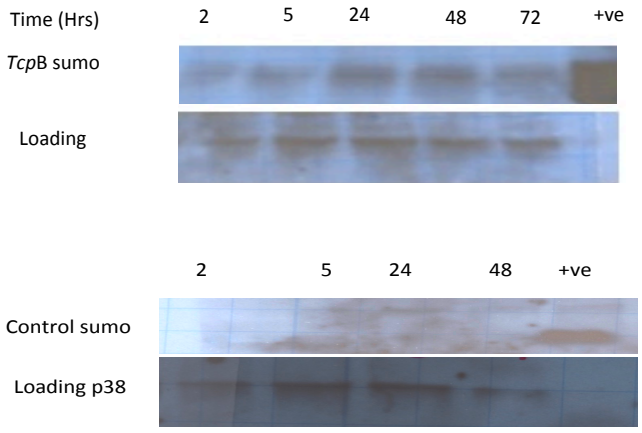
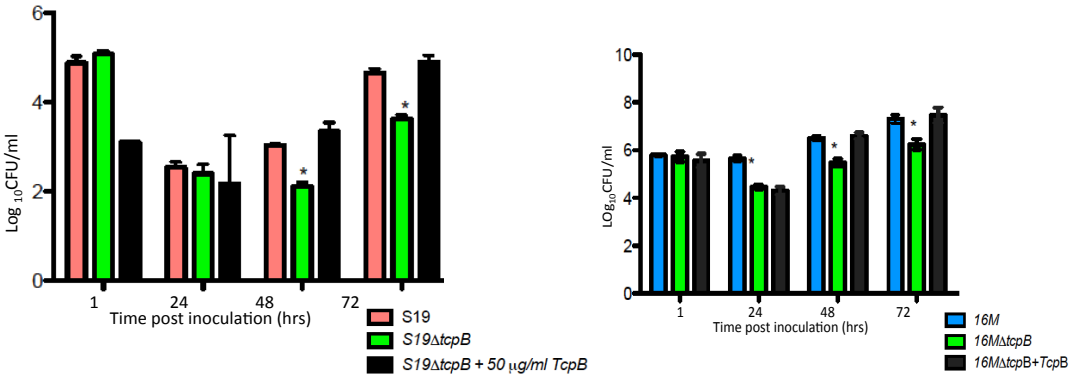


Fig. 7. Uptake of *Tcp*B By RAW264.7 macrophages.
Western blot analysis of RAW264.7 cells pre-incubated with purified *Tcp*B-Sumo or Sumo. Raw264.7 cells were incubated with 50 μg/ml of tcpB-sumo or Sumo-CAT and samples collected at various time points as described in materials and methods. The samples were subjected to Western blot analysis using mouse anti-His antibody.

2.5.6 The effect of *tcp*B deletion on intramacrophage burden of *Brucella*

Previous studies have indicated that *Brucella* Δ*tcp*B mutants exhibit little change in intracellular survival relative to the parental organism. However, a proposed Δ*tcp*B deletion from *Brucella abortus* S19 vaccine may offer an opportunity to enhance overall immune protection in a genetic background associated with reduced virulence, safety and genetic stability. To determine the effect of the *tcpB* deletion on the virulence of S19, the rate of growth of the mutant relative to the parent strain was evaluated *in vitro* in the murine leukemic monocyte-derived macrophage cell line RAW264.7. The cells were infected with either parental S19, or the S19Δ*tcp*B mutant, and the parental *B. melitensis* 16M or the 16MΔ*tcp*B mutant, and evaluated for the rate of replication and intramacrophage bacterial burdens evaluated at defined time points. For

complementation studies, the RAW264.7 cells were pre incubated with 50μg/ml of purified *Tcp*B for 24 hours. No significant differences in invasion or intracellular replication were noted between S19 and S19Δ*tcp*B mutant at 24 hours post infection. However, there were reduced bacterial burdens in macrophages infected with the mutant strains at 48 hours and 72 hours post infection ($p<0.05$). Similarly 16MΔ*tcp*B showed reduced intramacrophage burdens at 24, 48 and 72 hours post infection (see Fig 8) ($p<0.05$).



a) b)

Fig. 8. Evaluation of replication and intramacrophage burden of *Brucella* strains. a) S19 and S19Δ*tcp*B. b) *Brucella melitensis* 16M and 16MΔ*tcp*B. RAW264.7 cells were infected with S19 or S19Δ*tcp*B or B.m16MΔ*tcp*B. Bacteria burdens were determined by lysis with 0.5% (v/v) Tween 20 in water and plated on TSA plates. For complementation studies, RAW264.7 cells were pre-treated for 24 hours with *Tcp*B protein at a concentration of 50 µg/ml prior to infection. All experiments were done in triplicate and results are a representation of three independent experiments. Bacterial burdens were represented as the mean Log_{10}CFU/ml ±SD. Differences between groups were determined by analysis of variance (ANOVA) using Prism software (GraphPad). A p value <0.05 was considered statistically significant.

2.5.7 Cytotoxicity of RAW264.7 cells infected with *Brucella* strains

RAW264.7 cells infected with S19 or S19Δ*tcp*B were monitored for cytotoxicity by the cytotox96® non-radioactive cytotoxicity assay (Promega). While the cells manifested a cytotoxity of between 5-15% over the period of the experiment, there was no significant difference observed in cytotoxicity between cells infected by either S19 or S19Δ*tcp*B (Fig. 9).

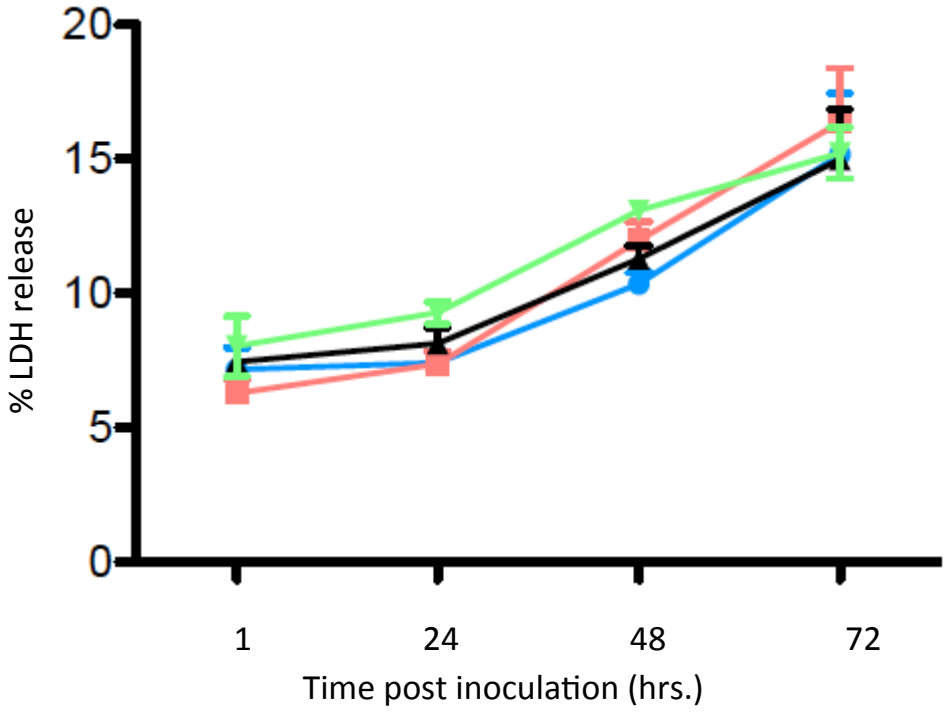


Fig. 9. LDH release by RAW264.7 cells infected with S19 or S19Δ*tcp*B. Supernatants were collected from RAW264.7 cells infected with S19, S19Δ*tcp*B and S19Δ*tcp*B+*Tcp*B. Cytotox96 non-radioactive assay was used to determine the levels of LDH released by the cells. ODs were measured at 530nm and % cytotoxicity calculated as LDH release release/Maximum LDH release. Percent cytotoxicity is expressed as mean of three results ±SD.

2.5.8 *tcp*B deletion enhances proinflammatory cytokine production in macrophages in culture

Brucella Δ*tcp*B mutants induced increased cytokine production (IL-1ß and TNFα) relative to the parental organism in IRF$^{-/-}$ mice [86]. To characterize cytokine production, RAW264.7 cells were infected with S19 and S19Δ*tcp*B at an MOI of 100. Supernatants were collected from the infected monolayers and sandwich ELISA was

performed to quantify cytokine expression. Consistent with the prediction based on the enhanced translocation of NF-κB and AK-1 activity, macrophages infected with S19Δ*tcp*B exhibited significantly elevated IL12 p40 at 72 hours and IL6 cytokine production at 48 and 72 hours in comparison to those infected with S19 and uninfected controls as shown in Fig. 10.

a

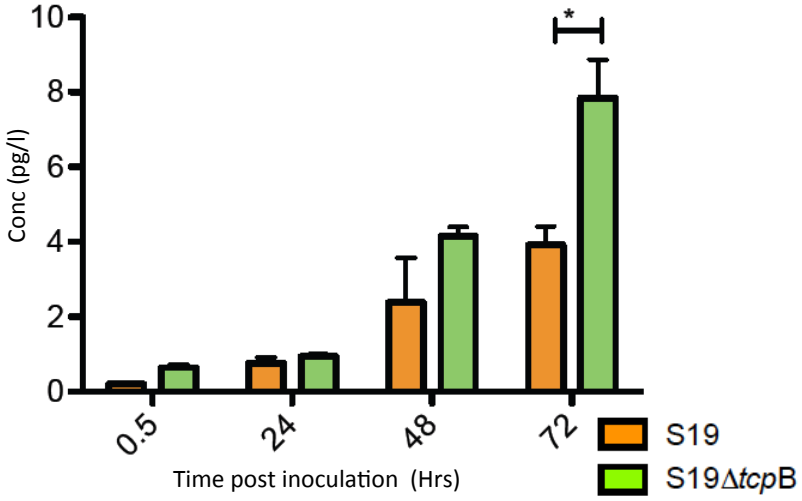


Fig .10. Expression of cytokines by RAW264.7 cells infected with *Brucella* strains. a) IL-12p40 and b) IL 6 by RAW 264.7 macrophages infected with S19 or S19Δ*tcp*B. Culture supernatants were collected and analyzed for the presence of IL-12p40 via sandwich ELISA. Data represent one of three independent experiments. Concentration levels are cytokine concentrations expressed as the mean pg/ml±SD plotted over time for each group. A p value <0.05 is considered significant. The significance differences between groups were determined by analysis of variance (ANOVA) using Prism software (GraphPad).

b

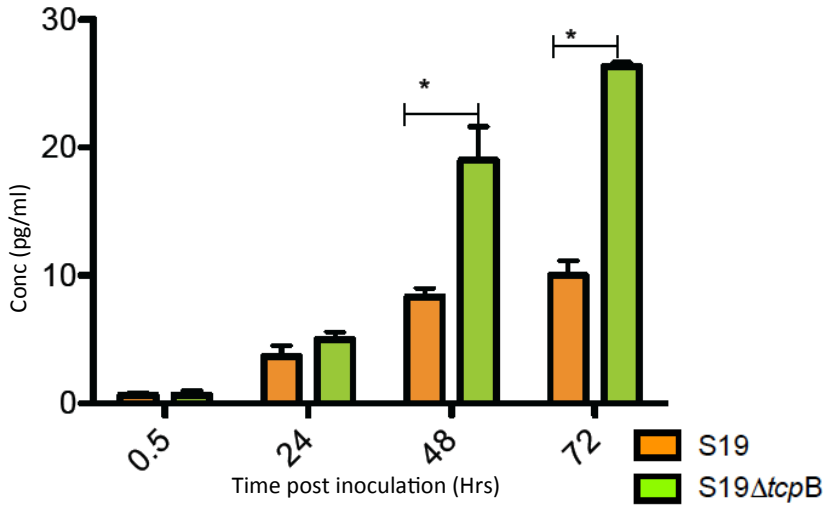


Fig. 10 continued

2.6 Discussion

Brucella invades and replicates in cells of the reticuloendothelial system, especially macrophages, dendritic cells and placental trophoblasts. Resistance to killing mechanisms expressed by these host cells has been suggested to be the factor controlling *Brucella* invasion, survival, persistence and virulence in the infected host. Generally referred to as a stealth pathogen, the *Brucella* elicits a weak innate immune response derived in part from the poor activation of Toll like receptor pathways. Additional mechanisms include expression of effectors that subvert downstream pathways to reduce the innate immune response. One such factor is the Toll interleukin receptor (TIR) domain containing protein *Tcp*B. Based of the published evidence of immune subversion, the *tcp*B gene was chosen as target of interest for deletion in a rational approach to improve *Brucella* LAV. *Tcp*B expression by *Brucella* has been suggested to abrogate TLR mediated MyD88 signaling thus limiting the host innate and subsequent

adaptive immunity that is essential in the control of brucellosis. In our experiments, constructed the *tcp*B mutant in S19 and *B. melitensis* 16M, we then evaluated the role of t*cp*B in *Brucella* infection, survival and replication in RAW264.7. Our experiments have shown that deletion of *tcp*B does not affect the growth rate of *Brucella* TSB media, indicating that the cultural characteristics of *Brucella* following *tcp*B deletion are unchanged outside the host (Fig 4). The results of intramacrophage replication however showed reduced bacterial burden of the mutant organism at about 48 and 72 hours post infection (Fig 9). We concluded that reduction in intramacrophage burdens was due to the interaction between tcpB and the host factors. We further examined the effect of tcpB mutation on *Brucella* induced host expression of proinflammatory cytokines (IL12 and IL6). It is therefore concluded that deletion of *tcp*B deletion is associated with increased proinflammatory activity, a mechanism that is proposed to enhance macrophage killing and reduced survival of *Brucella* as manifested by a reduction in bacterial burden. Interleukin 12 stimulates intracellular expression of IFN-γ by macrophages and dendritic cells via activity of transducer and activator of transcription 4 (STAT4) skewing the macrophage function toward the M1 phenotype [131]. M1 macrophages are characterized by increased microbicidal activity, due to secretion of high levels of pro-inflammatory cytokines such as TNFα and IL-6, production of reactive oxygen intermediates (ROI) and nitric oxide synthase-2 (NOS-2/iNOS), high antigen-presenting activity and increased production of IL-12 [132]. As the strong T-cell stimulating potential of the smooth attenuated *B. abortus* S19 *in vivo* has been documented before [133], we were interested in the possible exploitation of this

mechanism to enhance its vaccine potential. We hypothesized that increased inflammation may be exploited in the design of more effective *Brucella* vaccines. We exploited the immune upregulating role of a *tcp*B deletion to design a vaccine with higher efficacy. This study alone supports the possibility that a rationally designed *Brucella* vaccine may enhance the proinflammatory response to provide an adjuvant effect producing Th1 cytokines that drive the activation of effector and memory T cells. We also propose that a drawback to this increased proinflammatory response is the potential for enhanced pathology such as splenomegaly and will require additional animal trials to reconcile.

CHAPTER III

*TCP*B DELETION CONFERS IMPROVED IMMUNOGENICITY AND PROTECTIVE EFFICACY TO *BRUCELLA ABORTUS* S19 (S19)

3.1 Introduction

Brucella melitensis, B. abortus and *B. suis* are the three main etiological agents for human brucellosis. Their preferred natural hosts are sheep and goats, cattle, and swine, respectively [134.]. Although, live attenuated vaccines (LAVs) provide the best protection against challenge, their use is restricted due to several side effects including residual human virulence, and abortion in ruminants posing direct threats to public health [135,136]. Several *Brucella* strains have been attenuated and used as improved vaccines with varying immunogenicity and safety. S19, a spontaneously occurring attenuated form of *B. abortus,* has been used in the control of brucellosis in the U.S. and other parts of the world [137]. S19 is the vaccine of choice for the introduction of genetic attenuating mutations to enhance safety and reduce residual virulence for use in animals and to limit human exposure. This is supported by: i) previous evaluation in humans, ii) genetic stability in a wide variety of hosts, iii) cross protection against *B. melitensis,* the most prevalent cause of human brucellosis, and iv) greatly reduced human virulence [3,138-140]. Similarities in immune response with wild type strains also prevent differentiation of infected animals from S19 vaccinated animals (DIVA). To further improve the safety and efficacy of S19, the vaccine has been genetically modified by identifying and deleting virulence genes to provide vaccine strains with

significant immunogenic activity and enhanced protective efficacy while protecting the health and safety of the target species and the public [31,141]. *Brucella* TIR proteins bear significant homology to mammalian TIR [33,142]. Two TIR domain-containing proteins, *Tcp*B/Btp1 and Btp2 have been identified in *Brucella* spp. [32,33]. *Tcp*B has been shown to inhibit TLR2 and 4 mediated activity in HEK293 cells, and to bind to and promote the degradation of TIRAP resulting in abrogation of NF-kB mediated expression of proinflammatory cytokines [115]. This was validated in an experiment in which the secretion of TNF-α and IL-12p40 by bone marrow derived dendritic cells was impaired upon infection with wild type *B. abortus* in comparison with TcpB deficient strain [115]. Subcellular localization studies have indicated that *Tcp*B co-localizes with the plasma membrane and microtubules by binding to phosphoinositides [PI $(4,5)P_2$] [86]. It has been proposed that the binding of TcpB to PI $(4,5)P_2$ leads to a disruption of the action of the mTOR signaling pathway which limits effector $CD8^+$T and $CD4^+$T cell transition to long lived memory T cells [31,75]. As a result, infection with virulent *Brucella* has been proposed to restrict the number of T cells differentiating into memory cells, resulting in a lack of protective memory and reduced immune protection [36]. *Tcp*B plays an important role in reducing the *Brucella* induced secretion of cytokines as we have shown in our prior *in vitro* experiments in which proinflammatory cytokines (IL6 and IL12) were elevated levels in macrophages infected with *Tcp*B deficient strains of *B. abortus* S19. This agrees with an experiment in which cytokine profiles showed elevated levels of IL-1ß and TNFα in $IRF^{-/-}$ mice infected with a *Tcp*B deficient strain of *Brucella* [86]. As a proinflammatory cytokine, TNF-α drives secretion of IL-12 and

IFNγ by dendritic cells and macrophages, directing an acquired secondary response of the Th1 phenotype by $CD4_+$ and a Tc 1 phenotype by $CD8^+$ lymphocytes [143]. The Th1 immune response against *Brucella* leads to IFNγ secretion by antigen-specific T lymphocytes mostly CD4+T cells which have been shown to be the major producers of IFNγ [144]. IFNγ also activates the bactericidal action of macrophages, expression of antigen presenting and co-stimulatory molecules on antigen presenting cells, CTL mediated cytotoxicity, and potentiates apoptotic death of infected macrophages [145] (Fig 9). Recent data obtained in IFNγ and IL-12/β2-microglobulin knockout mice demonstrate that the lack of endogenous IFNγ is more important to the control of brucellosis than CTLs [126]. This underscores the crucial roles played by IFN-γ producing CD4+ T cells in the control of brucellosis. However, in chronic brucellosis T cells display an immune deficit that is associated with *Tcp*B, which inhibits CTL killing and memory T cell differentiation, constituting a novel effector of immune evasion [75]. *Tcp*B has also been associated with increased endoplasmic reticulum (ER) stress. TcpB, however, inhibits MyD88 mediated ER stress by abrogating the XBP-1 splicing, causing reduced cytokine expression [44]. *Tcp*B-deficient *B. melitensis* mutants have been observed to have deficient dissemination in $IRF^{-/-}$ mice [75]. In this study, we hypothesize that deletion of TcpB in the live attenuated vaccine, *B. abortus* S19, will improve innate as well as adaptive immunity resulting into enhanced protective efficacy. On vaccination, *Brucella* LAV are phagocytized by macrophages and dendritic cells where they are contained in the BCV [146]. *B. abortus* S19 activates the TLRs of the phagocytizing cells and causes induction of proinflammatory cytokines expression of

IL6 and IL12 [147]. IL12 acts on Th0 cells causing them to differentiate into Th1 cells that are crucial for expression of IFNγ [148]. IFNγ then activates intramacrophage killing of *Brucella* in addition to activating the cytotoxic activity of CD8 T cells [149]. Antigen presentation by infected macrophages to $CD4^+$ T cells in the context of MHC class II also leads to activation, differentiation, and subsequent effector functions including expression of IL2 that leads to clonal expansion of CD4+ and CD8+ T cells. IL6 produced by activated macrophages acts on regulatory T cells (T regs) suppressing their expression of Th2 cytokines [150]. The net result of vaccination with *B. abortus* S19 is up regulation of the Th1 response, (fig 11), a feature that is lacking in wild type *Brucella* that are responsible for activation of lowered Th1 phenotype and persistence of infection.

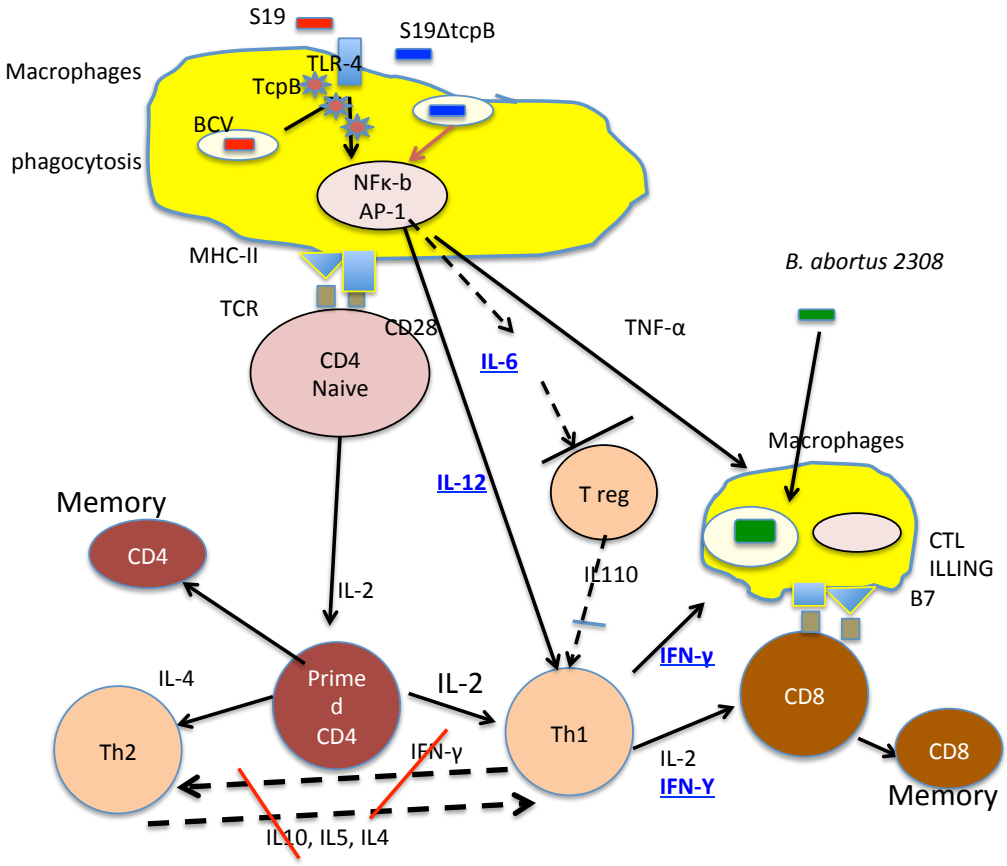


Fig. 11. Immunity in *Brucella* vaccination. Following vaccination, *Brucella abortus* S19 (red rectangles) are phagocytized by macrophages or other antigen presenting cells. *Brucella* are maintained in BCV where they replicate and avoid lyosomal degradation. Infected APCs traffic to the local LN where, *Brucella* antigens are processed and antigens are presented to naïve T cells in the context of MHC class II. The activated APCs produce IL-12 that skews the T cell phenotype to Th1. The Th1 cells then produce IFN-γ that activates macrophages to kill intracellular bacteria. IFNγ also activates the cytotoxic activity of $CD8^+$ T cells, which kill infected cells that present antigens in the context of MHC class I. IFN-γ produced by Th1 cells also down regulate Th2 cells. Activated macrophages also express IL-6, a cytokine that inactivates Tregs. This reduces IL10 production by T regs, hence enhancing differentiation of Th1 cells. S19 expresses *Tcp*B that binds to TIRAP and MyD88, abrogating NF-κb/AP1 mediated expression of pro inflammatory cytokines. S19Δ*tcp*B does not express *Tcp*B hence, no abrogation of NF-κb/AP1 mediated cytokine expression. Vaccination with S19ΔtcpB (Blue rectangles) is expected to up regulate NF-κb/AP1 mediated cytokine expression, adaptive immunity and protection against infection by wild type *Brucella* (green rectangles).

3.2 Materials and methods

3.2.1 Bacterial growth, immunization and challenge

Frozen aliquots of the vaccine preparations (*Brucella abortus* S19 *Δtcp*B and *Brucella abortus* S19*)* were obtained from -80°C storage and streaked on TSA plates. After three days growth, approximately 100 colonies of the vaccine preparations were grown to log phase. The bacterial were then adjusted to 10^6 CFU by use of a kletmemeter (Klett-summersson photoelectric colorimeter, industrial model 800-3) and resuspended in 100 ul of sterile PBS. At the time of immunization, the actual CFU inoculated was determined by serial dilution plate counts of the inoculum on TSA plates and counting the resultant colonies.

3.2.2 Mouse immunization and challenge

Eight-week-old male C57BL/6 female mice were purchased (Jackson Labs, Bar Harbor, ME) and housed in approved facilities under specific pathogen-free conditions. All experimental procedures and animal care were performed in compliance with humane approved Texas A&M University institutional animal care regulations (AUP 2012-156). Mice were randomly distributed into groups of five per cage and immunized via intraperitoneal inoculation with a single dose of vaccine containing $5x10^5$ CFU of *B. abortus* S19 or the TIR-deficient mutant strain S19Δ*tcpB* suspended in PBS. Control groups were inoculated intraperitoneally with PBS alone. Following vaccination, mice were monitored for 8 weeks prior to intraperitoneal challenge with 10^5 CFU wild type *B abortus* 2308. One week post-challenge, animals were placed in a chamber and 100%

carbon dioxide introduced at a fill rate of about 10% to 30% of the chamber volume per minute to render the mice unconscious This was followed by cervical dislocation. Spleens were harvested, weighed and homogenized in 1 ml of PBS using an Omni prep-sample homogenizer model 06-021 (Omni international). The sample were serially diluted and plated in duplicate onto Farrell's media. Three to five days post-inoculation, bacterial counts were enumerated. Results are represented as the mean CFU per gram of tissue ± SEM. Vaccine efficacy was evaluated by determining the $\log_{10}$CFU/gram of tissue of intraperitoneal challenge bacteria recovered from spleens following tissue homogenization and plating. The lower limit of detection was ≥5 CFU. Comparisons between groups were made by ANOVA followed by a Student's *t* test. A p value <0.05 was considered statistically significant

3.2.3 Determination of *Brucella* specific IgG2A and IgG1 levels

The levels of *Brucella* specific antibody in sera of mice vaccinated by S19, S19Δ*tcp*B and PBS control were determined by ELISA. Approximately100μl of blood was drawn from the tail vein of each mouse after 2, 4, 6 and 8 weeks post vaccination. The blood was clotted at 4^0C after which serum was separated by centrifugation. The serum was then used for determination of IgG1 and IgG2a levels by ELISA. Heat killed and sonicated *B. abortus* whole cell antigen was used to coat 96 well plates (Nunc-Immuno plates) at a concentration of 0.5μg/ml. Following overnight incubation at 4°C, plates were washed with 0.05% Tween in 1XPB (PBS-T), and blocked with 250 ul per well of blocking buffer [0.25% (w/v) BSA]. Mouse sera was diluted at 1:100 in blocking buffer

and added to the wells, which were then incubated for 1 hr at room temperature. The plates were then washed 3 times with wash buffer, after which goat anti-mouse IgG1 or IgG2a horseradish peroxidase (HRP) conjugate (eBiosciences) was added at 500ng/ml. Incubation was then continued for one hour. The plates were then washed plates and HRP substrate was added, and incubated with monitoring for up to 5 k times The reaction was stopped by addition of 100 μl of 0.5M NaOH and the absorbance measured at 450 nm (A450). One hundred µl of stop solution was added to each well and in absorbance read at 450 nm corrected for 570 nm. IgG levels elicited by vaccination are expressed as the mean A_{450}±SD for each group. The significance of differences between groups was determined by analysis of variance (ANOVA) using Prism software (GraphPad) and difference within groups determined by Student's *t* test. A p value <0.05 was considered statistically significant

3.2.4 Measurement of serum cytokine levels

Concentrations of cytokine in mouse serum were determined using commercial ELISA kits (eBiosciences) based on the method described by Finkelman et al., 1999 [151]. ELISA plates (Corning star ELISA 9018) were coated with 100 μ l per well of capture antibody (anti human mouse IL-5 or IFN-γ), in coating buffer (Ref# 00-0000-50 eBiosciences). The plates were then incubated overnight at 4°C. The plates were washed three times with wash buffer. The plates were inverted on absorbent paper to drain excess wash buffer, and the wells were then incubated in 200μl of blocking buffer at room temperature to prevent nonspecific binding. After one hour, the wells were

washed, followed by incubation in the presence of standards (IL-5 or IFN-γ recombinant protein) and sera added in two fold dilutions. Serum from vaccinated mice were diluted 1:100 in assay diluent and added to triplicate wells, and the plates were sealed with Para film. Following overnight incubation at 4°C, the supernatants were aspirated and the wells were washed five times with wash buffer. One-hundred μl of detection antibody (anti mouse IL-5 or anti mouse IFN- γ) was added to each well at a concentration of 0.1- 0.25 μg/ml. and the plates were incubated for an additional hour at room temperature. The wells were washed 5 times with wash buffer followed by the addition of 100μl per well of Avidin HRP diluted in 1X assay diluent buffer. The plates were sealed with parafilm and incubated for 30 minutes at room temperature. The plates were then washed with wash buffer and 100 μl of 1XTMB (3,3',5,5'-Tetramethylbenzidine) solution was added to each well. The plates were incubated at room temperature for 15 minutes after which 50 μl of stop solution was added to each well and absorbance at 450 nm was determined. A standard curve was plotted using recombinant protein standards provided in the kit to determine the cytokine concentrations over time and results expressed as the mean of concentrations ±SD for each group. The significance differences between groups per given time point was determined by analysis of variance (ANOVA) using Prism software (GraphPad) followed by Student's *t* test. A p value <0.05 was considered statistically significant.

3.2.5 Immuno-histochemistry and histopathology

Tissues from mice were assessed to determine the degree of pathology associated with challenge. Mice were challenged via intraperitoneal inoculation following vaccination with either S19Δ*tcp*B, S19 or PBS control. One week post challenge the mice were euthanized by CO_2 asphyxiation, spleens were harvested and one random sample cut from approximately a similar anatomical location, fixed in 10% buffered formalin, paraffin embedded, and stained with anti CD3 antibody (Dakocytomation) Carpentaria, CA, U.S.). Positive staining for CD3 as described with modifications after Randal *et al*., [152]. Areas stained by CD3 were determined by visualization of the areas staining brown with, the chromogen, DAB was used to label the antigenic sites. The positive cells are visualized as have dark brown staining of the cell membrane. The nuclei were counterstained with hematoxylin. The slides were blinded and presented to an independent pathologist/immuno-histochemist. Analysis of the tissue staining for CD3 was determined by visual examination and use of public domain image J soft ware. A total of 4 to 5 tissues sections were examined per group. Scores were assigned on a continuous scale of 1 to 5 (with 1 being the least T cell population and 5 being the highest T cell populations) as described by Rizzard *et al*. with modifications [153].

3.3 Statistical analysis

Calculations were done using GraphPad prism version 5.0 for Mac. Bacterial burdens in efficacy studies were expressed as CFU ± SD and presented graphically as $\log_{10}$ CFU of bacteria recovered per gram of tissue. Concentrations of cytokines in ELISA assays were

determined using a standard curve and averages for each group (n=5) expressed as pg/ml ± SD. Comparisons were made using ANOVA followed by a Student's *t* test. ($p<0.05$ were considered significant). For immunohistochemistry/histopathology, the stained slides were blinded and examined by two independent pathologist quantified the CD3 staining reaction as described above. Mean percentages of infiltrating T cells per group were expressed as % T cell score ± SD. Comparisons between groups were made by one way ANOVA followed by Student's *t* test with $p <0.05$ considered significant. .

3.4 Results

3.4.1 Immunity to *B. abortus* S19Δ*tcp*B

One of the early events in infection/vaccination with intracellular LAV bacteria is phagocytosis by resident macrophages and the release of cytokines by these cells. The release of proinflammatory cytokines early in vaccination is crucial in priming the adaptive immune reaction that is essential for the clearance of infection. The bulk serum levels of IFNγ were analyzed by ELISA. IFNγ levels were significantly elevated at two weeks post vaccination in the serums of mice immunized with the *S19ΔtcpB*, in comparison with the levels in the sera of mice vaccinated with S19 (Fig. 12). The levels waned with time. The levels of IFNγ in the sera of the control mice were at minimal levels throughout the vaccination period. This transient increase in pro inflammatory cytokines in *Brucella* infection has been described previously [29].

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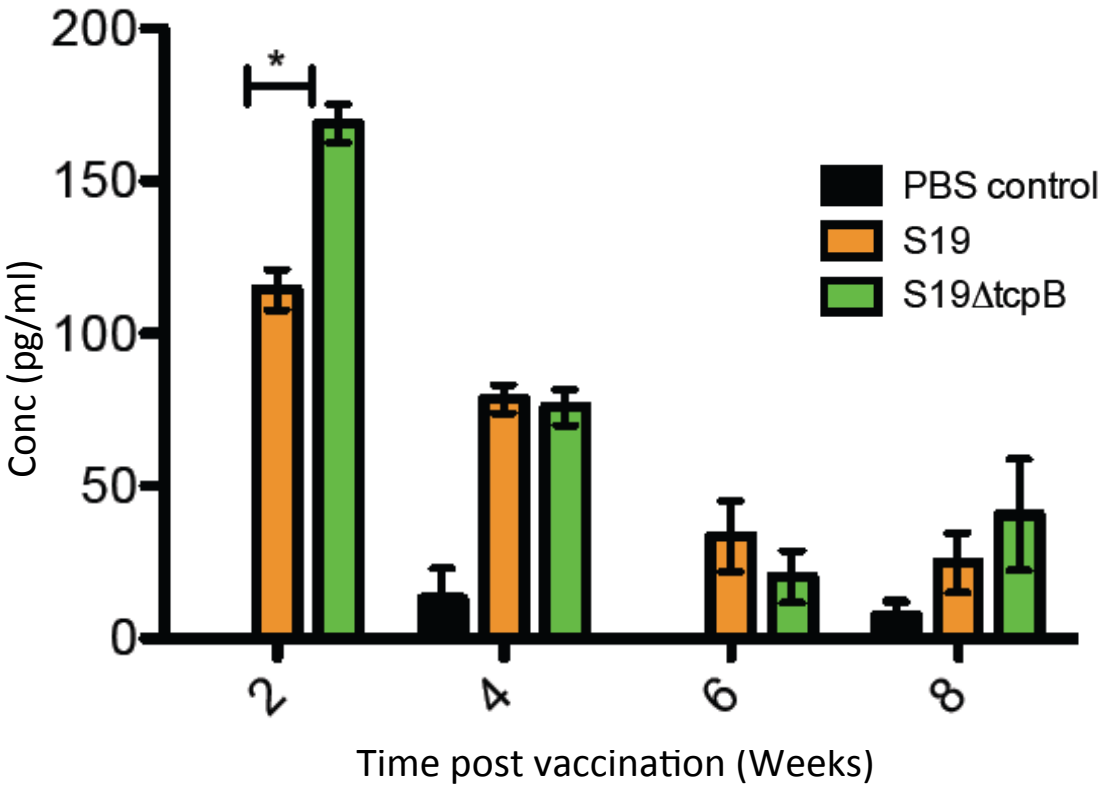


Fig. 12. IFN γ levels in serum of mice vaccinated with *B. abortus* S19 and *B. abortus* S19Δ*tcp*B**.** C57BL/6 mice were vaccinated with S19, S19Δ*tcp*B or PBS control. Serum was obtained from the mice at specified time points and levels of IFNγ determined by ELISA using a commercial kit (eBiosciences). The concentrations of cytokines were calculated for individual mice and the means of each group ± SEM plotted against time. The significance of differences between groups was determined by analysis of variance (ANOVA) using Prism software (GraphPad). A student's t test was used to determine the difference between means. A p value <0.05 was considered statistically significant.

3.4.2 IgG2A and IgG1 ratios and levels

The IgG2A and IgG1 ODs were obtained for the mice in each group in the vaccinated animals and plotted against time post vaccination (fig 13 b). Values obtained from ELISAs showed elevation of IgG2A values relative to IgG1 in mice vaccinated with *B. abortus* S19Δ*tcp*B as compared to the mice vaccinated with *B. abortus* S19 or PBS ($p<0.05$) Tukey's multiple comparison test). This elevation was observed towards the latter stages of the vaccination (Fig. 13 a).

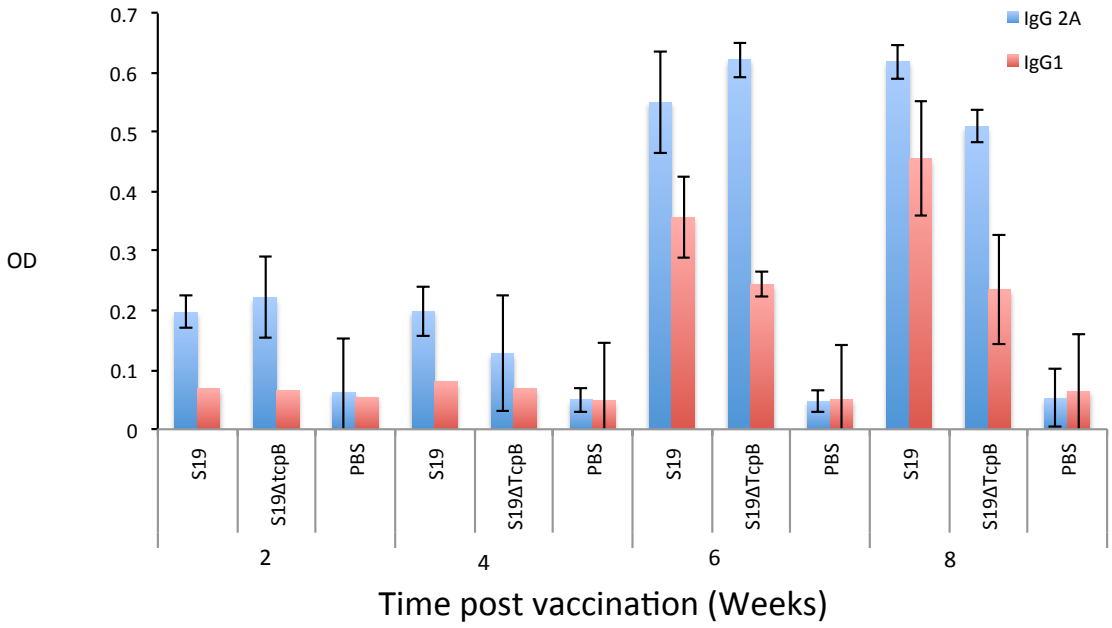


Fig. 13 IgG2a and IgG1 levels in sera from S19 and S19Δ*tcp*B vaccinated mice. C57/ BL6 mice (n=5/time point) were vaccinated with 1 $\times 10^6$ CFU/mouse of S19 or S19Δ*tcp*B. At 2, 4, 6, and 8 weeks post vaccination, sera were harvested from mice and IgG_{2a} and IgG_1 determined via ELISA. Values OD of IgG_{2a} and IgG_1 were then plotted for individual mice and the means of each group n=5 ± SD plotted at the selected time points.

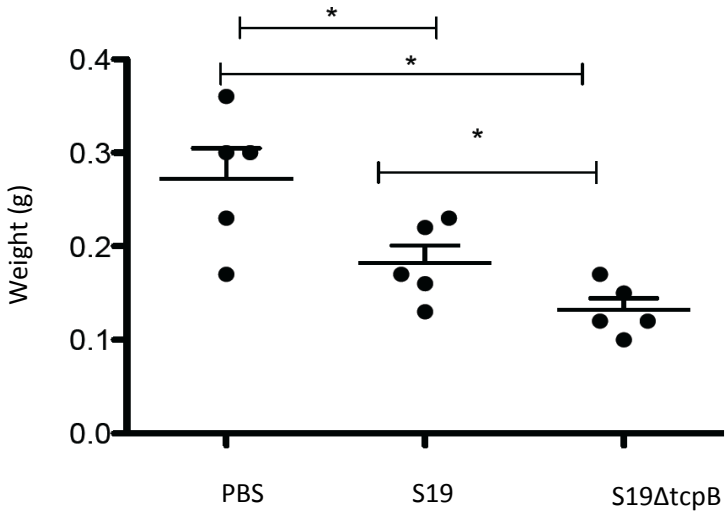


Fig 14. Spleen weights of mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS following challenge with *B. abortus* 2308. C57BL/6 mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS were infected intraperitonealy with 10^5CFU/mouse of wild- type *B. abortus* 2308. At one week post-infection, mice were euthanized and the spleens were weighed to determine the degree of splenomegaly post challenge. Results are expressed as the mean ± standard error of the mean. Differences between weights were determined by ANOVA followed by Student's *t* test (*$p<0.05$).

3.4.3 *B. abortus* S19Δ*tcp*B mutant protects against spleen colonization following challenge with *B. abortus* 2308

In order to determine whether *tcp*B deletion confers improved efficacy to a *B. abortus* S19Δ*tcp*B mutant LAV as compared to *B. abortus* S19 vaccine, the challenged mice were sacrificed after one week and evaluated for bacterial loads in the spleens. The results (Fig. 15) reveal a statistically significant 0.6 log reductions in the spleen bacterial load in mice vaccinated with S19Δ*tcp*B relative to those vaccinated with S19 ($p<0.05$). Both vaccines provided statistically significant protection ($p<0.05$) with respect to naïve mice of 2.2 logs of protection in S19 vaccinates and 2.8 logs of protection in S19Δ*tcp*B vaccinates (Fig.15). Reduced bacterial burdens following challenge were associated with

reduced splenomegaly. S19Δ*tcp*B vaccination therefore produced a better immune response as compared to S19 and PBS control that resulted in better protection against splenic colonization and inflammation protection as defined by the log difference between naïve and vaccinated (Fig. 15).

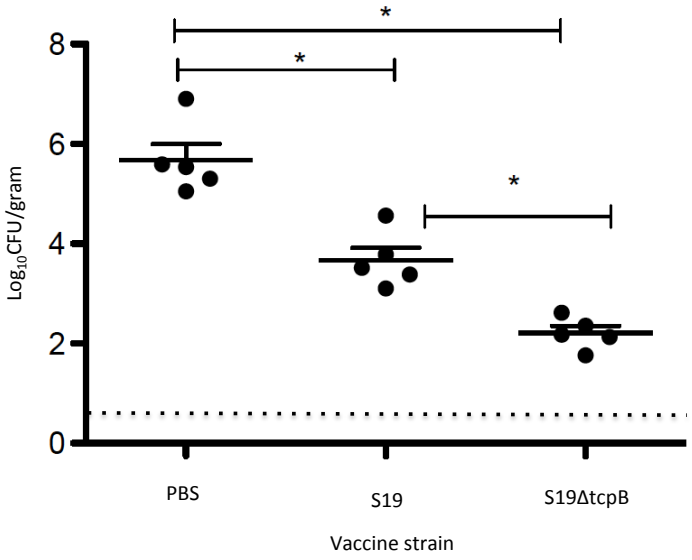


Fig. 15. Protection afforded to mice by *B. abortus* S19 and *B. abortus* S19Δ*tcp*B against *B. abortus* 2308 challenge. Mice were vaccinated with $1x10^6$ CFU *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS for 8 weeks. After the vaccination period, the mice were challenged with $1x10^5$CFU wild-type *B. melitensis* for 1 week. Mice were then euthanized, and spleens, extracted and homogenized in peptone saline. Homogenates were serially diluted to determine the CFU bacteria in the spleens. CFUs are expressed as the means of the $\log_{10}$ of individual mice ± standard error of the mean. Differences in colonization were determined by ANOVA and Student's *t* test comparing the (*P<0.05). The solid line represents the limit of detection which is ≥5 CFU).

3.4.4 T cell levels in spleens of mice vaccinated by *B. abortus S19Δtcp*B, S19 or PBS following challenge with *B abortus 2398*

Spleens were collected from mice challenged with *B. abortus* 2308 following vaccination with *B. abortus* vaccines S19, S19Δ*tcp*B or PBS (naïve control). Immunohistochemistry for spleen samples was performed on formalin-fixed paraffin embedded tissue sections stained with anti-CD3 polyclonal antibody. Higher levels of T cells were observed in spleens of mice vaccinated with S19Δ*tcp*B and challenged with *B. abortus* 2308 as measured by CD3 staining. In comparison, there were less CD3 T cells in spleens of mice vaccinated with S19 ($p \leq 0.05$) Students t test. Naïve mice challenged with *B. abortus* 2308 had lower T cell numbers (Fig. 16). Spleen weights of mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS following challenge with *B. abortus* 2308. C57BL/6 mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS were infected intraperitonealy with 10^5CFU/mouse of wild- type *B. abortus* 2308. At one week post-infection, mice were euthanized and the spleens were weighed to determine the degree of splenomegaly post challenge. Results are expressed as the mean ± standard error of the mean. Differences between weights were determined by ANOVA followed by Student's *t* test

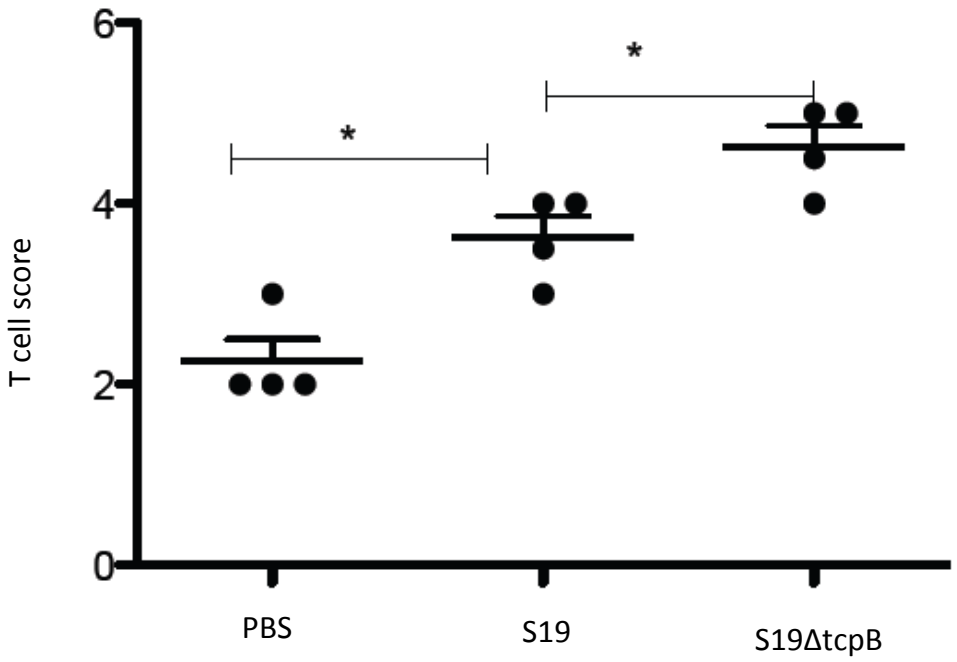


Fig .16. T cell levels in spleens of vaccinated mice following challenge with *B.abortus 2308*. CD3 T cell populations in spleens were stained with anti-CD3 antibody. Slides were scored and blinded by two independent pathologists and the averaged for each group. Comparisons were made between groups using ANOVA and Student's *t* test. (*$p<0.05$).

3.5 Discussion

Vaccination of mice with *B. abortus* S19 and *B. abortus* S19Δ*tcp*B stimulated expression of proinflammatory cytokines as were detected in serum. This has been previously evaluated in an experiment in which serum cytokines of mice vaccinated with S19 peaked at 2 weeks post vaccination coinciding with peak bacterial numbers [154]. Additionally we also observed increased expression of proinflammatory cytokines in mice vaccinated with S19Δ*tcp*B at early time points (Fig 12). The increased cytokine

responses following S19ΔtcpB may be crucial in recruiting dendritic cells (DC) early in vaccination. DCs then engulf the vaccine and transport it to the lymphoid organs where it is presented to naïve T cells. T cells then differentiate into a Th1 phenotype. The Th1 phenotype is associated with increased IgG2A levels, which was confirmed in our experiments (Fig 13). We also observed significantly larger populations of CD3 positive T cells in spleens of vaccinated mice as compared to naïve mice. More importantly, mice vaccinated with S19Δ*tcp*B had higher CD3 T cell levels than S19 vaccinates following challenge with *B. abortus* 2308 (Fig 16) . Although the CD3 cells could have been a heterogeneous population, they were associated with increased protection as evidenced by reduced levels of challenge organisms. Reduced splenomegaly and reduced splenic bacterial burden of *B. abortus* 2308 were observed in mice vaccinated with S19Δ*tcp*B relative to S19 following challenge with B. abortus 2308 (Figs 14 and 15). Collectively, these results suggest that deletion of *tcp*B from S19 enhanced Th1 mediated protective immunity against wild type challenge with *B. abortus* 2308.

CHAPTER IV

EFFECTS OF IMMUNE SPLENOCYTES ON INTRAMACROPHAGE SURVIVAL OF *BRUCELLA*

4.1 Introduction

The development of new vaccines against brucellosis has been hindered by a lack of knowledge of the immune mechanisms responsible for protection [25]. While it is known that cell mediated immunity and associated cytokines are responsible for controlling infection, specific correlates of immune protection have not been defined [76]. *Invitro* systems rarely reflect the overall complexity of the immune system while analysis in animal models do not always reflect the nature of disease observed in target species [155]. Furthermore, the use of animals and specialized housing under long-term expensive BSL-3 conditions is not always justified. Thus, the development of an *in vitro* system that models *in vivo* responses would facilitate the development of *Brucella* vaccines and the study of anti-brucellosis control mechanisms while reducing dependency on animal models. For example, consider utilizing PBMC from vaccinated ruminants to evaluate immune protection derived from enhanced intracellular killing. The development of an *invitro* preclinical assay to measure *Brucella* vaccine efficacy has not been reported. In order to accelerate development of *Brucella* vaccines, studies were initiated studies targeting immune splenocyte mediated control of *Brucella abortus* growth within macrophages and evaluating alterations in the composition and

distributions of cellular populations in the spleens of vaccinated compared to unvaccinated mice.

4.1.1 Splenocyte mediated inhibition of intramacrophage bacterial growth

The primary sites of *Brucella* multiplication are cells of the reticuloendothelial system, e.g. macrophages and dendritic cells [156]. *Brucella* establishes a replicative niche in these cells for long term survival, a factor that is partially responsible for the chronicity of the infection [157]. *Brucella* have adapted to survive and persist within the macrophage, and their survival strategies include subversion of macrophage bactericidal activities [158]. Virulent *Brucella* block various macrophage inflammatory functions including decreased expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines like IL4 and IL10 [159]. Reduction of intramacrophage replication caused by immune lymphocytes obtained from vaccinated animals could potentially be used as a biomarker for vaccine efficacy. We propose that an *in vitro* system of co-culture of splenocytes from vaccinated mice with infected macrophages will enhance macrophage activity and reduction of intracellular *Brucella,* serving as useful biomarkers of protective immunity. Such a system could be an effective alternative to animal challenge models and used as a predictive tool for vaccine efficacy.

Determination of protective efficacy of *Brucella* vaccines depends on vaccine-induced resistance to challenge by wild type strains as determined by reduction in splenic colonization relative to naïve mice. Additional parameters used to define immune protection include; T cell proliferation following *in vitro* antigen stimulation and

cytokine production via ELISA or ELISPOT. However, individually these parameters have provided poor correlation with protective efficacy [160,161] . In order to extrapolate to potential immune protection in humans, a thorough knowledge of the correlates of protection is necessary. A feature of the immune system is the capacity of memory T cells to mediate a faster, stronger and more effective response to secondary pathogen challenge than naïve T cells. While CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration, CD62L expression by antigen experienced T cells corresponds to effector memory CD62Llo and central memory CD62Lhi cells. A direct *in vitro* cytotoxicity assay measuring the effect of immune lymphocytes on intra macrophage growth of bacteria is expected to mimic *in vivo* vaccine activity to permit side-to-side comparison of different vaccines, and to provide a reliable predictive measure of vaccine efficacy. We hypothesize that reduced intramacrophage growth of *B. abortus* and patterns of composition and distributions of cellular populations in the spleens will potentially serve as candidate markers for protective immunity. To test this hypothesis we used S19*Δtcp*B vaccine and S19 to vaccinate mice and evaluated T cells ($CD8^+$, $CD4^+$) populations in the spleen in response to vaccination. We further infected autologous bone marrow derived cells with *B. abortus 2308* and co cultured them with splenocytes obtained from mice vaccinated with either S19, S19Δ*tcp*B or naïve mice.

4.2 Materials and methods

4.2.1 Experimental animals and design

In this study, we vaccinated mice with two *Brucella* vaccines, S19 and S19Δ*tcp*B. The mice were monitored for a period of 8 weeks after which splenocytes were harvested and used in a splenocyte macrophage co-culture assay. In this assay, autologous bone marrow derived macrophages were infected with *B. abortus* 2308. Immune splenocytes were then incubated with infected macrophages and the ability to restrict *Brucella* replication compared. It is hypothesized that splenocytes from mice vaccinated with S19Δ*tcp*B will exhibit enhanced macrophage killing activity as a result of increased inflammatory response associated with the failure to express *Tcp*B. Killing was defined as the reduction in the number of colony forming units (CFUs) measured at the end of the culture period, compared to the input CFUs. In this study, we used S19*Δtcp*B vaccine and S19 to evaluate T cells ($CD8^+$, $CD4^+$) populations in the spleen in response to vaccination.

4.2.2 Establishment of the bone marrow derived macrophages (BMDM)

Autologous bone marrow were ascetically derived macrophages (BMDM) were prepared from C57BL/6 mice euthanized by CO_2 asphyxiation followed by cervical dislocation as described [162,163] with minor modifications. An incision, made in the midline of the abdomen exposed the hind limb bones. The bones were freed by clipping muscle from the bones and by severing the bones at both ends. The femur was separated from the tibia at the knee joint and bone marrow was flushed out with DMEM using a 5-mL

syringe and a 25-gauge needle. A single cell suspension was formed by vigorous pipetting, and the cells were collected using a cell strainer, washed with 5 mL of DMEM and counted using a hemacytometer. A final count of live cells was done by use of Trypan blue exclusion. The cell suspension was adjusted the to 2×10^6 live cells/mL in complete DMEM complete supplemented with 10% (v/v) L-929-conditioned medium, 0.2 mM L-glutamine, and 0.1 mM nonessential amino acids and 100 U penicillin / 0.1 mg/ml streptomycin. The cells were cultured in 12 mL in a 10-cm culture dish with DMEM media supplemented with L-929-conditioned medium, and the media was replaced after every two to three days. Morphological characterization of the macrophages was done by observation though an inverted microscope (Nikon TMS®) As large adherent cells with large cytoplasm which is vacuolated. The nucleus tends to be pushed to the edge of the cell and the cytoplasm may have ingested materials present. Viability was determined at various points by trypan blue exclusion. At the end point of culture seven days, the cells were trypsinized in 05% Trypsin-EDTA (Invitrogen) and transferred into 24 well plates. The cells were seeded at $2X10^5$ per well and cultured for 24 hours before infection with *Brucella*.

4.2.3 Macrophage infection and splenocyte co-cultivation

Confluent adherent macrophage monolayers (BMDM) in 24 well tissue culture plates were infected at a multiplicity (MOI) of 1:100 (bacterium-to-BMDM) with *B abortus* 2308 suspended in DMEM and incubated for 30 minutes. Following uptake, the media was removed, the monolayer was rinsed with antibiotic-free DMEM, and media

supplemented with 50μg/ml gentamicin was added to kill extracellular bacteria and incubation continued for an additional 30 minutes. At this point the cell monolayers were rinsed as above and replaced with antibiotic-free DMEM supplemented with 30μg/ml gentamicin. Single cell suspensions of splenocytes derived from vaccinated mice were added to the monolayers 30 minutes later at $1x10^7$ cells per well. Cellular viability was determined by trypan blue exclusion staining and values above 95% were considered to be acceptable. On days 0, 1, 2 and 3 the monolayers was washed three times with PBS and lysed with 0.5% (v/v) aqueous Tween-20 and plated on TSA plates to enumerate the number of intracellular bacteria. Cell viability was measured using cytotox 96 LDH release assay (Promega). To generate immune splenocytes, we vaccinated mice with two *Brucella* vaccines, S19 and S19Δ*tcp*B. The mice were monitored for a period of 8 weeks after which splenocytes were harvested using standard techniques as described by De Pascalis [164] and used in a splenocyte macrophage co-culture assay.

4.2.4 Preparation of lymphocytes and flow cytometry

Single-cell suspensions of spleens were generated for *in vitro* culture and flow cytometry by standard techniques, as previously described [164]. No bacterial CFU were detected in organ homogenates at the time of harvest. The absence of vaccine strain (LAV) in organ homogenates at the time of harvest was verified by serial plating and bacterial culture of TSA. Cell viability was assessed by exclusion of trypan blue. Multiparameter

analyses were performed using a panel of murine cell surface markers with a Becton, Dickinson LSR II flow cytometer (San Jose, CA) and analyzed using FlowJo software (TreeStar, Inc) [164].

4.2.5 Flow cytometry for identification of splenic T memory cells

Mice were vaccinated intraperitonealy with *B. abortus* S19Δ*tcp*B, *B. abortus* S19 or PBS control and euthanized eight weeks post vaccination by CO_2 asphyxiation followed by cervical dislocation according to AUP number 2012-156. Single cell suspensions of splenocytes prepared as described above were aseptically harvested and immediately stained with a combination of anti-CD8 (PerCP-cy5.5 BD-Biosciences) anti CD62L (FITC clone MEL-14, BD Bioscience, CA, USA), anti CD44 (APC clone IM-7, BD Bioscience, CA, USA), and pacific blue conjugated rat anti-mouse CD4a (clone RM4-5, BD Bioscience, CA, USA) to determine the fraction of memory T cells ($CD4^+CD44^+CD62L^+$) among the $CD4^+$ T cell population of vaccinated mice. Stained splenocytes were analyzed on a BD Aria II FACS (CA, USA) acquiring 10,000 events, and the output was analyzed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad software, LA Jolla, CA).

4.2.6 Evaluation of cytokine levels in splenocyte-macrophage co cultures

Supernatants recovered from *in vitro* co-cultures were assayed using standard sandwich ELISA, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Quantification of IFNγ and IL-5, were assessed by comparison using recombinant

protein standards (BD Pharmingen). Standards provided in the kit were used in the ELISA alongside the test supernatants. ODs obtained from standards were used to plot a standard curve of OD against concentrations. A trend line was derived from the curve, providing a function that was used to calculate the concentrations. The concentrations were expressed as mean pg/ml ±SD. Differences between means were determined by ANOVA followed by a student's t test and values of $p \leq 0.05$ were considered significant.

4.2.7 Measurement of cell viability (the LDH assay)

LDH release from the co-culture assays was measured by the cytotox 96®Non-cytotoxicity assay according to the manufacture's instructions (Promega). Supernatants were collected from wells with splenocyte-macrophage co-cultures, at 24, 48 and 72 hours post infection. Control wells left uninfected (Spontaneous Release) or incubated with lytic reagent (Maximum Release). Supernatants were incubated with 50μl of the CytoTox 96® incubate at room temperature, protected from light, for 30 minutes. 50μl of the Stop Solution was added and absorbance read at 490nm. The percentage of cytotoxicity was calculated as (Infection-induced Release – Spontaneous Release/ Maximum –Spontaneous Release), and presented as the average (n=5) of % cytotoxicity ± SD.

4.3 Results

4.3.1 Evaluation of T cell percentages in isolated splenocytes

Ex vivo isolated splenocytes stained for the percentage of $CD8^+$ and CD4 T^+ cells and analyzed revealed elevated bulk CD8+T cells in splenocytes of mice vaccinated intraperitonealy with either *B. abortus* S19 or *B. abortus* S19Δ*vjb*R relative to the control mice. However there was no significant difference in the population of CD8+T in mice receiving either vaccine strain (Fig. 17 a). No significant difference was detected in the overall CD4+ T cell population in splenocytes derived from either vaccinated or control mice. However, analysis of the memory CD4+ T cells revealed an increase within the CD4+ T cell fraction (Fig. 17 b). In addition, the memory CD4 T cell fraction was significantly elevated in splenocytes obtained from mice vaccinated with the S19Δ*tcp*B mutant relative to the mice vaccinated with S19 (Fig 18 a and b).

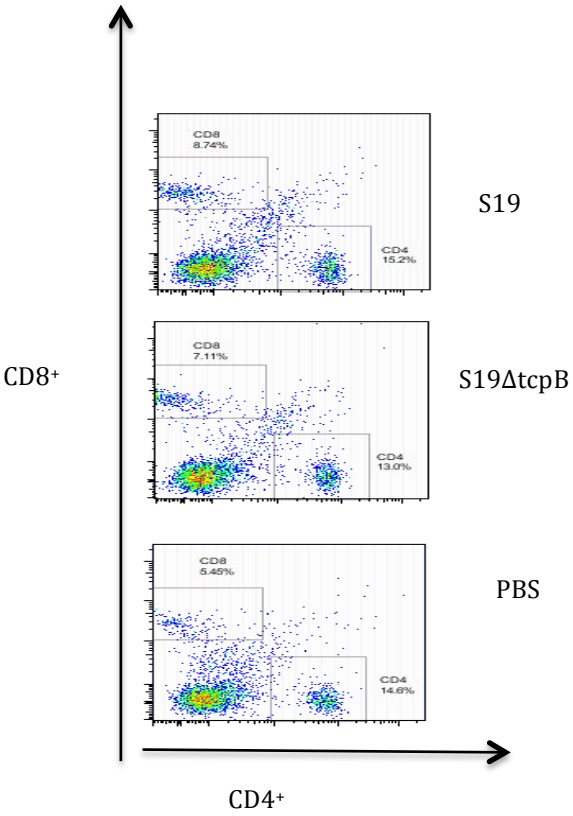


Fig. 17 a) Gating of total $CD4^+$ and $CD8^+$ T cells in splenocytes obtained from vaccinated mice**.** Mice were immunized with *B. abortus* S19 or *B. abortus* S19Δ*vjb*R at $1X10^5$ CFU per mouse. After 8 weeks, splenocytes were harvested and stained with flourochrome-conjugated antibodies specific for $CD4^+$ and $CD8^+$ T cells. a) Gating strategy for phenotypic analysis of CD4 and CD8 T cells from splenocytes of vaccinated mice. Cells were gated using Aria flow cytometer and their percentages expressed as a total percentage of T Cells.

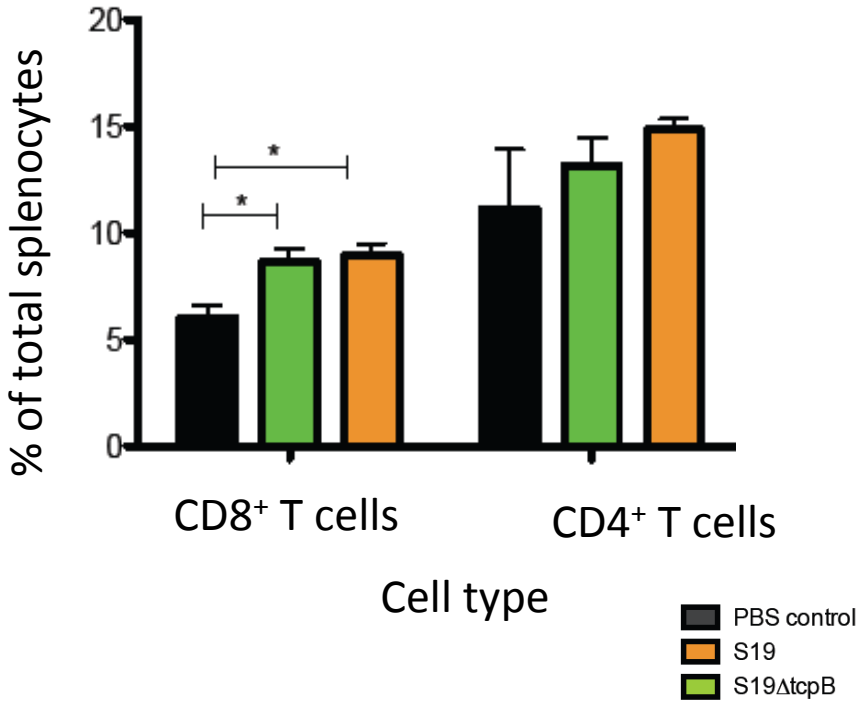


Fig. 17 b) Percentages of $CD4^+$ and $CD8^+$ T cells in splenocytes obtained from vaccinated mice**.** Graphical representation of total $CD4^+$ and $CD8^+$ T cells from spleens of vaccinated mice (n=5). Data were expressed as means ± SD of samples and comparisons made between treatment groups using ANOVA and analyzed using Student's *t* test (*p-values <0.05)

4.3.2 Analysis of the memory T cell population within CD4+ T cell fraction of splenocytes from vaccinated mice

Splenocytes harvested from mice vaccinated intraperitonealy with *B. abortus* S19, *B. abortus* S19Δ*tcp*B or PBS were evaluated for presence of memory T cells. The markers used did not allow differentiation between hi and lo memory cells. Elevated percentage increases in memory cells bearing $CD44^+$ $CD62L^+$markers were found in splenocytes of mice vaccinated with *B. abortus* S19Δ*tcp*B mutants (ANOVA and Student's *t* test $p<0.05$) in comparison to those vaccinated with *B. abortus* S19.

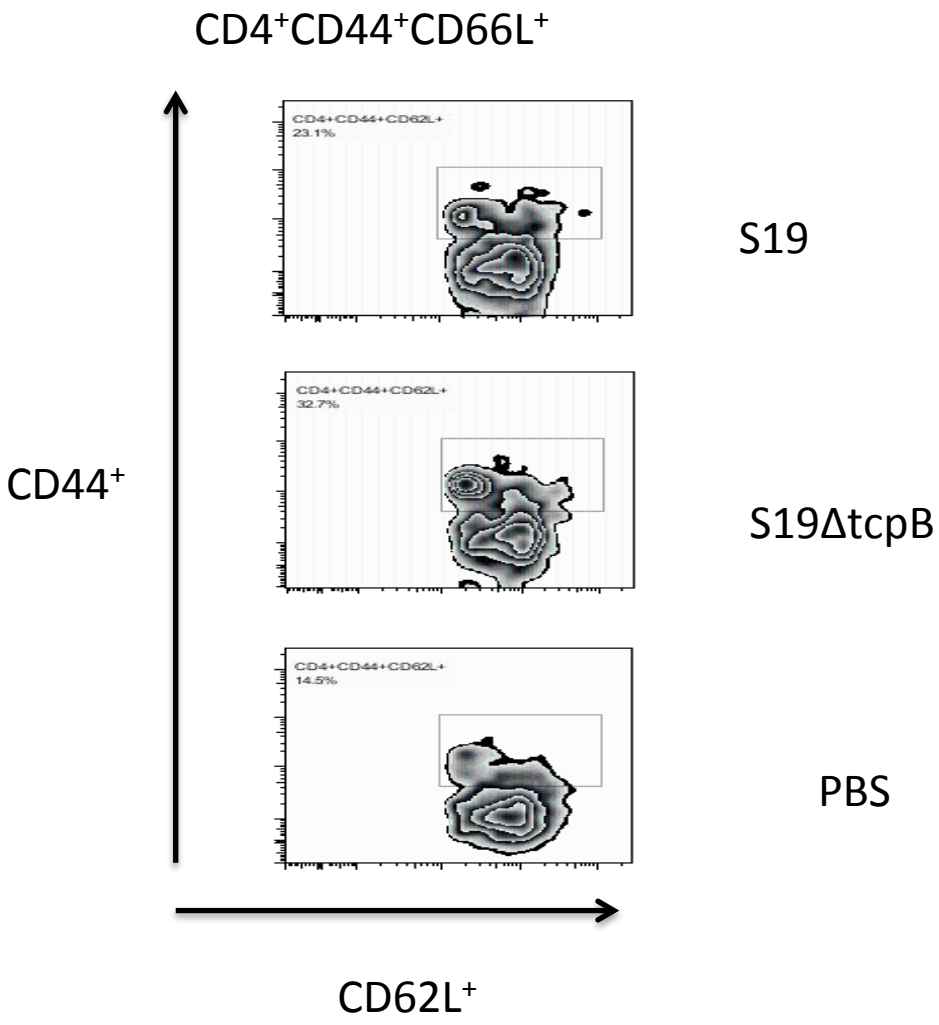


Fig. 18 a) Gating strategy for phenotypic analysis of CD44+ and CD62L+ cells in the CD4 fraction of splenocytes from vaccinated mice. Cells were gated using Aria flow cytometer and their percentages expressed as a total percentage of T Cells.

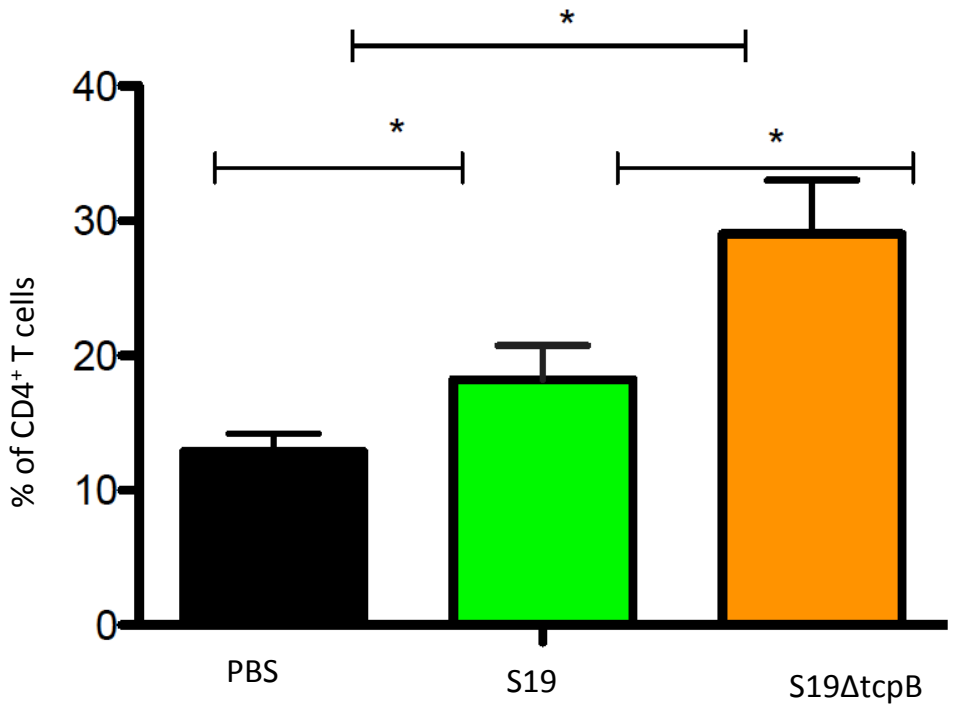
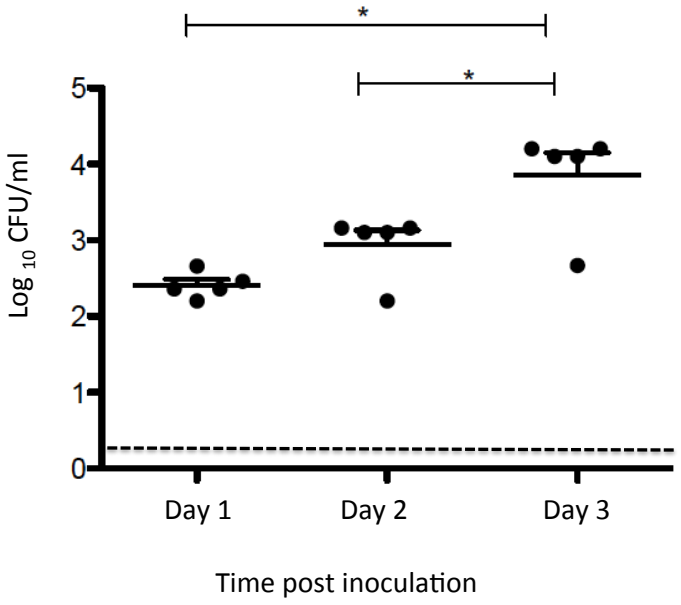


Fig. 18 b) Analysis of the memory $CD4^+CD44^+CD66L^+$ T cell population within $CD4^+$ T cell fraction of splenocytes from vaccinated mice. Individual mice were Immunized with *B. abortus* S19 or *B. abortus* S19Δ*vjb*R with 10^5 CFU. Splenocytes were harvested 8 weeks post-vaccination and stained with flourochrome-conjugated antibodies against $CD4^+$, $CD44^+$ and $CD66L^+$. $CD4^+CD44^+CD66L^+$ cells were gated using Aria flow cytometer and expressed as a percentage of $CD4^+$ T cells.
Data are expressed as means ± SEM of n=5 and comparisons made between treatment groups using ANOVA and the Student's *t* test (p-values <0.05)

4.3.3 Splenocyte mediated killing of intracellular *Brucella*

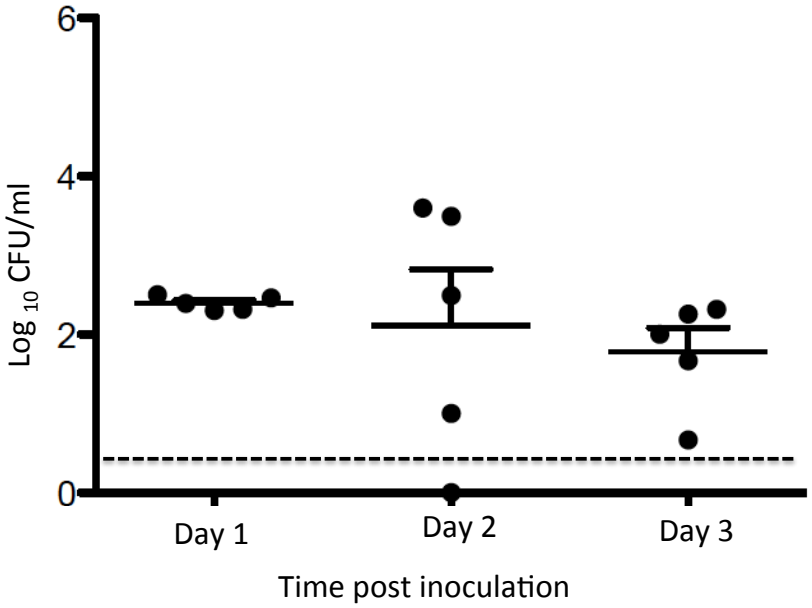
Splenocytes isolated from mice vaccinated intraperitonealy with *B. abortus* S19, B. abortus S19Δ*tcp*B or PBS were co-cultured with BMDM infected *in vitro* with *B.*

abortus 2308. In addition, the intracellular bacterial burdens of BMDM cultured without addition of splenocytes (Blanks) were monitored. *B. abortus* 2308 replicated efficiently in BMDM, and the addition of immune splenocytes accelerated intramacrophage inhibition of replication of wild type Brucella (Fig. 19). Enhanced killing of *B. abortus* 2308 occurred in co-cultures using splenocytes derived from mice vaccinated with *B abortus* S19Δ*tcp*B relative to splenocytes obtained from *B. abortus* S19 vaccinated mice (P ±0.05). Naïve splenocytes did reduce the killing of intra-macrophage bacteria.



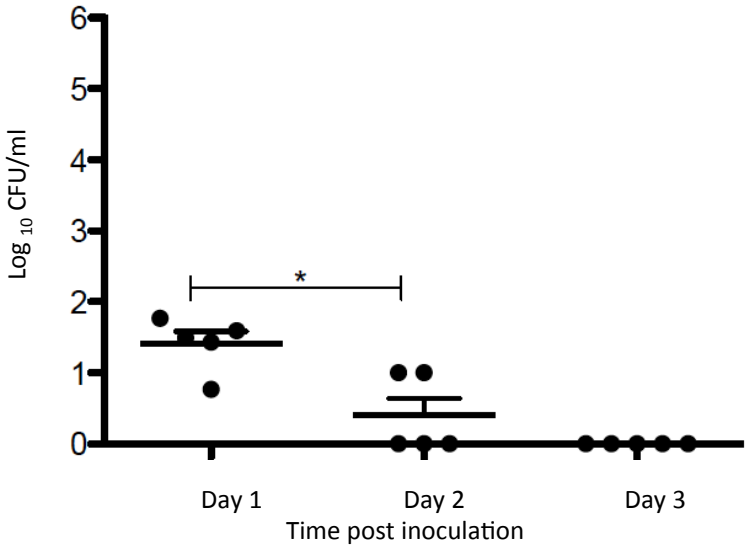
a)

Fig. 19: Effect of splenocytes from vaccinated and non-vaccinated mice on intramacrophage survival of *B. abortus* 2308**.** BMDM were generated from C56BL/6 mice and plated at $2X10^5$ in 24 well plates. The BMDMs were infected with wild type *B. abortus* 2308 for one hour at an MOI of 100. Splenocytes obtained from age matched C57BL/6 mice were added at 1, 2 and 3 days, (spleenocyte cells were obtained from mice vaccinated with a) PBS, b) S19 c) S19Δ*tcp*B) the cells were lysed with 0.5% tween 20 in sterile water. The lysed cells were serially diluted and plated on TSA plates. The plates were incubated at 37°C and numbers of colonies enumerated. The bacterial burdens were expresses as log $_{10}$CFU/ml and plotted against time. Values are the means of individual mice were expressed as CFU ± SD. The doted line represents the limit of detection which is ≥5 CFU.



b)

Fig. 19 (Continued)



c)

Fig 19. Continued

4.3.4 IFNγ expression by sensitized splenocytes

To identify a potential correlate of immune protection, the BMDM monolayer supernatants were evaluated for IFNγ content. Supernatants from the co-culture assay were harvested at various times, and the IFNγ level was evaluated using ELISA. Higher levels of IFNγ were detected in cultures that received splenocytes from mice vaccinated with *B. abortus* S19Δ*tcp*B as compared with those vaccinated with S19 ($P \leq 0.05$) Although attempted, no Th2 cytokine IL5 was detected by T cells in the co-culture assays.

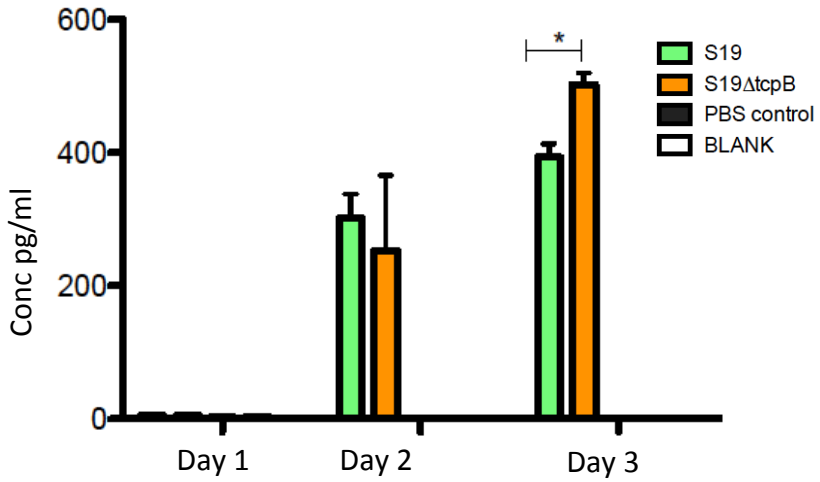


Fig. 20. Concentrations of IFNγ from *in vitro* cultures of immune splenocytes and infected macrophages. BMDM from C56BL/6 mice were plated at $2X10^5$ in 24 well plates and infected with wild type *B. abortus* 2308 for one hour at an MOI of 100. Splenocytes obtained from age matched C57BL/6 mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B, PBS or media without splenocytes were added. At 1, 2 and 3 days, supernatants were collected and analyzed for levels of IFNγ. Data were expressed as means ± SD for each group (n=5) and comparisons made between treatment groups using two way ANOVA analyzed using Tukey's Multiple Comparison test (p-values <0.05=*) were considered significant.

4.3.5 Measurement of LDH Release

LDH release from cells is a measure of cytolysis as a result of necrosis, apoptosis or pyroptosis. The LDH assay was used to determine the percentage viability of the cells in the spleenocyte macrophage co-culture assay. Although cell lysis in the assays was minimal (less than 15%), more cell lysis was observed in co cultures incubated with splenocytes obtained from S19 and S19 vaccinated mice than the macrophages with no splenocytes. (ANOVA and Tukeys Multiple comparison Test $p \leq 0.05$). This difference

may be attributable to multiple factors including, the cytotoxic activity of the sensitized splenocytes for infected cells or natural attrition.

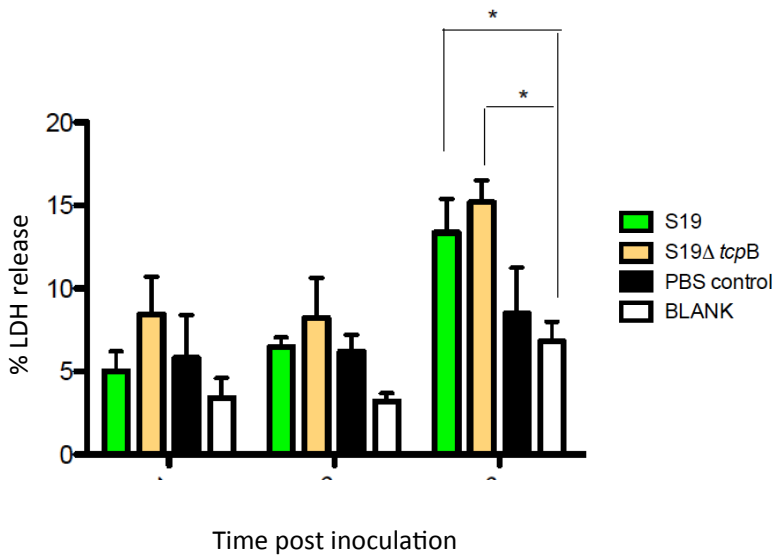


Fig. 21. LDH release from splenocyte-macrophage culture assays.
Supernatants were collected from co-cultures of splenocytes from mice vaccinated with *B. abortus* S19, *B. abortus* S19Δ*tcp*B, or PBS and macrophages only co-cultures. Cytotox96 non-radioactive assay was used to determine the levels of LDH released by the cells. ODs were measured at 530nm and % cytotoxicity calculated as LDH release release/Maximum LDH release. Percent LDH release is expressed as mean of three results ± SD. Comparisons between means were analyzed by ANOVA followed by Tukeys multiple comparizon test (* P≤ 0.05)

4.4 Discussion

Previously published results indicate that mice vaccinated with *Brucella* LAV mount an immune response that results in differentiation of splenic T cells [36]. Upon a second

encounter with the pathogen, T cells proliferate and express pro-inflammatory cytokines and effector mechanisms that enhance the intramacrophage killing or inhibition of replication of the pathogen [165]. CD4+CD44^{+}CD62L^{+} are memory CD4 T cell obtained in the spleen and respond robustly following secondary encounter with the pathogen. Memory CD4^{+} T and CD8^{+}T cells have been classified into effector (CD44CD62L^{Lo}) and central (CD44+CD62L+ hi) [166]. Central memory cells express CD44 homing receptors that locate them in the spleen and lymph nodes, from where they undergo robust proliferation on second encounter with the antigen, this gives long term protective memory to the host[166].

Our data confirmed that vaccination with the *B. abortus* S19Δ*tcp*B LAV mutant induced the highest frequency of CD4^{+} T cells in the spleen (Fig. 18), which in turn may provide the benefit of long-term protection against *B. abortus 2308* challenge. Flow cytometry of mice vaccinated with PBS did not have a significant increase in memory T cells; hence, the high numbers of T cells was attributed to response following vaccination. This may suggest that *tcp*B deletion results in up regulated transition of CD4 T cells to memory cells and is consistent with overall immune protection.

Splenocytes obtained from mice vaccinated with *Brucella* LAV vaccines accelerated the killing activity, which may be attributed to the increased IFN-γ detected, although alternative explanations are possible and require further investigation (Figs 19 and 20). Splenocytes obtained from vaccinated mice are expected to contain high levels of sensitized T cells in addition to other cells like NK cells, gamma delta cells. These may participate in specific and on specific killing of intramacrophage bacteria.

From our experiments, we determined the overall cell lysis in the assays, more cell lysis observed in cultures of splenocytes obtained from S19 and S29 tcpB vaccinated mice, this was however minimal (Fig 21) and may be attributed to among other factors, the presence of sensitized T cells. Although the less lysis was observed in cultures with splenocytes obtained from PBS vaccinated mice, it was not significant. In general, this is explained by the principle that splenocytes isolated from vaccinated mice are rich in pathogen specific T cells following a prior encounter with the pathogen, however other factors may also be responsible for the killing, including, NK cells, γδ cells [167]. Infected macrophages process and present *Brucella* pathogen to these sensitized splenocytes in the context of MHC class II. The splenocytes are activated and secrete IFNγ, which in turn activates the macrophages to kill the intracellular bacteria [168]. This assay could be further developed and used to quantitate the efficacy of the vaccine. Factors that may be considered to improve the assay include correcting for death of effector and/or targets by attrition. Our studies also provide evidence that splenocytes obtained from mice vaccinated with S19Δ*tcp*B are associated with enhanced intramacrophage killing of *Brucella* infected macrophages. These studies provide evidence that vaccines of unknown efficacy may be evaluated by infecting macrophages with wild type *Brucella* and used as co-cultured targets with immune splenocytes from LAV vaccinated animals.

CHAPTER V

CONCLUSIONS AND SUMMARY

Live attenuated vaccines against *Brucella* provide elevated protection against wildtype challenge, but exhibit varying levels of safety [25]. As a result, there remains a significant level of concern related to their use both in animals and humans, and as a result concerted efforts are needed to improve their safety and effcicacy [26]. These efforts include the construction of stable deletion mutants lacking virulence and the potential to revert to virulent forms [26] . Although these goals are now achievable, they can restrict immune potential if not carefully formulated. An alternative line of investigation to supplement the approach is to define the host immune response with the intent of identifying protecive mechanisms and to target pathway components in an effort to enhance protection. As a stealth pathogen, *Brucella* enters the cell with minimal activation of the host innate immune response [57].

In this study, we compared the ability of two LAV *Brucella* vaccines, *B. abortus* S19 and the *B. abortus* S19Δ*tcp*B deletion to stimulate host immune reaction. We evaluated their capacity to stimulate host innate and adaptive immune responses in addition to promoting T cell memory differentiation and protective efficacy. It was expected that the mutant would induce a stronger host innate as well as adaptive immune respnses and provide an enhanced immune protection. Following vaccination, there was a significant elevation of inflammatory IFNγ in the serum of mice vaccinated by the *B. abortus*

S19Δ*tcp*B mutant as compared to the parent *B. abortus* S19 (Fig. 12). The increased serum IFNγ, however, waned with time. This could be explained by the reduced tissue bacterial loads. The initial increased inflamation, could be attributed to the enhanced inflammatory properties of the mutant. This initial inflamation could play a critical role in providing the adjuvant effect that boosts the hosts immune reaction to the LAV vaccine and recruiting antigen presenting cells to the site of the vaccine. A significantly elevated IgG_{2a} isotype was observed late post vaccination in mice vaccinated with *B. abortus* S19Δ*tcp*B in comparison with mice vaccinated with *B. abortus* S19 and the non-vaccinated controls (Fig. 13). Since Th1 immunity is associated with elevated IgG2a titers, it was concluded that the mutant skewed the immune response toward early T cell differentiation, which is critical in development of the important memory T cells. Indeed, we also evaluated the levels of IFNγ in serums of *Brucella* LAV vaccinated mice by ELISA. We observed that there were elevated levels of IFNγ in serum of mice vaccinated by *Brucella* LAV. These levels were further elevated in mice vaccinated with *B. abortus* S19Δ*tcp*B vaccine. Since *Brucella* is an intracellular pathogen, it replicates inside the macrophages, activating the MyD88 signaling pathways. This activation may be downstream of TLR9, TLR2 or TLR4. However, greater effects have been observed in MyD88 mediated pathways, as evidenced in reduced bacterial clearance in myD88 knock out mice. TLR activation enhances the expression of IL12, IL6 and other IFNγ cytokines. These cytokines act on T cells causing differentiation into the Th1 phenotype. Th1 T cells produce IFNγ which acts on macrophages to enhance intracellular killing of the intracellular *Brucella* [144]. This provides a plausible explanation for the elevated

IFNγ levels in *Brucella* LAV vaccinated mice. *Brucella,* however, expresses *Tcp*B, a protein that subverts TLR signaling and reduces expression of proinflammatory cytokines [33]. Deletion of *tcp*B from the S19 vaccine strain reduced the immune subversive effects and resulted in up regulation of IFNγ. Splenocytes from mice inoculated with the *B. abortus* S19Δ*tcp*B deletion mutant had increased $CD4^+$ T memory cells. Since *Tcp*B has been implicated in the disruption of mTOR signaling pathway, its absence may be expected to enhance memory cell differentiation [36], and the observed increase in the number of $CD4^+$ memory T cells is consistent with this prediction. Splenocytes obtained from LAV vaccinated mice also exhibited higher killing activity in a co-culture with *B abortus* 2308 infected bone marrow derived macrophages. Splenocytes obtained from mice immunised with S19Δ*tcp*B had enhanced killing activity towards BMDMs infected with *B. abortus* 2308. This difference could be attributed to elevated levels and/or improved performance of memory T cells among the splenocytes derived from *B. abortus* S19Δ*tcp*B vaccinated mice. Reduced spleen weights occurred in *B. abortus* S19Δ*tcp*B vaccinated mice challenged with *B. abortus* 2308. This suggests that vaccine *B*. abortus S19Δ*tcp*B afforded a better protection against *B. abortus 2308* than *B. abortus* S19. This suggests that Δ*tcp*B deletion confers improved immunogenicity to *B. abortus* and better protective efficacy against virulent *B. abortus* 2308.

The high $CD4^+$T cell memory cell population in the spleens observed in this study is consistent with high T cell levels in mice vaccinated with *B. abortus* S19 *tcp*B and challenged with *B. abortus* 2308. Importantly, the significantly higher levels of $CD4^+$T

memory cells observed in mice vaccinated with *B. abortus* S19Δ*tcp*B corresponded with the degree of splenomegaly in these mice relative to those vaccinated with *B. abortus* S19 or in the naïve controls. This effect is attributed to $CD4^{+}$T memory cells as it has been previously reported that IFN_{γ} secretion is related to $CD4^{+}$T cells levels [169]. This evidence strongly suggests that the presence of *Tcp*B restricts vaccine efficacy and deletion of *Tcp*B should be pursued to improve vaccine efficacy. Reduced spleen bacterial burden following *B. abortus* 2308 challenge in *B. abortus* S19Δ*tcp*B vaccinated mice is consistent with improved protective efficacy of the LAV candidate.

A second TIR protein encoded by the locus (BruAbi0725), designated Btp2 has recently been identified in *Brucella*, and has been found to bind to and abbrogate NF-κB mediated cytokine production [32]. The mechanism of action has been defined as inhibition of binding to MyD88 and subverting TLR signaling function [32]. Btp-2 deletion mutants in wild type *B. abortus* exhibited increased expresion of proinflammatory cytokiens in immunocompromised mice [32,86]. This extends the impact of TIR protein modulation of inflammatory response, a key factor in vaccine efficacy. However, deletion of TIR in wild type strains does not significantly alter the virulence of bacteria [58]. *tcp*B deletion enhances the immunogenic properties of LAV while preserving most of the pathogen invasive properties. This, phenomena can be exploited in live attenuated vaccines to improve efficacy while maintaining safety. Further studies need to be performed to determine the effect of Btp2 gene deletion in live attenuated bacterial vaccines.

While it is known that cell mediated immunity and associated cytokines are responsible for controlling *Brucella* infection, the correlates of protection have not been adequately defined. In addition it is difficult to study numerous interactions of the components of the immune system *in vivo*. Current studies to identify new vaccine candidates involve use of animals housed in expensive BSL-3 conditions that are challenged with virulent organisms after vaccination. The development of an *in vitro* system that models *in vivo* responses would facilitate the development of *Brucella* vaccines and the study of brucellosis control mechanisms besides reducing the number of animals and cost of vaccine trials. The development of *in vitro* preclinical assays that mimic in *vivo* efficacy of *Brucella* vaccines not been developed. In order to accelerate development of *Brucella* vaccines, we have initiated development of an *in vitro* functional assay as a correlate of protective activity of *Brucella* vaccines. In this assay, *Brucella* protection was evaluated by targeting the reduction of *Brucella abortus* 2308 growth within macrophages. Splenocytes obtained from mice vaccinated with vaccines *B. abortus* S19 or *B. abortus* S19ΔtcpB or naïve mice were used as effectors and co-cultured with *B. abortus* 2308 infected macrophages. We observed that following one to three days of incubation; the intramacrophage replication of *Brucella* was dramatically reduced. Importantly, splenocytes obtained from mice vaccinated with *B. abortus* S19Δ*tcp*B exhibited enhanced killing activity against *B. abortus* 2308 infected macrophages as compared to those obtained from animals vaccinated with *B. abortus* S19. This result suggests that the splenocyte killing activity may be associated with *in vivo* protective ability. Supernatants obtained from the assays were also evaluated for levels of pro-

inflammatory cytokine Th1 cytokine IFNγ and anti-inflammatory Th2 cytokine IL5. Supernatants obtained from the co-cultures that exhibited reduced bacterial burdens had higher levels of IFNγ cytokines and virtually undetectable levels of IL5 cytokines. This is in agreement with the role of IFNγ in promoting intramacrophage killing of *Brucella* and the subsequent low levels of IL-5. Inhibition of intramacrophage burdens of Brucella was significantly reduced on day one following pre incubation with splenocytes despite minimal levels of IFN- γ in the supernatants, pointing towards an alternative killing mechanism deployed in the assay. A possibility of other mechanisms includes expression of other pro inflammatory cytokines and chemokines.

5.1 Future directions

*Tcp*B occupies a central control point in modulating the LAV *Brucella* induced host immune responses. There is need to quantitate the relative contribution of each action of TcpB to immune subversion. This will aid in explaining the pathogenesis of *Brucella* and assist in more logically designed vaccines and chemotherapeutic agents. Additionally, other *Brucella* effectors like T4SS VceC seem to act in contravention to TcpB by activating the UPR and enhancing cytokine expression [97]. It may be possible that the timing and balance of expression between these effectors is different so that each activity is designed to achieve a specific goal at a certain point in the cycle of the pathogen. The overall mechanism by which TcpB causes ER restructuring and subsequent activation of UPR has not been experimentally determined. Paclitaxel, a cancer drug also causes microtubule restructuring at low concentrations, like TcpB

[170]. A comparison of the two on the effects of the cell cycle could shed light on the mechanism of TcpB action. In conclusion, tcpB deletion provides a good secondary deletion that can be made in the current available *Brucella* LAV as a means of improving efficacy. This will remove the immune subversive action that may compromise the vaccine as well as provide the much needed adjuvant effect to enhance efficacy and longevity of protection. In addition, the inherent safety of the vaccine will not be compromised. The results of the co culture assay may demonstrate a practical and reproducible way of assessing *Brucella* vaccines. Vaccinated animals can serve as a source of effector cells that can be used to probe activity against wide range of targets (macrophages infected with a variety of bacteria). Vaccine specific effector cells can then be clonally expanded and stored. The spectrum of effector cells can then be used in future to probe multiple targets as a test for vaccine efficacy. Clonally expanded T cells can be used to identify specific epitope targets obtained from pathogen peptides in an effort define the responding MHC class I or class II epitopes. These epitopes could then be designed as potential peptide vaccines. Further development of this assay will be necessary for testing multiple vaccines to enhance application of the system to *Brucella* LAV. Such an assay could then be used to predict vaccine efficacy while reducing the time and cost of vaccine testing.

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