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Construction, characterization, and evaluation of CLH001 as a vaccine candidate against respiratory glanders

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Construction, characterization, and evaluation of CLH001 as a vaccine candidate against respiratory glanders

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Dedication

To my mom, Harry, and Karin, your unwavering love, support, and encouragement have helped sustain me through this journey.

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Construction, characterization, and evaluation of CLH001 as a vaccine candidate against respiratory glanders

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Burkholderia mallei is the causative agent of glanders, an incapacitating disease with high mortality rates in respiratory cases. Its endemicity and ineffectual treatment options emphasize its public health threat and highlight the need for a vaccine. In this study, we constructed and characterized *B. mallei* Δ*tonB* Δ*hcp1* (CLH001), a strain deficient in iron uptake and type six secretion functions, and investigated its ability to protect against acute respiratory glanders infection. When compared to wild-type (wt), CLH001 exhibited decreased growth kinetics in both culture media (LBG) and RAW 264.7 murine macrophages. Additionally unlike wt, CLH001 was deficient in Hcp1 production and was unable to induce multinucleated giant cells (MNGC) formation in both phagocytic and non-phagocytic cell lines. Intranasal (i.n.) administration of CLH001 ($1.5x10^4$ CFU) to BALB/c and NSG mice resulted in 100% survival with no detectable colonization or abnormal histopathology in the lungs, liver or spleen of vaccinated mice. BALB/c mice immunized i.n. with $1.5x10^5$ CFU of CLH001 in a prime/boost regimen showed full protection post-

challenge with $1.5x10^4$ CFU of *B. mallei lux* wt strain. Organs from surviving mice were clear of bacterial colonization and histopathological abnormalities. Immunized mice showed high *B. mallei*-specific IgG serum titers and a Th1-biased response (IgG2a:IgG1 ratio = 4.0), a good predictor of protection. Additionally, pre-challenge sera displayed significant bactericidal activity over naïve serum (p=0.0062). Vaccinated BALB/c mice were also significantly protected (87.5% survival; p= < 0.0001) against higher dose ($3.5x10^5$ CFU) of *B. mallei* 23344 challenge. Our studies show that CLH001 is attenuated and safe, and effective at providing protection against lethal *B. mallei* challenge. CLH001 is not only a viable vaccine platform for advancement into pre-clinical studies, but also represents the first Tier 1 Select Agent-excluded *B. mallei* strain.

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wt	wild-type
Mb	megabase pair
ORF	open reading frame
GC	guanine-cytosine
IS	insertion sequence
SSR	simple sequence repeat
LPS	lipopolysaccharide
T3SS	type III secretion system
T6SS-1	type VI secretion system cluster 1
OIE	World Health Organization for Animal Health
CPE	cytopathic effect
HSV	herpes simplex virus
CMV	cytomegalovirus
HIV	human immunodeficiency virus
HHS	Department of Health and Human Services
FDA	Food and Drug Administration
s.c.	subcutaneous
DD3008	*Burkholderia mallei* 23344 Δ*wcbB* capsular mutant strain
i.p.	intraperitoneal
LD_{50}	lethal dose 50%
CFU	colony forming units

IgG	immunoglobulin G
Th2	T helper cell 2
IgG2a	immunoglobulin G subclass 2a
IgG1	immunoglobulin G subclass 1
Th1	T helper cell 1
IL-12	interleukin 12
IFNγ	interferon gamma
TNFα	tumor necrosis factor alpha
Q12h	every 12 hours
CpG ODN	cytosine-phosphate-guanine oligodeoxynucleotide
ISCOM	immune-stimulating complex
i.n.	intranasal
AuNP	gold nanoparticle
AuNP-LPS	AuNP conjugated to purified LPS
IgM	immunoglobulin M
AuNP-LPS-FliC	AuNP-LPS coupled with recombinant *Burkholderia pseudomallei* FliC
NHP	non-human primate
PBS	phosphate buffered saline
BALF	bronchial alveolar lavage fluid
LEE	linear expression elements
SR1	*B. mallei* 23344 ΔBMAA0437-BMAA0497, sucrose-resistant strain
ILV1	*B. mallei* SR1 Δ*ilvI*, branched chain amino acid auxotroph
TMM001	*B. mallei* 23344 Δ*tonB*, iron acquisition deficient strain

CSM001	*B. mallei lux* strain
T6SS	type VI secretion system
Hcp	hemolysin co-regulated protein
VgrG	valine-glycine repeat protein G
gp	gene product
MNGC	multinucleated giant cell
LB	Luria-Bertani
CDC	Centers for Disease Control and Prevention
BSL3	biosafety level 3
USDA	U.S. Department of Agriculture
ABSL3	animal biosafety level 3
LBG	LB containing 4% glycerol
LBG with $FeSO_4$	LBG containing 200μM $FeSO_4$
CLH002	*B. mallei* Δ*hcp1*, T6SS deficient mutant
CLH001	*B. mallei* Δ*tonB* Δ*hcp1*, iron acquisition and T6SS deficient mutant
Km	kanamycin
YT	yeast extract and tryptone
OD_{600}	optical density at a wavelength of 600nm
ANOVA	analysis of variance
M9G	M9 minimal salts broth supplemented with 0.4% glucose
SDS	sodium dodecyl sulfate
PVDF	polyvinyl difluoride
TBS-T	Tris-buffered saline with Tween 20

HRP	horseradish peroxidase
ECL	enhanced chemiluminescence
RAW 264.7	mouse leukemic monocyte macrophages
HeLa	human cervical carcinoma cells
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
MOI	multiplicity of infection
Gm	gentamicin
AG	aminoguanidine
NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice
TMB	tetramethylbenzidine
OD	optical density
mAb	monoclonal antibody

INTRODUCTION

Chapter 1. *Burkholderia mallei*

THE PATHOGEN

Burkholderia mallei, the causative agent of glanders, is a Gram-negative, non-motile, facultative intracellular, obligate mammalian pathogen. Over time this bacterium has undergone several genera re-classifications including: *Bacillus*, *Corynebacterium, Mycobacterium, Loefflerella, Pfeifferella, Malleomyces, Actinobacillus,* and *Pseudomonas*. Its current genus, *Burkholderia*, was assigned in 1992 based on a number of criteria including: biochemical properties, cellular lipid and fatty acid composition, DNA-DNA homology, and 16S rRNA sequencing (Yabuuchi *et al*, 1992).

B. mallei possesses a 5.8 megabase pair (Mb) genome comprised of two large chromosomes. These chromosomes contain 5,535 protein-encoding open reading frames (ORFs) and are rich (68.5%) in guanine-cytosine (GC) residues. Chromosome 1 is 3.5 Mb and is mostly comprised of housekeeping genes related to metabolism, macromolecule biosynthesis and replication; whereas chromosome 2 is slightly smaller (2.3 Mb) and primarily contains accessory function genes (Holden *et al*, 2004; Nierman *et al*, 2004). Its virulence genes are distributed across both chromosomes.

Genomic comparison demonstrates that, in addition to sharing the same genomic organization (2 circular chromosomes), *B. mallei* has a high degree of sequence homology with *B. pseudomallei* and *B. thailandensis*, which are described as having 99.1 and 94.0% genomic homology, respectively (Godoy *et al*, 2003; Nierman *et al*, 2004; Kim *et al*, 2005). Based on their genomic similarities, it is theorized that these three *Burkholderia* species

recently evolved from a common ancestor through a two-part divergent evolutionary process. *B. thailandensis* is thought to have split from the *B. mallei*/*B. pseudomallei* common ancestor, in a process resulting in the loss of virulence genes and pathogenicity. Later, when *B. mallei* split from *B. pseudomallei*, it is thought to have undergone reductive evolution, losing or disabling genes required for environmental survival, therefore limiting its reservoirs to mammalian hosts only (Kim *et al*, 2005).

The continuing divergence between *B. mallei* and *B. pseudomallei* in response to adaptive pressures has been attributed to the fact that the *B. mallei* genome encodes for a large number of insertion sequences (ISs) and simple sequence repeats (SSRs). ISs are linked with insertion, deletion, and inversion mutations, whereas SSRs are prone to frameshift mutations. Taken together, these account for a large percentage of the approximately 1,430 *B. pseudomallei* genes that are either not present or are mutated in the *B. mallei* genome (Holden *et al*, 2004; Nierman *et al*, 2004). In fact, key phenotypical differences between *B. mallei* and *B. pseudomallei*, including *B. mallei*'s loss of chemotaxis and motility, are attributed to an SSR frameshift in a chemotaxis gene and IS insertions in flagellar *fliP* and *motB* genes (Nierman *et al*, 2004).

Virulence in *B. mallei* is described as being multifactorial. Currently, the function has not been determined for a significant portion (~40%) of *B. mallei* genes, therefore it is likely that not all virulence genes/factors have been identified. Known virulence factors in this organism include: antibiotic resistance (Nierman *et al*, 2004), extracellular capsule (DeShazer *et al*, 2001), lipopolysaccharide (LPS) O-antigen (Burtnick *et al*, 2002), quorum sensing (Ulrich *et al*, 2004), the animal pathogen-like type III secretion system (T3SS)

(Ulrich & DeShazer, 2004; Ribot & Ulrich, 2006), and the type VI secretion system cluster 1 (T6SS-1) (Schell *et al*, 2007; Burtnick *et al*, 2010).

Chapter 2. Glanders

B. mallei is the etiological agent of glanders, a disease primarily affecting solipeds (e.g., horses, donkeys, and mules), but with rare cases occurring among humans (Neubauer *et al*, 2005; Galyov *et al*, 2010; Van Zandt *et al*, 2013). Glanders is considered one of the oldest known diseases in solipeds, with anecdotal accounts of equine glanders infection being captured in the writings of Aristotle and Socrates as early as 400 BC. *B. mallei* was first identified as the causative agent of glanders in 1882 by Loeffler and Schutz, and its depiction in numerous historical writings from various cultures suggest that the disease had a worldwide distribution. In 1890, Helman, Kalning, and Pearson are attributed with developing the mallein test, a purified protein derivative test, which was the first serodiagnostic test for glanders detection in solipeds (Hagebock *et al*, 1993). Mallein testing was quickly adopted in numerous countries and became an integral part of internationally coordinated "test and slaughter" methods. These methods are credited with the eradication of glanders in Great Britain in 1925, the US in 1934, Canada in 1938, and Western Europe in 1965 (Derbyshire, 2002; Khan *et al*, 2013; Verma *et al*, 2014). Worldwide eradication efforts coordinated by the World Organization for Animal Health (OIE), include mandatory testing of international imports and exports of equids and equid products, and mandatory reporting of glanders positive solipeds. However, despite these ongoing efforts for eradication, pockets of endemicity still exist in Africa, Asia, the Middle East, and South America (Khan *et al*, 2013). In light of recent equid outbreaks in India (Malik *et al*, 2012), Pakistan (Hornstra *et al*, 2009) and Bahrain (Scholz *et al*, 2014); glanders has been classified as a re-emerging disease. Because of the potential for false

negative tests in pre-symptomatic equids, international trade of equids remains a possible contributor to disease outbreaks by reintroducing glanders into disease free regions (Khan *et al*, 2013; Verma *et al*, 2014). Although rare, naturally acquired human cases occur in endemic areas, particularly among persons with prolonged exposure to solipeds (M'Fadyean, 1904; Neubauer *et al*, 2005; Malik *et al*, 2012). However, a small number of cases have also been reported among laboratory workers (Howe & Miller, 1947; Srinivasan *et al*, 2001).

Glanders is a debilitating disease that is often fatal in both humans and solipeds. The major routes of transmission in glanders are cutaneous and respiratory. Disease course and severity is route dependent (M'Fadyean, 1904; Gregory & Waag, 2007; Whitlock *et al*, 2007; Van Zandt *et al*, 2013). Cutaneous cases typically result in a more gradual progression of clinical symptoms, often beginning with the appearance of mucopurulent papules or abscesses at the infection site. In unresolved cutaneous cases, the bacteria are able to utilize the lymphatic system to disseminate to soft tissues, resulting in organ abscesses and, in some cases, septicemia. Respiratory infection is characterized by a more rapid onset of symptoms, including: fever, wasting, lymphadenopathy, pulmonary abscesses, pneumonia, disseminated infection of organs and ultimately septicemia (Howe & Miller, 1947; Srinivasan *et al*, 2001; Van Zandt *et al*, 2013; Waag & DeShazer, 2005; Robbins, 1906). Because of the high incidence of septicemia following respiratory *B. mallei* infection, fatality rates in human cases of respiratory glanders have been estimated at 90% without treatment and 50% with aggressive antibiotic therapy (Batts-Osborne *et al*, 2001; Currie, 2010; Van Zandt *et al*, 2013). Although human cases are rare, the recommended post-exposure treatment regimen for glanders is long term antibiotic therapy

consisting of 10-14 days of intravenous administration of ceftazidime, meripenem, or imipenem; followed by 12-20 weeks of orally administered trimethoprim-sulfamethoxazole, doxycycline, or amoxicillin-clavulanate (Van Zandt *et al*, 2013).

B. mallei has been shown to exhibit a tropism for lymph nodes, lung, liver, and spleen; this has been demonstrated in autopsies of human glanders cases as well as necropsies following naturally acquired and experimentally induced animal cases. Abscesses in the lung, liver and spleen are described as being similar to those observed in miliary tuberculosis; however, abscesses resulting from *B. mallei* infection undergo more rapid necrosis. Multiple historical accounts describe glanders tubercles in these tissues as having a necrotic center ringed with epithelioid and lymphoid giant cells (M'Fadyean, 1904; Robbins, 1906; Duval & White, 1907). Although giant cell formation is a common cytopathic effect (CPE) in viral infections including herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency virus (HIV), this phenomenon is rare among bacteria and has only been described in *B. mallei*, *B. pseudomallei*, *B. thailandensis*, and *Mycobacterium tuberculosis* infections (Kespichayawattana *et al*, 2000; Suparak *et al*, 2005; Allwood *et al*, 2011).

The use of *B. mallei* as a bioweapon was documented in World War I and II (Wheelis, 1998; Hawley & Eitzen, 2001; Lehavi *et al*, 2002; Riedel, 2004; Waag & DeShazer, 2005; Gregory & Waag, 2007). More recently, it was allegedly used by the Former Soviet Union against the Mujahedeen in Afghanistan (Alibek & Handelman, 1999). Its historical use as a bioweapon, amenability to aerosolization, low infectious dose, ability to cause debilitating illness, and high-level, intrinsic antibiotic resistance make *B. mallei* a top candidate for bioterrorism use (Hawley & Eitzen, 2001; Gregory & Waag, 2007;

Galyov *et al*, 2010; Silva & Dow, 2013). Because of its public health threat, the Department of Health and Human Services (HHS) has categorized *B. mallei* as a Tier 1 Select Agent and a priority for vaccine development (HHS and USDA, 2012).

Chapter 3. *B. mallei* vaccine history

B. mallei's potential for malicious use and lack of effectual treatment options highlight the need for an effective vaccine to prevent infection, particularly among military personnel deployed to endemic areas, first responders, and *Burkholderia* researchers. There are currently no Food and Drug Administration (FDA)-approved vaccines against *B. mallei.* A number of vaccine strategies, such as inactivated whole-cell killed, subunit, and live attenuated vaccines have been tested against *B. mallei* with limited success. While some protection can be generated against lethal infection, none of the vaccines tested have been shown to provide sterile immunity. The following is a summary of the glanders vaccine candidates that have been evaluated in animal models.

INACTIVATED WHOLE-CELL VACCINES

Inactivated *B. mallei*, whether heat-killed or irradiated, has been shown to provide significant protection against high dose challenge in terms of survival, but does not prevent organ colonization. Amemiya *et al* immunized BALB/c mice (with or without alhydrogel adjuvant) with two subcutaneous (s.c.) doses containing 100µl (1µg/µl) of heat-killed *B. mallei* 23344, irradiation-inactivated *B. mallei* 23344 or irradiation-inactivated *B. mallei* 23344 Δ*wcbB* capsular mutant strain (DD3008). Three weeks after the last vaccination, mice were challenged intraperitoneally (i.p.) with either a low dose (34 lethal dose 50% (LD_{50}) or $2.4x10^7$ colony forming units [CFU]) or a high dose (329-400 LD_{50} or $2.3\text{-}2.8x10^8$ CFU) of *B. mallei* 23344, and monitored for survival for 21 days. Irradiated *B. mallei* provided the greatest protection against high dose challenge, with 58% of mice surviving. At lower doses, survival rates were 80% for mice vaccinated with heat-killed *B. mallei* and

100% for both the irradiated and capsule mutant *B. mallei* vaccinated groups. At both doses, necropsy and CFU enumeration revealed that all surviving mice exhibited splenomegaly and had high splenic bacterial burdens. Analysis of serum from vaccinated mice showed that all three vaccine preparations generated weak immunoglobulin G (IgG) titers. The capsule and heat-killed *B. mallei* both produced a T helper cell 2 (Th2)-biased antibody response (immunoglobulin G subclass 2a (IgG2a): immunoglobulin G subclass 1 (IgG1) ratios of 0.25 and 0.5, respectively), whereas irradiated *B. mallei* produced a mixed T helper cell 1 (Th1)/Th2 response (IgG2a:IgG1 ratio =1). This Th2-bias was observed in mice immunized with and without the adjuvant alhydrogel; therefore, it was surmised that a Th1-like IgG subclass response was required for protection (Amemiya *et al*, 2002).

A follow-on study by this group examined the effects of adding varying concentrations (0.10, 0.25, 0.50, or 1.0 μg) of murine interleukin 12 (IL-12) to their irradiated *B. mallei* vaccine formulation (100μl irradiated bacteria + 100μl alhydrogel). In this study, groups of BALB/c mice were given two s.c. doses of the irradiated *B. mallei* vaccine with and without murine IL-12, prior to i.p. challenge with 143 LD_{50} ($1x10^8$ CFU) of *B. mallei* 23344. Survival was monitored for 21 days, and, at the challenge dose tested, all animals receiving irradiated *B. mallei* + alhydrogel succumbed to infection. However, increased survival (ranging from 20-60%) was observed in animals receiving vaccines supplemented with IL-12. Supplementation with 0.1 and 0.5 μg of IL-12 provided the best protection, with 60% survival in both groups. However, it is important to note that while this vaccine formulation provided significant protection against challenge, the organ pathology and bacterial colonization of surviving animals were not determined. Interestingly, although IgG2a:IgG1 ratio was not increased dramatically, animals given

supplemental IL-12 exhibited a two-fold increase in total IgG over animals receiving irradiated *B. mallei* + alhdrogel only. Re-stimulated splenocytes from animals receiving IL-12 supplementation also displayed a significant increase in proliferation and IFNγ production in comparison to animals vaccinated without IL-12 supplementation (Amemiya *et al*, 2006). Taken together these results support the idea that both cell mediated and humoral responses are required for protection against *B. mallei* infection.

Whitlock *et al* also evaluated the efficacy of a heat-killed *B. mallei* vaccine. BALB/c mice receiving a single i.p. vaccination of $1x10^5$ CFU heat killed *B. mallei* exhibited a 40% survival at 10 days post-challenge with $2x10^7$ CFU (~20 LD_{50}) of *B. mallei* 23344. This study also evaluated the role of major cell populations ($B220^+$ B cells, $CD4^+$ or $CD8^+$ T-cells) and key cytokines (interferon gamma (IFNγ) and tumor necrosis factor alpha [TNFα]) in survival. Depletion studies showed that while only a 50% increase in mortality was observed in mice where $CD4^+$ or $CD8^+$ T-cells were depleted, 100% mortality was observed in mice where B220+ B-cells or cytokines IFNγ or TNFα were depleted (Whitlock *et al*, 2008).

Finally, a study by Waag *et al* examined the combined effects of vaccination with heat killed *B. pseudomallei* followed by post-exposure administration of antibiotic therapy in protection against lethal aerosol challenge with glanders. In this study, groups of BALB/c mice were administered i.p. prime and boost vaccinations of 100 μg of heat-killed *B. pseudomallei* 1026b. Vaccinated mice were aerosol challenged 6 weeks post-boost with ~3 LD_{50} *B. mallei*, and survival was monitored 21 days. During the post-challenge period, one group of mice remained untreated while the other groups were administered one of three antibiotics recommended for glanders treatment (azithromycin (15 mg/kg; every 12

hours (Q12h); i.p), moxifloxacin (16 mg/kg; Q12h; i.p.), or sulfamethoxazole (40 mg/kg)/trimethoprim (40 mg/kg; Q12h; i.p.). The heat-killed only control group exhibited survival rates ranging from 40-80%, but all surviving mice showed splenic colonization. The survival and splenic colonization of treated mice was dependent on the antibiotic received, time at which therapy was administered, and length of treatment. Survival rates in animals receiving post exposure therapy ranged from 70-90% and between 25-50% of these animals had no detectable splenic colonization. Specifically, vaccinated mice given moxifloxacin at 3 days post exposure for 3 or 5 days exhibited the highest level of protection (100% survival), with 50% of animals showing no detectable bacteria in their spleens (Waag, 2015). This study showed that post-exposure antibiotic administration could be utilized to increase the effectiveness of a partially protective vaccine.

SUBUNIT VACCINES

B. mallei subunit vaccines have also been tested, but to date, no subunit vaccine has generated complete protection. Whitlock *et al* tested various combinations of recombinant proteins (BimA, BopA, Hcp1, and FliC) coupled with the adjuvants cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN) and immune-stimulating complex (ISCOM). Mice immunized intranasally (i.n.) with recombinant proteins exhibited significant protection against i.n. challenge with a 2 LD_{50} dose ($1x10^5$ CFU) of wild-type *B. mallei.* Groups receiving BimA or BopA proteins displayed the greatest protection, with 100% survival at day 21. However, *B. mallei* was recovered in the spleens of all animals indicating incomplete clearance (Whitlock *et al*, 2010).

Gregory *et al* developed and evaluated a series of gold nanoparticles (AuNPs) conjugated to purified LPS (AuNP-LPS) and covalently linked to one of three recombinant

carrier proteins (TetHc, Hcp1, or FliC) to be tested as vaccines in the respiratory murine glanders model. Different combinations of the AuNPs were delivered i.n. to BALB/c mice prior to a lethal challenge with *B. mallei* 23344. At a low challenge dose (~2 LD_{50}), LPS, AuNP-LPS and AuNP-LPS coupled to TetHc, Hcp1, or FliC provided similar levels of protection; however, at the highest challenge dose (6.5 LD_{50}) groups receiving AuNP coupled to TetHc, Hcp1, or FliC conferred increased protection, and had significantly lower splenic bacterial burdens. Further, sera from the protein-coupled nanoglycoconjugate groups contained significantly higher LPS-specific IgG and immunoglobulin M (IgM) levels than other control groups (Gregory *et al*, 2014).

A follow up study by Torres *et al* evaluated the protective capacity of AuNP-LPS coupled with FliC (AuNP-LPS-FliC) in a non-human primate (NHP) model of respiratory glanders. Groups of NHPs were vaccinated with either phosphate buffered saline (PBS) or AuNP-LPS-FliC prior to aerosol challenge with $1.4x10^4$ CFU of *B. mallei* 23344. AuNP-LPS-FliC vaccinated NHPs demonstrated higher survival rates (50% survival) compared to NHPs given PBS alone (33.3% survival). Organs extracted from surviving control animals contained significant bacterial burden; however, no detectable bacteria were present in either the bronchial alveolar lavage fluid (BALF) or organs (spleen, liver and lungs) of AuNP-LPS-FliC vaccinated animals. Serum LPS-specific IgG titers were significantly increased in AuNP-LPS-FliC vaccinated NHPs over those control animals. These results demonstrated that the AuNP-LPS-FliC vaccine was immunogenic and provided protection against aerosolized glanders infection (Torres *et al*, 2015).

While subunit vaccines tested to date have significantly increased survival rates in mice and NHPs challenged via the respiratory route, none have provided complete

protection. It is likely that successful subunit vaccination will require a multivalent approach in which many antigens, including ones not yet characterized, will need to be included for sterilizing immunity (Titball, 2008; Silva & Dow, 2013).

DNA VACCINES

Whitlock *et al* constructed linear expression elements (LEEs) from 5,760 of *B. mallei*'s ORFs and ensured genetic activity by attaching promoter and terminator sequences. These ORFs were combined into 144 pools using a two dimensional matrix strategy that would allow for a determination of which single ORFs were responsible for protection in a single animal experiment. Each ORF pool was bound to positively charged AuNPs and delivered via gene gun into the ear pinnae of BALB/c mice using a prime and boost strategy. Twelve weeks post-boost, the mice were i.n. challenged with $2LD_{50}$ ($1x10^5$ CFU) of *B. mallei* 23344 and their survival was monitored for 21 days. Eight of the ORF pools provided survival rates of 50% or greater. By analyzing the ORF pool composition, twelve genes associated with increased survival were identified. Based on a bioinformatics analysis, five of these genes were predicted as being potential antigens (BMAA0712, BMAA0768, BMA2821, BMA2804, and BMA0816). Recombinant proteins from each of these five genes were expressed, purified and administered i.p. (concentration/dose not specified) to groups of BALB/c mice. Three weeks post-boost the mice were challenged with 2 LD_{50} of *B. mallei* 23344 and survival was monitored for 8 days. Only one recombinant protein (BMAA0768) extended survival to day 8 (25% survival); however, two additional proteins (BMA2821 and BMA0816) did exhibit extended time to death over controls. While immunization with ORF pools provided significant protection against challenge, it is unclear what effects this vaccination strategy had on colonization, as

bacterial burden was not determined. While the vaccination approaches described here did not provide full protection, this study is significant because it was the first to utilize a global approach to screen for potential vaccine candidates, and the first to evaluate the efficacy of a DNA vaccine in a mouse model of respiratory glanders (Whitlock *et al*, 2011).

LIVE ATTENUATED VACCINES

Live attenuated vaccines are currently regarded as the most viable vaccine strategy against *B. mallei* due to their ability to elicit both cellular and humoral immune responses (Bondi & Goldberg, 2008; Mott *et al*, 2011; Choh *et al*, 2013; Silva & Dow, 2013). DeShazer *et al* constructed a *wcbB* capsule mutant in *B. mallei* 23344 (DD3008). This strain was found to be avirulent in Golden Syrian hamsters and BALB/c mice when administered via i.p. and aerosol challenge, respectively. However, this attenuated strain provided little protection against challenge: BALB/c mice vaccinated with 9.2×10^3 to 7.67×10^5 CFU of DD3008 exhibited only 20% survival at day 21 against challenge with 21 LD_{50} (1.8×10^4 CFU) of *B. mallei* 23344. Although it is not clear what dose(s) provided protection, the study concluded that a single dose of DD3008 was not effective as a live attenuated vaccine (DeShazer *et al*, 2001).

Ulrich *et al* evaluated the protective capacity of three *B. mallei* mutants deficient in quorum sensing. The mutant strains included two *B. mallei luxI* mutants (*bmaI1* and *bmaI3*) and one *B. mallei luxR* mutant (*bmaR5*). In this experiment BALB/c mice were received a prime and two boost aerosol vaccination with 1.0×10^4 CFU of *bmaI1*, *bmaI3*, or *bmaR5* at 9 days post second boost mice were aerosol challenged with 10 LD_{50} (1.0×10^4 CFU) of *B. mallei* 23344 and monitored for survival for 11 days. Neither the *bmaI1*, nor *bmaR5* provided any protection against B. *mallei* challenge as mean time to death was no different

from what was observed in unvaccinated mice, but bmaI3 was found to be partially protective as 30% of the mice survived to the 11 day experimental end point (Ulrich *et al*, 2004).

A subsequent study by Ulrich *et al* performed a head-to-head vaccination study of DD3008 and a Δ*ilvI* branched chain amino acid auxotroph (ILV1) constructed in *B. mallei* 23344 ΔBMAA0437-BMAA0497, sucrose-resistant strain (SR1). In this study, BALB/c mice were given a prime and single boost aerosol dose of either of $7.4x10^4$ CFU ILV1 or $1.2x10^3$ CFU DD3008. Bacteria were undetectable in the organs at 7 days post-boost, suggesting that the vaccine strains had been cleared from the organs of both groups. At day 21 post-boost, the remaining mice were challenged with a high, whole body aerosol dose of *B. mallei* 23344 (340 LD_{50} for DD3008 vaccinated group and 440 LD_{50} for ILV1 vaccinated group) and monitored for 30 days. While all mice immunized with DD3008 died by day 5 post-challenge, mice vaccinated with ILV1 had survival rates of 45% and 25% at days 14 and 30 post challenge, respectively. However, necropsy and CFU enumeration of surviving ILV1 mice revealed that hepatic and splenic abscesses were present in all mice and that their spleens were heavily colonized (>$1x10^5$ CFU of challenge strain). A subsequent analysis of serum showed that while both groups developed strong post boost *B. mallei*-specific IgG titers (32,000 for DD3008 and 53,333 for ILV1), the groups differed in their IgG2a:IgG1 ratios. Vaccination with the DD3008 strain resulted in an IgG2a:IgG1 ratio of 0.3, whereas ILV1 vaccination resulted in a ratio of 4.7. Based on these findings, the authors concluded that complete clearance of vaccine strains by day 7 might not be sufficient for the generation of a comprehensive immune response.

Additionally, their findings supported the idea that a Th1-biased response (IgG2a:IgG1 ratio >1) is required for protection (Ulrich *et al*, 2005).

Bandara *et al* evaluated another live attenuated strain, a Δ*ctpA* bacterial cell envelope integrity deficient strain constructed in the *B. mallei* SR1 strain. This *ctpA* mutant vaccine strain was administered to CD1 mice at a dose of $4.4x10^5$ prior to i.p. challenge with 1.1 LD_{50} ($6.6x10^5$ CFU) of *B. mallei* 23344. A 75% survival rate was noted in Δ*ctpA* vaccinated mice at day 15; however, once again surviving mice exhibited significant splenomegaly and varying amounts of *B. mallei* splenic colonization. However, it is not clear whether this colonization was attributed to the vaccine or challenge strains, or both. Evaluation of serum revealed that Δ*ctpA* vaccinated mice developed an IgG2a:IgG1 ratio of 1, indicating a mixed Th1/Th2 response (Bandara *et al*, 2008). This, along with the low challenge dose and partial survival provided by Δ*ctpA* over the short post challenge period, supports the previous conclusion that a mixed Th1/Th2 response is not sufficient for protection.

Bozue *et al* evaluated another live attenuated strain, a Δ*tssN* type 6 secretion system cluster 1 (T6SS-1) deficient strain constructed in the *B. mallei* SR1 strain. In this study BALB/c mice were prime and single boost aerosol vaccinated with $1.3x10^5$ and $2.3x10^4$ CFU of *B. mallei* Δ*tssN*, respectively. At day 21 post-boost, the mice were aerosol challenged with $4.3x10^4$ CFU (43 LD_{50}) of *B. mallei* 23344 and monitored for 21 days. At 21 days post-challenge, a 67% survival rate was noted in Δ*tssN*-vaccinated mice. However, it was noted that mice exhibited a significant decrease in weight following each vaccination (1.7 g and 3.6 g, respectively) and that the lungs, livers, and spleens of mice surviving challenge exhibited extensive histopathology and heavy *B. mallei* colonization. Further, it

was noted that prime and boost vaccination with Δ*tssN* generated a weak humoral response (mean reciprocal *B. mallei*-specific IgG total titer = 400), and a modest cellular response (Bozue *et al*, 2016).

Recently, *B. mallei* 23344 Δ*tonB, iron* acquisition deficient strain (TMM001) was constructed and evaluated as a live attenuated vaccine candidate in an acute inhalational glanders model. BALB/c mice were i.n. immunized with the TMM001 strain and i.n. challenged three weeks later with 22 LD_{50} ($1.5x10^5$ CFU) of *B. mallei lux* (CSM001). Mice vaccinated with TMM001 at doses of 1.5×10^5 and 1.5×10^4 CFU had survival rates of 100% and 75% at 50 days post challenge, respectively. However, necropsy and CFU enumeration of organs indicated that all mice had splenomegaly and splenic abscesses due to heavy colonization by the TMM001 vaccine strain. Analysis of serum from TMM001-vaccinated mice found that the vaccine generated a strong *B. mallei*-specific IgG titer with a Th1 bias (IgG2a:IgG1 ratio > 1.0) (Mott *et al*, 2015). This study was significant because it describes the first attenuated strain to provide 100% survival against *B. mallei* challenge. However, the persistence of TMM001 poses a significant safety concern. To address this, my project focused on incorporating an addition gene deletion into the TMM001 backbone in an effort to facilitate host clearance and reduce vaccine strain persistence while still maintaining immunogenicity.

Chapter 4. Type VI secretion system

Less than a decade ago, a novel bacterial secretion system, known as the type VI secretion system (T6SS) was described in *Pseudomonas aeruginosa* (Mougous *et al*, 2006) and *Vibrio cholera* (Pukatzki *et al*, 2006). Subsequent phylogenetic analyses revealed that this secretion system was highly conserved among Gram-negative bacteria, as T6SS clusters were identified in more than 25% of the genomes surveyed; additionally, many bacteria were found to contain multiple T6SS clusters (Bingle *et al*, 2008; Boyer *et al*, 2009). T6SSs have been shown to play a number of different roles in the pathogenesis of various pathogens. For example, in both *Aeromonas hydrophila* and *Vibrio cholerae*, the Vas T6SS has been implicated in macrophage killing as well as inhibition of classical activation of macrophages (Pukatzki *et al*, 2006; Suarez *et al*, 2010). In *Escherichia coli* K1, the Evf T6SS has been shown to be involved in adherence, cytoskeletal rearrangement, and invasion of human brain microvascular endothelial cells and has been implicated in this strains ability to cause meningitis (Zhou *et al*, 2012). In a mouse model, the Sci T6SS of *Salmonella enterica* serovar Typhimurium, was shown to be involved in dissemination. Additionally, this T6SS has been shown to be involved in intracellular replication and survival in macrophages *in vivo* (Mulder *et al*, 2012). Finally, as will be discussed later, T6SSs have also been shown to play a role key role in the virulence of *B. mallei* and *B. pseudomallei* (Schell *et al*, 2007). Although not as well studied, T6SSs have also been shown to play an important role in the interbacterial interactions required for niche adaptation. These interactions vary from symbiotic to competitive in both bacterial pathogens and non-pathogens (Jani & Cotter, 2010; Russell *et al*, 2014). The T6SS is a contact dependent, one-step secretion system that resembles an inverted bacteriophage-like

tail in both structure and function. Like bacteriophages, the T6SS machinery protrudes past the bacterial cell wall to puncture and inject effectors into target cells (Coulthurst, 2013). Interestingly, although numerous T6SSs have been studied in detail, effector molecules have only been identified in a small number of these (Silverman *et al*, 2012).

The T6SS is comprised of 13 conserved or core subunits required for functionality and a compliment of accessory proteins which varies by bacterial species (Zheng & Leung, 2007). The core components can be broken into four categories: 1) Membrane-embedded components which anchor the secretion system into the bacterial cell wall (TssJ, TssL, and TssM), 2) Proteins with bacteriophage structural homology (hemolysin co-regulated protein (Hcp), valine-glycine repeat protein G (VgrG), TssB (also known as VipA), TssC (also known as VipB), and TssE), 3) energizing protein (ClpV) and 4) Proteins of unknown function (TssA, TssF, TssG, and TssK) (Silverman *et al*, 2012; Coulthurst, 2013; Zoued *et al*, 2014). The structural similarities between bacteriophage and T6SS phage tail components, along with the similarities in assembly and organization, provide strong evidence that they co-evolved and indicate that Gram-negative bacterial genomes may have incorporated genes introduced by bacteriophages (Pell *et al*, 2009; Records, 2011). Because the structural and organizational similarities between bacteriophage and the T6SS phage tails (Illustration 1) have been used to develop the current model of T6SS function, these will be decribed briefly.

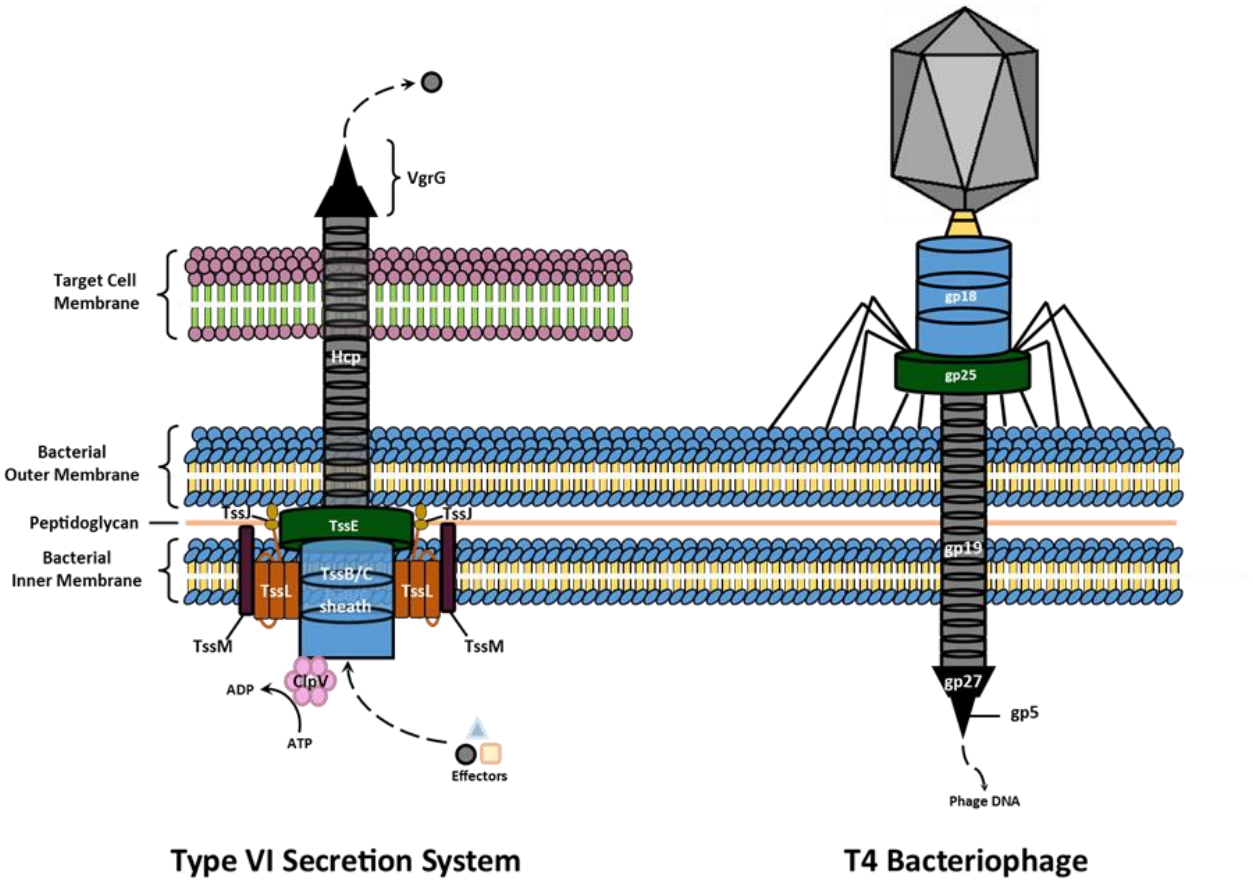


Illustration 1: Structural homology between Type VI Secretion System (T6SS) and T4 bacteriophage tail components. A side-by-side schematic drawing of a T6SS and a T4 bacteriophage. The core components of both the T6SS and the T4 bacteriophage are labeled with their gene products. T6SS proteins with structural and functional homology to phage proteins are colored the same as their T4 phage counterparts. Figure adapted from source (Records, 2011).

TssE forms an oligomer which attaches to the bacterial membrane and potentially serves as a scaffold for both TssB/TssC sheath complex and Hcp nanotube assembly. The TssE complex bears structural homology to the T4 baseplate, which is a complex of gene product (gp) 25 (Boyer *et al*, 2009). Although crystallographic comparison has not been performed, electron microscopy revealed that TssB and TssC assemble to form a large

tubular complex reminiscent of the T4 tail sheath (formed by polymerized gp18). The TssB/TssC tubule has an inner diameter that is large enough (10 nm) to accommodate the Hcp tube. The threaded topography of its inner ring is thought to be involved in propelling the Hcp tube and VgrG spike toward target cells like an auto injector upon contraction (Bonemann *et al*, 2009; Bonemann *et al*, 2010; Filloux, 2009). Hcp automatically hexamerizes into a ring-like structure with an inner diameter of 4 nm and an outer diameter of 8.5 nm. These hexamers stack in a head-to-tail fashion to form a nanotube with structural homology to the T4 phage tail tube, which is comprised of assembled gp19 subunits (Ballister *et al*, 2008; Leiman *et al*, 2009). Although it is widely accepted that this Hcp nanotube serves as a conduit for effector protein delivery, a recent study by Silverman *et al* suggest its role in effector secretion may be more active than previously thought. In this study it was shown that key residues on the tube's inner surface are involved in determining which proteins are secreted as well as aiding in the secretion process. (Silverman *et al*, 2013).

The current model of T6SS activity describes a four step process: 1) contact with target cell triggers assembly of VgrG-tipped Hcp nanotube and TssB/TssC sheath on TssE baseplate; 2) contraction of TssB/TssC sheath extends VgrG-tipped Hcp nanotube through cell membrane allowing VgrG to penetrate target cell; 3) effector delivery, and 4) ClpV recruitment. At the conclusion of this process, ATP hydrolyzing activity restores TssB/TssC complex to its resting state by retracting the Hcp nanotube (Basler *et al*, 2013; Zoued *et al*, 2014).

Chapter 5. Type VI Secretion System Cluster 1 and its Role in *B. mallei* Pathogenesis

The genomes of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* each have multiple type six secretion system (T6SS) clusters. *B. pseudomallei* has six clusters and *B. mallei* and B. thailandensis each have unique sets of five of the six clusters contained in *B. pseudomallei*. Four of these, (clusters 1, 2, 3, and 6) are conserved among all three species (Table 1). However, only the T6SS cluster 1 (T6SS-1) has been shown to be essential for pathogenesis in all three *Burkholderia* species. Schell *et al* demonstrated the role of the T6SS-1 in virulence by making a series of single, in-frame deletions in four highly conserved *B. mallei* T6SS-1 genes: *tssB*, *hcp1*, *tssD*, and *tssE*. When the mutants were administered i.p. to Syrian hamsters at ~100 LD_{50}, all survived (Schell *et al*, 2007).

Table 1: Type VI secretion system (T6SS) gene clusters present in *B. mallei*, *B. pseudomallei*, and *B. thailandensis*.

T6SS Cluster	*B. mallei*	*B. pseudomallei*	*B. thailandensis*
1	**Present**	**Present**	**Present**
2	**Present**	**Present**	**Present**
3	**Present**	**Present**	**Present**
4	**Present**	**Present**	**Absent**
5	**Absent**	**Present**	**Present**
6	**Present**	**Present**	**Present**

Table adapted from source (Schell *et al*, 2007)

In a similar study performed by Burtnick *et al*, *B. pseudomallei* K96243 T6SS *hcp* mutants were constructed and administered i.p. to Syrian hamsters. These *in vivo* studies showed that only Δ*hcp1* was highly attenuated (LD_{50} $>10^3$ CFU), whereas Δ*hcp2*-Δ*hcp6* mutants maintained wild-type lethality (LD_{50} <10 CFU) (Burtnick *et al*, 2011).

Finally, a study by Schwarz *et al* indicated that only T6SS-1 was required for virulence in *B. thailandensis*. In this study, five constructs containing multiple gene deletions within each T6SS cluster (1, 2, 3, 5, and 6) were generated in *B. thailandensis* E264. C57BL/6 mice were challenged with 10^5 CFU of wild-type or T6SS mutants and survival was monitored for 10 days. Only mice administered the T6SS-1 mutant survived. Additionally, when administered a construct in which all the T6SS clusters (except for T6SS-1) were mutated, all mice succumbed, demonstrating that T6SS clusters 2, 3, 5 and 6 were dispensable with respect to *B. thailandensis* virulence in mice (Schwarz *et al*, 2010).

Another important line of evidence supporting the role of the T6SS-1 in pathogenesis lies in its unique regulation. The expression of the T6SS, unlike the other 4 T6SS clusters, is regulated by VirAG a two-component sensor kinase system, and to a lesser extent, BMAA1517, an AraC-type regulator (Schell *et al*, 2007). While the T6SS-1 genes are expressed at low levels in nutrient rich media, they are upregulated following uptake by macrophages (Schell *et al*, 2007; Burtnick *et al*, 2010). This upregulation is dependent on internalization, a fact that has been demonstrated in numerous experiments in *B. mallei* and *B. pseudomallei*, including: co-localization assays (Burtnick *et al*, 2010), *in vitro* expression technology (Shalom *et al*, 2007), and measurement of gene transcription using real-time PCR following infection of macrophages with and without cytochalasin D treatment (Chen *et al*, 2011). These findings suggest that host conditions facilitate the

upregulation of the T6SS-1 during infection. A recent study by Burtnick *et al* showed that T6SS-1 expression is down-regulated in nutrient rich media, but upregulated in media containing low iron and zinc. It is therefore postulated that similar host conditions may result in T6SS-1 upregulation *in vivo*, as both of these divalent ions are maintained at low levels intracellularly (Burtnick & Brett, 2013).

Chapter 6. *hcp1* as an Additional Gene Deletion Target to Further Attenuate Vaccine Backbone Strain TMM001

Hcp serves as both a structural component and a secreted protein in the T6SS apparatus. Additionally, Hcp has been identified as having an important role in T6SS functionality and pathogenesis, and has been considered as a potential vaccine/therapeutic target in T6SS expressing pathogens (Mougous *et al*, 2006; Pukatzki *et al*, 2006; Bingle *et al*, 2008). As previously mentioned, Schell *et al* and Burtnick *et al* demonstrated that *hcp1* mutants in *B. mallei* and *B. pseudomallei* were avirulent in Syrian golden hamsters (Schell *et al*, 2007; Burtnick *et al*, 2011). Additionally, Burtnick *et al* demonstrated that deletion of *B. mallei's* tssE gene, a component of the T6SS apparatus required for Hcp1 secretion, resulted in a mutant that exhibited significant decreases in intracellular growth and intra- and intercellular spread. This mutant was also unable to form multinucleated giant cells (MNGCs) in RAW 264.7 macrophages (Burtnick *et al*, 2010).

A similar study by this group revealed that the *B. pseudomallei hcp1* mutant also displays decreased intracellular growth and an inability to form MNGC in infected macrophages *in vitro*. This mutant was also highly attenuated in a Syrian golden hamster infection model (Burtnick *et al*, 2011). Together, these findings indicate that the *hcp1* gene is essential for virulence and MNGC formation in *Burkholderia* species.

MNGC formation is characteristic of both *B. mallei* and *B. pseudomallei* infections, and has been detected both *in vitro* and *in vivo* (Duval & White, 1907; Harley *et al*, 1998; Burtnick *et al*, 2011) (Brett *et al*, 2008; Burtnick *et al*, 2010). MNGCs are believed to be involved in these organisms' ability to establish persistent infections by allowing intracellular spread and immune evasion (Kespichayawattana *et al*, 2000; Galyov *et al*,

2010; Burtnick *et al*, 2011; Boddey *et al*, 2007; Schwarz *et al*, 2014) . Based on its essential role in virulence, *hcp1* was considered an ideal candidate to target for gene deletion. Using the TMM001 vaccine platform, an additional gene deletion within the *hcp1* gene will be performed in an effort to potentially reduce vaccine strain persistence while still maintaining immunogenicity.

Objectives of this dissertation study

Burkholderia mallei is the causative agent of glanders, which, when inhaled in low doses, can cause a severe disease of high mortality in both humans and horses (M'Fadyean, 1904; Howe & Miller, 1947; Neubauer *et al*, 2005; Gregory & Waag, 2007; Van Zandt *et al*, 2013). *B. mallei*'s potential for bioterrorism use, its high-level of antibiotic resistance, and the lack of treatment alternatives make it a significant public health threat and highlight the need for an effective vaccine (Gregory & Waag, 2007; Galyov *et al*, 2010; Silva & Dow, 2013; Van Zandt *et al*, 2013). As such, the long-term objective of this research is to develop a vaccine that provides sterilizing immunity against *B. mallei* respiratory infection. Live attenuated vaccines are currently considered the most efficacious approach to immunizing against *B. mallei* infection due to their ability to generate both cellular and humoral immunity, but to date single gene deletion mutants have not provided complete protection (Bondi & Goldberg, 2008; Mott *et al*, 2011; Choh *et al*, 2013; Silva & Dow, 2013). Recently, *B. mallei* Δ*tonB* (TMM001), a strain deficient in iron acquisition, was shown to provide significant protection against wild-type challenge when used as a live attenuated vaccine. Although vaccinated mice showed 100% survival, they also demonstrated enlarged spleens containing abscesses from which the vaccine strain was recovered (Mott *et al*, 2015). The persistence demonstrated in this model raises a significant safety concern regarding its utility as a vaccine candidate and suggests that introduction of an additional gene deletion may be required to facilitate increased host clearance. Literature regarding the pathogenic role of hemolysin co-regulated protein1 (*hcp1*), a *B. mallei* cluster 1 type six secretion system (T6SS-1) gene, make it an ideal candidate. An *hcp1* gene deletion in *B. mallei* was

attenuated in Syrian golden hamsters (Schell *et al*, 2007). Additionally, an *hcp1* gene deletion in a closely related species, *B. pseudomallei* exhibited an inhibition of MNGC formation and decreased intracellular growth and spreading in cell culture (Burtnick *et al*, 2011). The deletion of both *hcp1* and *tonB* genes should produce a *B. mallei* phenotype that is metabolically attenuated due to its inability to acquire iron from bound sources, and that is more susceptible to host clearance due to its inability to spread cell-to-cell via MNGCs. Thus, I believe that introduction of an *hcp1* gene deletion in the *B. mallei* TMM001 backbone vaccine strain will decrease its persistence in the host while maintaining its ability to confer protective immunity against lethal challenge with respiratory glanders. I have tested my central hypothesis by completing the following two aims: 1) Construct and Characterize the *B. mallei* CLH001 strain and perform experiments to evaluate its *in vitro* fitness and virulence potential compared to wt *B. mallei*; and 2) Evaluate its *in vivo* attenuation, safety, susceptibility to host clearance, and ability to protect against lethal challenge with respiratory glanders in murine models.

MATERIALS AND METHODS

IN VITRO STUDIES

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2. *E. coli* were grown in Luria-Bertani (LB) media at 37ºC. All manipulations of *B. mallei* strains were conducted in Centers for Disease Control and Prevention (CDC)-approved and registered biosafety level 3 (BSL3) or CDC/ U.S. Department of Agriculture (USDA)-approved and registered animal biosafety level 3 (ABSL3) facilities at the University of Texas Medical Branch and experiments were performed in accordance with Select Agent standard operating practices. *B. mallei* strains were taken from freezer stocks, plated on LB agar containing 4% glycerol (LBG) and LBG with 200 μM $FeSO_4$ (LBG with $FeSO_4$) and incubated 37ºC for 3 days. For liquid cultures, 2-3 colonies were inoculated into 20 mL of LBG broth. Liquid cultures were then incubated overnight (18h) at 37ºC with agitation (200 rpm). Challenge and vaccination doses were prepared from overnight LBG cultures and diluted in PBS in a total volume of 50 μL (25 μL/ nare).

Table 2: Bacterial strains used in dissertation research

Strains	Relevant features	Reference
***B. mallei* ATCC 23344**	Human clinical isolate; $Km^{S}Pb^{R}$	(Yabuuchi *et al*, 1992)
***B. mallei* CSM001**	*B. mallei* ATCC 23344 with a mini-Tn*5*::*lux*Km_2; $Km^{R}Pb^{R}$	(Massey *et al*, 2011)
***B. mallei* TMM001**	*B. mallei* ATCC 23344 with an unmarked intragenic deletion in BMAA1801 (Δ*tonB*)	(Mott *et al*, 2015)
***B. mallei* CLH001**	*B. mallei* TMM001 with unmarked intragenic deletion in BMAA0742 (Δ*hcp1*)	This study
***B. mallei* CLH002**	*B. mallei* ATCC 23344 with an unmarked intragenic deletion of BMAA0742 (Δ*hcp1*)	This study
***B. pseudomallei* ATCC K96243**	Human clinical isolate; Km^{R} Gm^{R} $Zeo^{R}Pb^{R}$	(Holden *et al*, 2004)
***E. coli* S17-1 (pMo130-ΔBPSS1498)**	Donor strain containing pMo130--ΔBPSS1498 plasmid; $Sm^{R}Tp^{R}Pm^{S}$ Km^{R}	(Burtnick *et al*, 2011)

Construction of *B. mallei hcp1* mutants CLH001 and CLH002

B. mallei Δ*hcp1* (CLH002) and *B. mallei* Δ*tonB* Δ*hcp1* (CLH001) were constructed using a donor strain and plasmid strain donated by Dr. Mary Burtnick (University of South Alabama). The donor strain was a chemically competent *E. coli* S17-1 λ *pir* strain containing a pMo130ΔNX plasmid designed to introduce a 162 base pair intragenic in-frame deletion in the *hcp1* gene (Burtnick *et al*, 2011). The CLH002 mutant was created by introducing the plasmid from the donor strain into *B. mallei* 23344 via bi-parental mating. Deletion mutants were isolated by selection on kanamycin (Km) agar plates, followed by counter selection on 5% sucrose yeast extract and tryptone (YT) agar supplemented with 200 μM $FeSO_4$ (Illustration 2). The CLH001 mutant was created by introducing the plasmid into *B. mallei* TMM001 via conjugal transfer and repeating the screening and confirmation process as described above. The Δ*hcp1* mutation was then confirmed via PCR amplification, followed by sequencing, of the *hcp1* gene using the following primers: Forward primer (ATG CTG GCC GGA ATA TAT CTC); Reverse primer (GCC ATT CGT CCA GTT TGC GG).

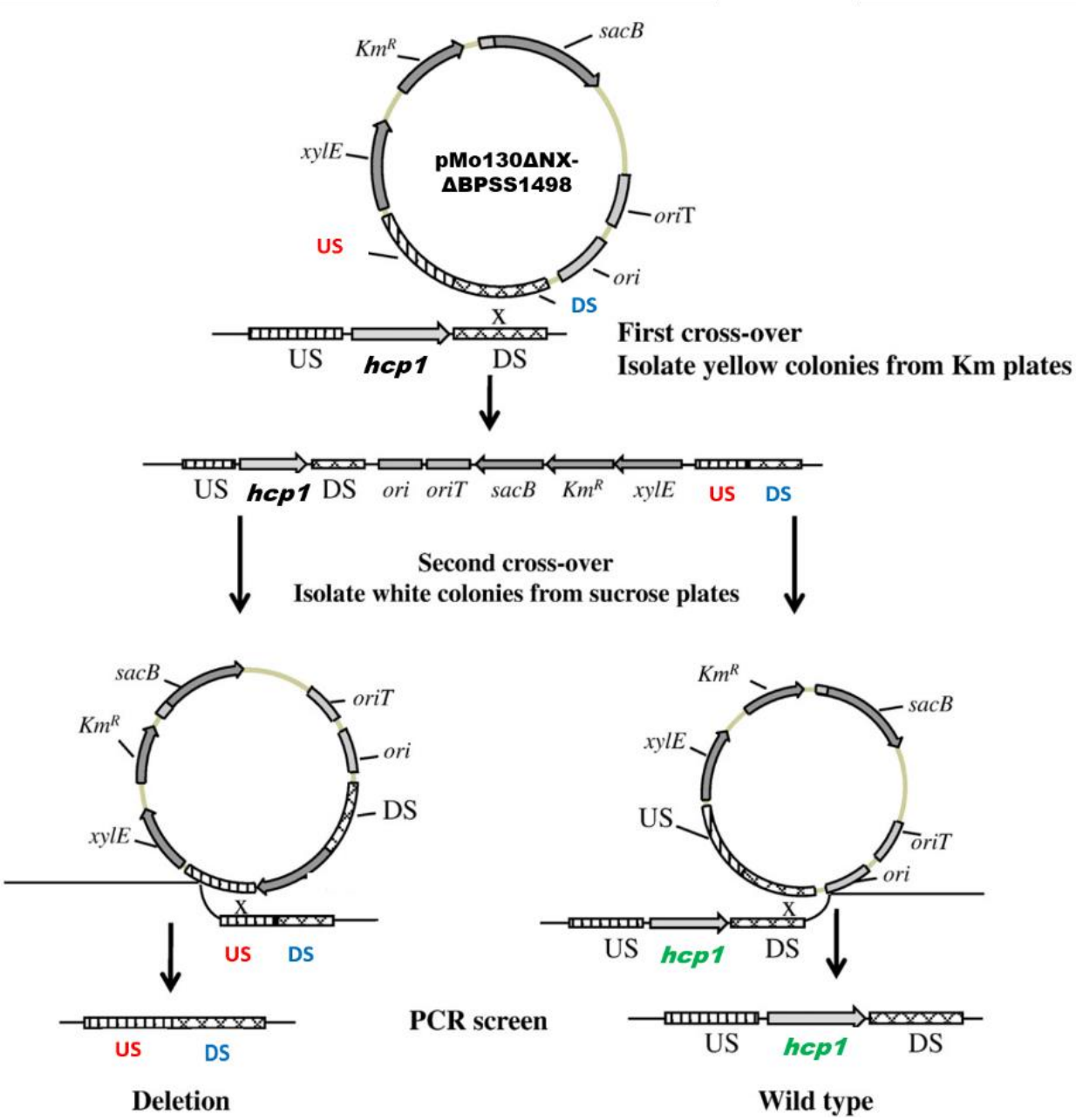


Illustration 2: Mutagenesis approach schematic. Diagramed are the pMo130 plasmid map and the processes used to generate an unmarked deletion of the *hcp1* gene via allelic exchange. Figure adapted from source (Hamad *et al*, 2009).

Growth kinetics

Overnight broth cultures of each strain (*B. mallei* 23344, TMM001, CLH002, and CLH001) were used to inoculate fresh 40 ml LBG and LBG with $FeSO_4$ broths at a uniform initial optical density at a wavelength of 600 nm (OD_{600}) of 0.05 at for each strain. Inoculated cultures were then incubated overnight with agitation at 37°C. At the indicated time points (0, 4, 8, 12, 16, 24, and 32 h), 1 mL aliquots from each culture were taken to measure optical density at 600 nm (using a spectrophotometer). Individual data points represent the OD_{600} mean ± standard deviation (SD) of three independent experiments. Significant difference due to treatment over time was ascertained via two-way analysis of variance (ANOVA). *S*ignificant differences of each OD_{600} reading at every time point compared to wild-type were determined by one-way ANOVA followed by Dunnett's multiple comparisons test.

Hcp1 Expression Assay

Using the methodology described by Burtnick and Brett (Burtnick & Brett, 2013), three colonies of LBG with $FeSO_4$ plate growth for each *B. mallei* strain (*B. mallei* 23344, CSM001, TMM001, CLH002, and CLH001) was used to inoculate 20 ml M9 minimal salts broth supplemented with 0.4% glucose (M9G) and were incubated overnight with agitation at 37°C. An OD_{600} reading was taken for each culture, in order to ensure standardization, this reading was used to calculate the volume of each bacterial suspension required to equal an OD_{600} of 1 (volume required = (OD_{600}=1/OD_{600} actual reading x 1 ml)). The resulting volumes of bacterial suspensions for each strain were pelleted by centrifugation at 16,000 x g for 5 minutes. Pellets were then re-suspended in 0.2 ml of 1X Tris-glycine sodium dodecyl sulfate (SDS) sample buffer with β-mercaptoethanol and boiled for 10 min. Samples were loaded and run on a 12% SDS PAGE gel and then electrophoretically

transferred to a PVDF (polyvinyl difluoride) membrane. After blocking for 1 hour in Tris-buffered saline with Tween 20 (TBS-T) and 5% non-fat dry milk, the membrane was placed in TBS-T with a 1:3,000 dilution of polyclonal rat anti-*B. mallei* Hcp1 serum and rocked for 1 hour at room temperature. After washing twice in TBS-T, the membrane was transferred to TBS-T with a 1:5,000 dilution of goat anti-rat IgG horseradish peroxidase (HRP) conjugated antibodies and again rocked for 1 hour at room temperature. After three ten-minute TBS-T wash steps, the membranes were treated with enhanced chemiluminescence (ECL) western blotting substrate (Pierce Biotechnology, Rockford, IL) and analyzed by using the GE Image Quant LAS 400 imaging system (GE Healthcare Bio-Sciences, Pittsburg, PA).

Cell lines and culture conditions

Mouse leukemic monocyte macrophages (RAW 264.7) and human cervical carcinoma cells (HeLa) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37°C with 5 % CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1µM L-glutamine, sodium pyruvate and penicillin/streptomycin.

Multinucleated Giant Cell Formation Assay

As previously described (Harley *et al*, 1998; Burtnick *et al*, 2010; Hopf *et al*, 2014), 12mm glass coverslips were aseptically placed in four of the wells of two six-well plates. The glass coverslip containing wells of one plate was seeded with RAW 264.7 macrophages and the other was seeded with HeLa cells at 1 x 10^6 cells/well and incubated overnight at 37°C with 5% CO_2. Overnight liquid bacterial cultures of *B. mallei* 23344, TMM01, CLH002, and CLH001 were grown in LBG at 37ºC with agitation (200 rpm).

Following overnight incubation, the cells were washed three times with PBS and provided fresh DMEM supplemented with FBS. A bacterial suspension of *B. mallei* 23344, TMM001, CLH002, or CLH001 was added to the each of the four wells containing RAW 264.7 macrophages and Hela cells at a MOI of 1 ($1x10^6$ CFU/well). Once infected the RAW 264.7 cells and HeLa cells were incubated at 37°C with 5% CO_2 for 12h and 16h, respectively. Following incubation, each plate was washed twice with PBS. The coverslip containing wells were then methanol fixed, stained with 5% Geimsa and rinsed with distilled water. After rinsing, the coverslips were carefully removed from the plates and allowed to dry completely. Once dry, the coverslips were mounted cell side down on microscope slides and evaluated for MNGC formation using light microscopy at 40X magnification. Images of 15 random fields per coverslip were captured using an Olympus BX53 upright microscope outfitted with a DPT-3 imaging system and Cell Sens Dimension version 1.7 software (Olympus Inc., Center Valley, PA). For enumeration at least 1,000 cells per coverslip were counted, and percent MNGC formation was calculated as follows: (number of MNGC/total number of cells counted) x 100. As in previous studies, a MNGC was defined as a cell containing 3 or more nuclei within the same cell boundary. The experiment was performed in triplicate. A significant difference in percent MNGC formation between groups for each cell line was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

Macrophage Uptake and Survival Study

As previously described (Whitlock *et al*, 2009) (Brett *et al*, 2008), RAW 264.7 macrophages were seeded in a 24-well plate at 5 x 10^5 cells/well and incubated overnight at 37°C with 5% CO_2. Overnight liquid bacterial cultures of *B. mallei* 23344, TMM01,

CLH002, and CLH001 were grown in LBG at 37ºC with agitation (200 rpm). Following overnight incubation, macrophages were washed three times with Hank's balanced salt solution (HBSS) and provided fresh DMEM supplemented with FBS. A bacterial suspension of *B. mallei* 23344, TMM001, CLH002, or CLH001 was added to the each of the four wells containing RAW 264.7 macrophages at a multiplicity of infection (MOI) of 10 (5.0×10^6 CFU/well) and then centrifuged for 2 minutes at 800 x g to facilitate contact. After a 1h incubation at 37°C at 5 % CO_2, infected macrophages were washed three times with HBSS to remove non-adherent bacteria and incubated in new media supplemented with 50 μg/ml of gentamicin (Gm) and 200 μg/ml of aminoguanidine (AG) for 1hr to kill extracellular bacteria and prevent macrophage killing of intracellular bacteria, respectively. At time points 2, 8, 12, 16, 24 and 32 h, infected macrophages were washed once with HBSS and the lysed with 250 μL of 1% Trition X-100 in HBSS. Intracellular bacteria were quantified by plating serial dilutions of the lysate on LBG with $FeSO_4$. Percent uptake was calculated as number of bacteria recovered at 2 h for each as follows: number of bacteria recovered per strain/number of bacteria input (5.0×10^6) x 100. Percent survival at each time point was determined as follows: number of bacteria recovered at time point/number of bacteria recovered at 2 h time point for that strain x 100. Each experimental group was assayed in triplicate. Significant differences in strain specific survival at each individual time point was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

IN VIVO STUDIES

Animal Studies

All animal experiments were performed using female, 6- to 8-week-old, BALB/c mice or NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice obtained from Charles River (Wilmington, MA, USA). Mice were housed in microisolator cages under pathogen-free conditions, provided with rodent feed and water *ad libitum*, and maintained on 12 h light cycle. To allow adequate acclimation, mice were housed within the animal facility for 1 week prior to experimentation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Protocol Number: 0503014B).

Survival and Persistence Study

Anesthetized BALB/c mice (n = 11) were challenged i.n. with $1.5x10^4$ CFU of *B. mallei* luminescent reporter strain (CSM001) or isogenic mutants TMM001, CLH002, or CLH001. At day 2 post-infection, mice (n = 3) were euthanized and their lungs, livers, and spleens were aseptically harvested for CFU enumeration. The remaining BALB/c mice (n = 8) were monitored for survival for 21 days. Survival curves were generated and analyzed using the Kaplan-Meier method and a significant difference in survival curves was ascertained via a log-rank (Mantel-Cox) test. At the 21-day experimental end point, the surviving animals (n = 8 for TMM001, CLH001, CLH002 groups) were euthanized and the lungs, livers, and spleens were aseptically harvested. The lungs, livers, and spleens were plated for CFU enumeration (n = 5) or histopathological evaluation (n = 3).

Immunocompromised Mouse Survival Study

Anesthetized NSG mice (n = 10) were i.n. challenged with either $1.5x10^4$ CFU of CSM001 (n = 4) or CLH001 (n = 6). The mice were monitored for survival for 21 days. Survival curves were generated and analyzed using the Kaplan-Meier method. A significant difference in survival curves was ascertained via a log-rank (Mantel-Cox) test. At the 21-day experimental end point, the surviving animals (n = 6 for CLH001) were euthanized and the lungs, livers, and spleens were aseptically harvested. Organs were plated for CFU enumeration (n = 4), or histopathological evaluation (n = 2).

Single Dose Vaccination Study

Anesthetized BALB/c mice (n = 11) were administered a single i.n. vaccination consisting of 50 µl PBS or $1.5x10^4$ CFU of TMM001, or CLH002. At day 21 post-vaccination, mice (n = 3) were anesthetized and retro-orbital blood was collected for antibody analysis. These mice (n = 3) were then euthanized and lungs, livers, and spleens were plated for CFU enumeration. The remaining BALB/c mice (n = 8) were i.n. challenged with $1.5x10^4$ CFU of CSM001 and BALB/c mice were monitored for survival for 14 days. Survival curves were generated and analyzed using the Kaplan-Meier method. A significant difference in survival curves was ascertained via a log-rank (Mantel-Cox) test.

Prime and Boost Vaccination Study

Anesthetized BALB/c mice (n = 11) were administered a series of three i.n. vaccinations consisting of 50 µl PBS, $1.5x10^4$ CFU or $1.5x10^5$ CFU of CLH001 at two week intervals (days -49, -35 and -21 days pre-challenge). At days -35, -21, and -1 pre-

challenge, mice (n = 3) were anesthetized and retro-orbital blood was collected for antibody analysis. On day 0 prior to challenge, mice (n = 3) were euthanized and lungs, livers, and spleens were aseptically removed. Organs from the PBS-vaccinated mice were submitted for histopathological analysis and the organs from the CLH001 vaccinated groups were plated for CFU enumeration. The remaining BALB/c mice (n = 8) were i.n. challenged with $1.5x10^4$ CFU of CSM001. BALB/c mice were monitored for survival for 35 days. Survival curves were generated and analyzed using the Kaplan-Meier method. A significant difference in survival curves was ascertained via a log-rank (Mantel-Cox) test. At the 35-day experimental end point, the surviving animals (n = 5 for CLH001 [$1.5x10^4$ CFU] and n = 8 for CLH001 [$1.5x10^5$ CFU]) were euthanized and the organs were aseptically harvested. Lungs, livers, spleens were plated for CFU enumeration (n = 3 for CLH001 [$1.5x10^4$ CFU] and n = 5 for CLH001 [$1.5x10^5$ CFU]) and submitted for histopathological evaluation (n = 2 for CLH001 [$1.5x10^4$ CFU] and n = 3 for CLH001 [$1.5x10^5$ CFU]).

High Dose *B. mallei* ATCC 23344 Challenge Prime and Boost Vaccination Study and *B. pseudomallei* Cross-protection Study.

Anesthetized BALB/c mice (n = 11) were administered a series of three i.n. vaccinations consisting of 50 µl PBS, or $1.5x10^5$ CFU of CLH001 at two week intervals (days -49, -35 and -21 days pre-challenge). At days -35, -21, and -1 pre-challenge, mice (n = 3) were anesthetized and retro-orbital blood was collected for antibody analysis. On day 0 prior to challenge, mice (n = 3) were euthanized and their lungs, livers, and spleens were aseptically removed and plated for CFU enumeration. The remaining mice (n = 8) were i.n. challenged with $3.5x10^5$ CFU *B. mallei* ATCC 23344 (high dose *B. mallei* ATCC 23344

challenge prime and boost study) or $9.36x10^2$ CFU *B. pseudomallei* ATCC K96243 (cross protection study). BALB/c mice were monitored for survival for 35 days. Survival curves were generated and analyzed using the Kaplan-Meier method. A significant difference in survival curves was ascertained via a log-rank (Mantel-Cox) test. Additionally, at the 35-day experimental end point, the surviving animals were euthanized and the lungs, livers, and spleens were aseptically harvested and plated for CFU enumeration.

Organ CFU Enumeration

The lungs, liver and spleen were homogenized in 1 mL of PBS using a tissue grinder (Covidien, Mansfield, MA). Ten-fold serial dilutions of the homogenate were made in PBS and 100 µl of each dilution was plated on 100 mm LBG with $FeSO_4$ plates. For each organ, 500 µl of the remaining organ homogenate was plated on a 150 mm LBG with $FeSO_4$ plate to increase culture sensitivity. The resulting plates were incubated 96 h at 37 ^oC, enumerated by standard plate counts, and reported as CFU per organ. The sensitivity of the plating method for bacterial detection was approximately $\geq$10 CFU per organ.

Histopathological Evaluation.

At the indicated time points, anesthetized BALB/c mice were euthanized and lungs, livers and spleens were collected. Organs were placed in 10% formalin, paraffin-embedded, and processed for histopathology. Hematoxylin and eosin stained slides were examined for presence/absence of perivascular and peribronchial infiltrates, necrosis and microabscesses in lungs; granulomas and necrosis in liver; and inflammation and necrosis in spleens, and scored by a pathologist blinded to treatments based on the severity using the follow scale: 0 (unremarkable), 1 (minimal), 2 (mild), 3 (moderate) and 4 (severe).

Student's *t* test was performed to ascertain a significant difference in histopathological score between individual treatment as compared to the PBS-treatment control or naïve mice for each organ.

Analysis of *B. mallei*-specific IgG, IgG2a and IgG1

Whole blood was collected via retro-orbital bleeding of anesthetized BALB/c mice. The blood was stored in microvette tubes without anti-coagulant and incubated at room temperature for 20 min to permit clotting. Following centrifugation, serum was collected and stored at -80°C. Samples were inactivated by γ-irradiation using a JL Shepherd Model 109–68 Cobalt-60 Research Irradiator (JL Shepherd & Associates, San Fernando, CA 91340). Samples were irradiated on dry ice until 5 MRAD of exposure was reached and sterility was verified by plating 10% of the serum volume on LBG with $FeSO_4$. Irradiated serum from PBS or CLH001-vaccinated BALB/c mice was evaluated for *B. mallei* specific IgG total, IgG2a, and IgG1 using an ELISA performed in 96-well Costar High Binding microplates (Corning, Inc., Corning, NY). Briefly, irradiated *B. mallei* was diluted to a concentration of 10 µg/ml in 1x PBS and wells were coated with 100 µl/well of diluted suspension and incubated overnight at 4°C. Wells were washed twice with wash buffer (1x PBS containing 0.05% Tween-20) and incubated with 250 µl of blocking solution (1x PBS, 1% bovine serum albumin, 0.05% Tween-20) for 2 h at room temperature (RT). After blocking, plates were washed twice with wash buffer. Two-fold dilutions of mouse sera were made with sample diluent (1x PBS, 0.5% bovine serum albumin, and 0.05% Tween-20) in triplicate. After serum addition, plates were incubated for 1h at RT and then washed four times with wash buffer. One hundred µl of diluted sera along with 100 µl of 1:10,000 anti-Ig class or subclass horseradish peroxidase conjugate (Southern Biotechnology

Associates, Inc., Birmingham, AL) was added to sample wells and plates were incubated at RT for 2 h. The plates were washed four times with wash buffer prior to addition of 100 µL of Tetramethylbenzidine (TMB) substrate solution (eBioscience, Inc., San Diego, CA). After a 15 min, 100 µL of stop solution (2N H_2SO_4) was added and the wells were read at 450nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). The results were reported as the reciprocal of the highest titer giving an optical density (OD) reading of at least 0.1, which was at least twice the background ± 1 SD. All assays were performed in triplicate, and results were reported as the mean reciprocal endpoint titer ± SD.

Serum Bactericidal Assay

An overnight culture of *B. mallei* ATCC 23344 was diluted 1:100 in fresh LBG and grown to log phase (OD_{600} of 0.6). The bacterial concentration was adjusted to 1 x 10^5 CFU/well in a 96-well plate and incubated with heat inactivated (56°C for 1 h), pooled 1:100 diluted CLH001 strain prime and boost vaccinated serum (n = 3), or 5 µg/ml anti-*B. mallei* LPS monoclonal antibody (MAb) 3d11 (AbD Serotech, Raleigh, NC) in LBG broth containing 22 µl guinea pig complement (Sigma-Aldrich, St. Louis, MO). Bacteria incubated in LBG broth containing heat inactivated pooled 1:100 diluted naïve serum (n = 3) and 22 µl guinea pig complement was used as a negative control. After 6 h of incubation (37°C with 135 rpm), 10-fold serial dilutions were plated on 100 mm LBG agar and incubated for 72 h at 37°C. The bacterial counts were reported as CFU/ml. Each experimental group was assayed in triplicate. A significant difference in bacterial survival between groups was determined using one-way ANOVA.

Passive Vaccination

As previously described (Silva *et al*, 2013), anesthetized BALB/c mice (n = 3) were administered a series of three i.n. vaccinations consisting of 50 µl PBS, or $1.5x10^5$ CFU of CLH001 at two week intervals. At 3 weeks after administration of the last vaccination, mice were anesthetized and whole blood was collected via terminal cardiac puncture. The blood was stored in microvette tubes without anti-coagulant and incubated at room temperature for 20 min to permit clotting. Following centrifugation, serum was collected and stored at -80°C. Samples were inactivated by γ-irradiation using a JL Shepherd Model 109–68 Cobalt-60 Research Irradiator (JL Shepherd & Associates, San Fernando, CA 91340). Samples were irradiated on dry ice until 5 MRAD of exposure was reached and sterility was verified by plating 10% of the serum volume on LBG with $FeSO_4$. Non-immune sera was collected from unvaccinated BALB/c mice (n = 3) in a similar manner. Sera from each respective group was pooled and *B. mallei*-specific IgG tiers were determined via ELISA prior to transfer as previously described. Recipient mice (n = 3) were anesthetized and administered 250 µl of naïve sera, PBS-vaccinated sera or CLH001-vaccinated sera via the i.p. route. 24 hours later mice were i.n. challenged with $2.1x10^3$ CFU of CSM001 and monitored for survival. Survival curves were generated and analyzed using the Kaplan-Meier method.

IFNγ ELISA

At day 0 prior to challenge and day 2 post-challenge BALB/c mice (n =3) were anesthetized whole blood was collected via retro-orbital bleeding of anesthetized BALB/c mice and then humanely euthanized and lungs were aseptically removed. The blood was stored in microvette tubes without anti-coagulant and incubated at room temperature for

20 min to permit clotting. Following centrifugation, serum was collected and stored at -80°C. The lungs were homogenized in 1 mL of PBS using a tissue grinder (Covidien, Mansfield, MA). Following centrifugation, the lung supernatant was removed and stored at -80°C. Samples were then inactivated by γ-irradiation as previously described and sterility was verified by plating 10% of the sample volume on LBG with $FeSO_4$. Irradiated sera and lung supernatant from PBS or CLH001-vaccinated BALB/c mice were evaluated for IFNγ using the Mouse IFN gamma ELISA Ready-SET-Go! (eBioscience, Inc., San Diego, CA) and assays were performed following the manufacturers recommended protocol. Sera was reported as pg/ml and lung lysates were reported as pg/g of lung and data values are representative of the results of 3 mice. One-way ANOVA was performed to ascertain a significant difference in IFNγ production between PBS- and CLH001-vaccinated groups for each sample type.

Chapter 7. Characterization of *B. mallei* CLH001

INTRODUCTION

As previously described, the CLH002 and CLH001 mutants were constructed via allelic exchange of intragenic in-frame deletion of the *hcp1* gene into the TMM001 background. Once truncation of the *hcp1* gene had been confirmed, the resulting mutants were characterized *in vitro.* First, growth kinetics were evaluated in nutrient rich media (with and without $FeSO_4$ supplementation) and Hcp1 expression was measured from supernatants of bacteria cultured in M9G minimal media, Additionally, CLH001 was assessed based on the ability to induce MNGC formation in both HeLa and RAW 264.7 cells. Lastly, the uptake and survival of the CLH001 mutant in RAW 264.7 cells was evaluated. In these *in vitro* studies, a wild-type *B. mallei* strain (*B. mallei* 23344 or CSM001) was used for comparison. Additionally, isogenic mutants containing each of the single gene deletions present in CLH001 (TMM001 and CLH002) were included to aid in determining the contribution of the individual gene deletions. These assays were performed to assess the viability and *in vitro* attenuation of the mutants, and to evaluate CLH001's suitability for advancement to *in vivo* testing in a mouse model.

RESULTS

Growth Rate Studies

To demonstrate that CLH002 and CLH001 mutant strains were viable and that CLH001 retained the iron acquisition deficiency metabolic attenuation of its parent strain, TMM001, *B. mallei* 23344, TMM001, CLH002, and CLH001 were grown in LBG and LBG with $FeSO_4$ and assessed for growth kinetics (Figure 1A and Figure 1B, respectively).

CLH002 exhibited growth characteristics that closely mirrored those of *B. mallei* 23344 at all time points in both LBG and LBG with $FeS0_4$. TMM001 and CLH001 mirrored *B. mallei* 23344 at the 4 h time points in both media, but then exhibited significantly decreased growth kinetics from the 8 hour time point until the 32 hour time point ($p < 0.05$) in LBG (Figure 1A). Mean bacterial doubling times for *B. mallei* 23344 and CLH002 grown in LBG were 199.1 minutes and 191.0 minutes, respectively; while those in TMM001 and CLH001 were 243.35 minutes and 241.12 minutes, respectively (data not shown). In LBG with $FeSO_4$, the growth kinetics of TMM001 and CLH001 strains were restored to wild-type levels and no significant differences in growth OD_{600} readings were detected at any time point (Figure 1B). Additionally, mean doubling time was equivalent for all 4 strains when grown in LBG with $FeSO_4$; 184.2 minutes for *B. mallei* 23344, 189.0 minutes for CLH001, 191.22 minutes for TMM001, and 188.41 minutes for CLH002 (data not shown).

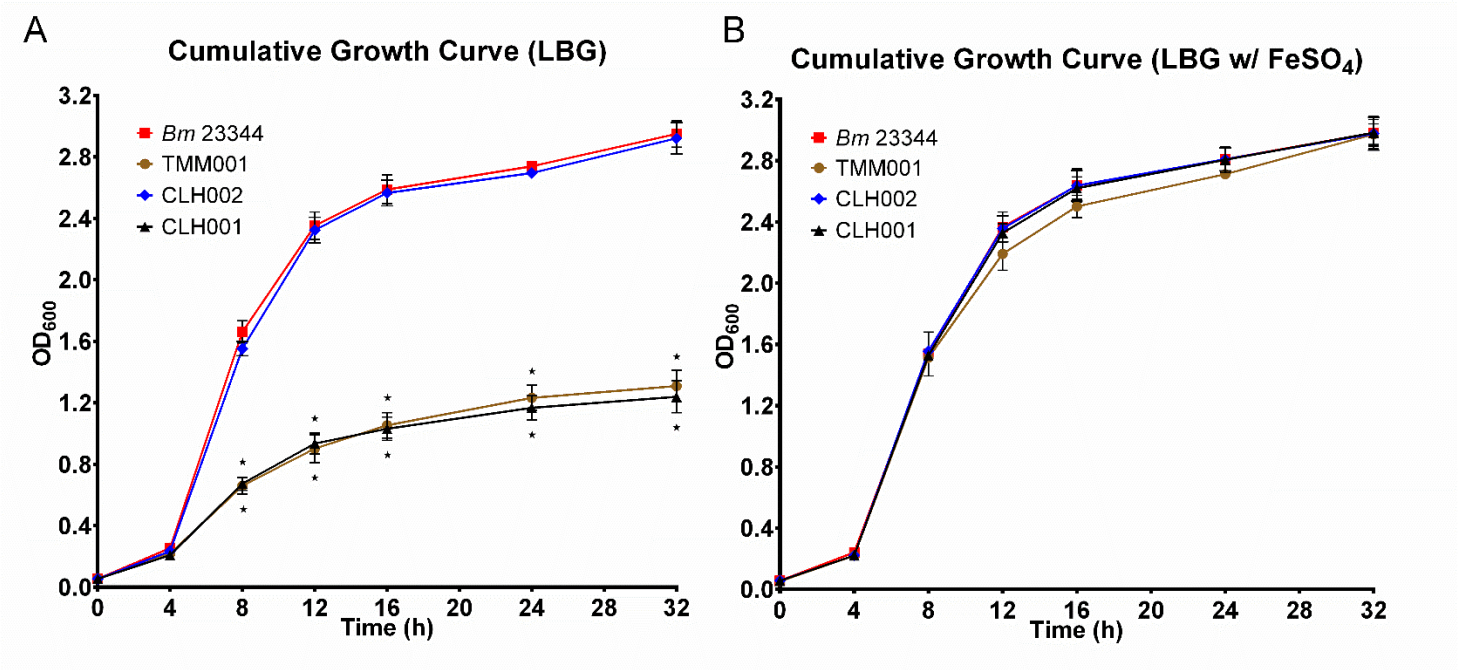


Figure 1: Decreased growth kinetics observed in *B. mallei tonB* mutants TMM001 and CLH001 grown in LBG are restored to wild- type levels when grown in LBG with $FeSO_4$. Overnight cultures of *B. mallei* 23344 (■), TMM001(●), CLH002 (◆), and CLH001 (▲) were diluted to an OD_{600} of 0.05 in 40 ml of LBG (Figure 1A) and duplicated in LBG with 200 µM $FeSO_4$. At the indicated time points, OD_{600} readings of all strains were measured. The means plotted with SD are representative of three independent experiments. Statistical significance was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.⋆, $P < 0.05$.

Hcp1 Expression Assay

Next the strains were assessed based on the ability to express Hcp1. Whole cell lysates of overnight cultures were grown in M9G were run on a 12% SDS PAGE gel, transferred to a PVDF membrane and analyzed using western blot (Figure 2). While ≈ 23 KDa bands corresponding to Hcp1 production were present in 0.1 μg recombinant *B. mallei* Hcp1, TMM001, CSM001, and *B. mallei* 23344 (Figure 2A, B, C, and E, respectively), bands were absent in CLH002 and CLH001 (Figures 2D and E, respectively).

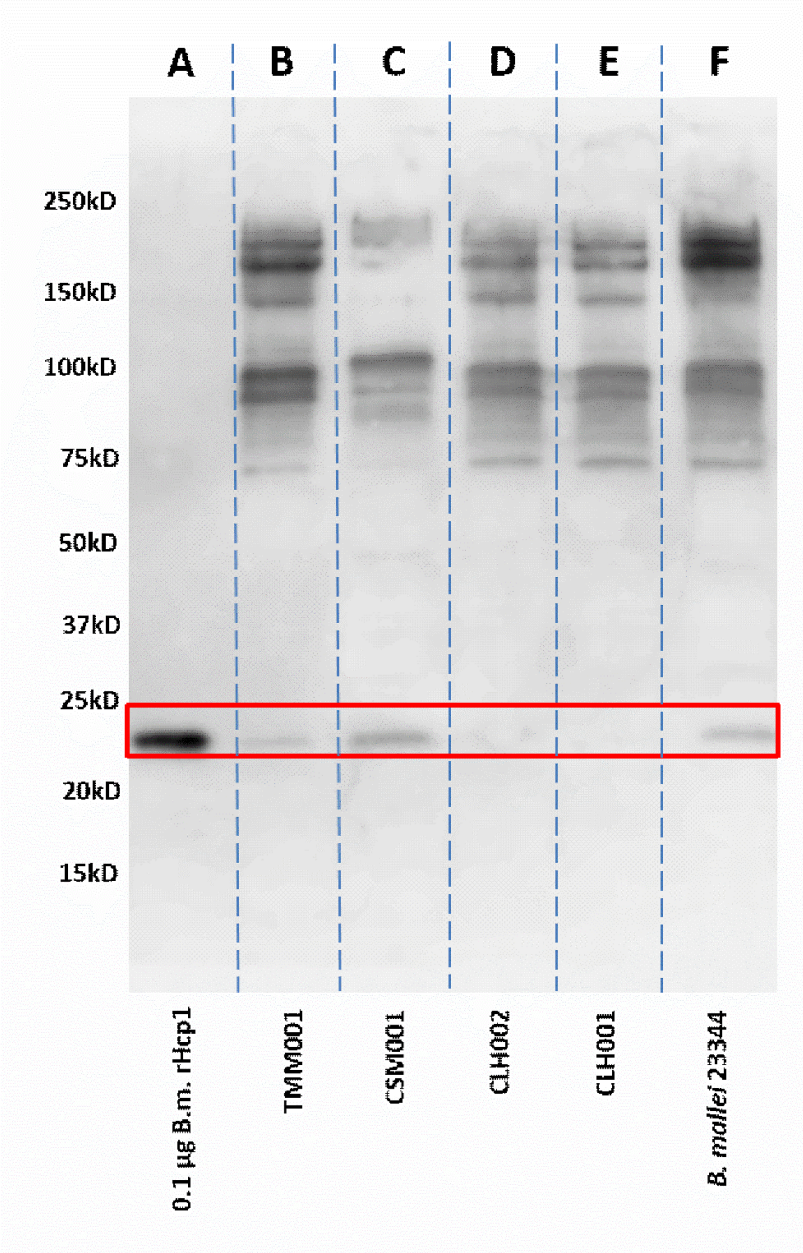


Figure 2: Hcp1 expression is absent in *hcp1* mutants CLH001 and CLH002. Lane (A) was loaded with ladder and lane (B) was loaded with 0.1 µg or recombinant *B. mallei* Hcp1 protein. The remaining lanes were loaded with whole cell lysates prepared from overnight cultures of (C) TMM001, (D) CSM001, (E) CLH002, (F) CLH001, and (G) *B. mallei* 23344 grown in M9G, and then assayed for Hcp1 production by western blot using anti-*B. mallei* Hcp1 polyclonal rat serum followed by goat anti-rat IgG HRP conjugated antibodies. The area of the membrane corresponding to Hcp1 (≈ 23 kDa) is indicated by a red rectangle.

Multinucleated Giant Cell Formation Assays

Since it had previously been shown that MNGC formation was absent in RAW 264.7 murine macrophages infected with *B. pseudomallei* Δ*hcp1* (Burtnick *et al*, 2011), we performed a similar assay to see if the same was true of our *B. mallei hcp1* mutants, CLH002 and CLH001. RAW 264.7 cells were infected were infected an MOI of 1 with *B. mallei* 23344, TMM001, CLH002, or CLH001 at 12 h post-infection monolayers were fixed, Geimsa stained and observed at 40x using light microscopy. MNGCs were defined as cells containing 3 or more nuclei in the same cytoplasm and the percent MNGC was calculated for each treatment as follows; number of MNGCs/ number of cells present in 15 random fields x 100. A minimum of 1000 cells were counted per treatment. The percent MNGC observed in cells infected with mutant strains was then compared to the percent MNGC observed in *B. mallei* 23344 infected cells. MNGCs were present in RAW264.7 cells infected with *B. mallei* 23344 or TMM001 (Figure 3A and 3B, respectively), but were absent in cells infected with CLH002 or CLH001 (Figure 3C and 3D, respectively). There was no significant difference in percent MNGC formation in RAW 264.7 cells infected with *B. mallei* 23344 vs. TMM001 (2.31% vs. 2.06%, respectively) (Figure 3E). However, since no MNGCs were detected in RAW 264.7 cells infected with CLH002 or CLH001 (Figure 3C and 3D, respectively) there was a statistical significance in percent MNGC formation between cells infected with these groups and those infected with *B. mallei* 23344 (**, $p < 0.01$) (Figure 3E).

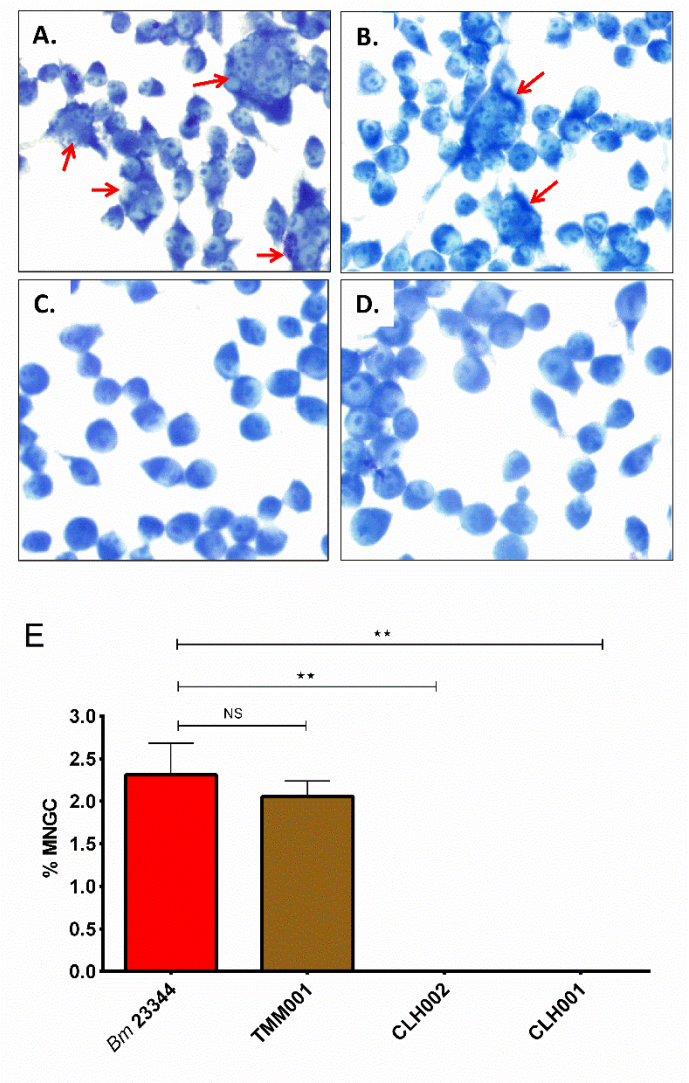


Figure 3: MNGC formation is prevented in RAW264.7 cells infected with *hcp1* mutants CLH001 and CLH002. RAW 264.7 murine macrophages were seeded at $1x10^6$ cells/well and infected at an MOI of 1 were fixed at 12 h post-infection, Geimsa stained and examined at 40x using light microscopy. Representative images monlayers infected with (A) *B. mallei*, (B) TMM001, (C) CLH002 or (D) CLH001. (E) Percent MNGC formation was calculated for each strain. Error bars represent the SD of three independent experiments and significant difference at each individual time point was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.**, $p < 0.01$.

Since this phenomenon had also been described in non-phagocytic cell lines, such as HeLa and Vero infected with *B. mallei* (Harley *et al*, 1998), we repeated this assay to see if *B. mallei hcp1* mutants also exhibited a deficiency in MNGC formation in non-phagocytic cells. Again, we noted that MNGC formation was present in HeLa cells infected with *B. mallei* 23344 and TMM001 (Figure 4A and 4B, respectively) and absent in HeLa cells infected with CLH002 or CLH001 (Figure 4C and 4D, respectively). Likewise, we found that there was no significant difference in percent MNGC formation between *B. mallei* 23344 and TMM001 infected HeLa cells (2.62% vs. 2.62%, respectively), but that there was a statistical significance between HeLa infected with CLH001 and CLH002 vs. those infected with *B. mallei* 23344 (**, $p < 0.01$) (Figure 4E).

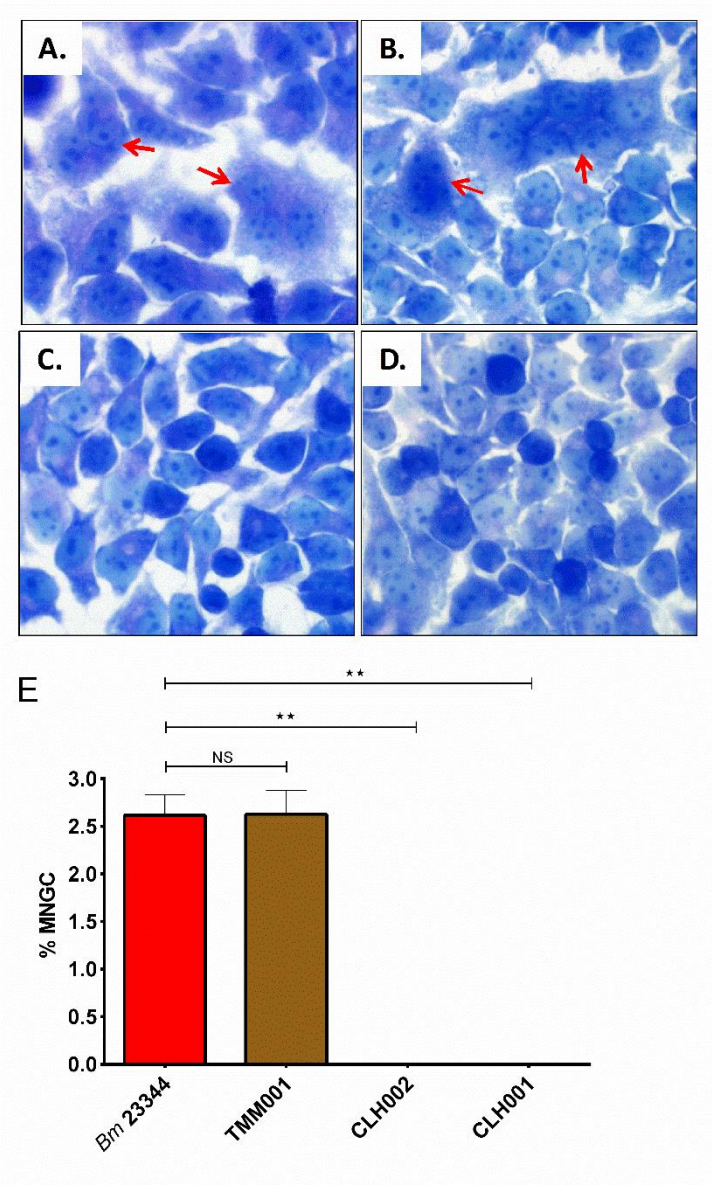


Figure 4: MNGC formation is inhibited in HeLa cells infected with *hcp1* mutants CLH001 and CLH002. HeLa cells were seeded at $1x10^6$ cells/well and infected at an MOI of 1 were fixed at 16 h post-infection, Geimsa stained and examined at 40x using light microscopy. Representative images monlayers infected with (A) *B. mallei*, (B) TMM001, (C) CLH002 and (D) CLH001. (E) Percent MNGC formation was calculated for each strain. Error bars represent the SD of three independent experiments and significant difference at each individual time point was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.**, $p < 0.01$.

Macrophage Uptake and Survival Assay

B. mallei is a facultative intracellular pathogen, which is known to infect and take up residency in phagocytic cells, such as macrophages early in infection (Harley *et al*, 1998) (Harley *et al*, 1998; Ribot & Ulrich, 2006). Uptake and survival characteristics in macrophages have been widely used as a tool for assessing attenuation of *B. mallei* mutant strains. As such, we performed an uptake and survival assay in RAW264.7 murine macrophages. Twenty-four well plates containing RAW 264.7 cells were infected with *B. mallei* 23344, TMM001, CLH002, or CLH001 at an MOI of 10, with 200 μg/ml of AG to inhibit iNOS production and prevent macrophage killing. After 1 h of incubation, the cells were treated with gentamicin for extracellular bacterial killing. At 2, 8, 16, 24 and 32 hours, a well of infected monolayer for each cell was lysed and the lysate was plated for CFU enumeration. Percent uptake was assessed at 2 hours post infection and calculated as follows: number of bacteria recovered/ number of bacteria input x 100. Percent survival at each time point was calculated as follows: number of bacteria recovered at time point/ number of bacteria recovered at 2 h time point x100. There was no significant difference in percent uptake as about two percent of each strain was phagocytized by RAW 264.7 cells (Figure 5A). *B. mallei* 23344 exhibited steady increases in percent survival as the time course study progressed (8h to 32 h time points). Additionally, with the exception of CLH002 at the 8 and 16 h time points, the percent survival for each of the strains was significantly lower than those observed in *B. mallei* 23344. The changes in survival percentages that occurred between the 24 and 32 h time points seemed to provide the most important contrast between the mutants and their ability to survive intracellularly. Whereas TMM001 exhibited a substantial increase in percent survival between these time points

(2.5% to 444.9%), CLH002 and CLH001 exhibited a decline in percent survival (4.9% to 0.2% and 1.7% to 0%, respectively) (Figure 5B).

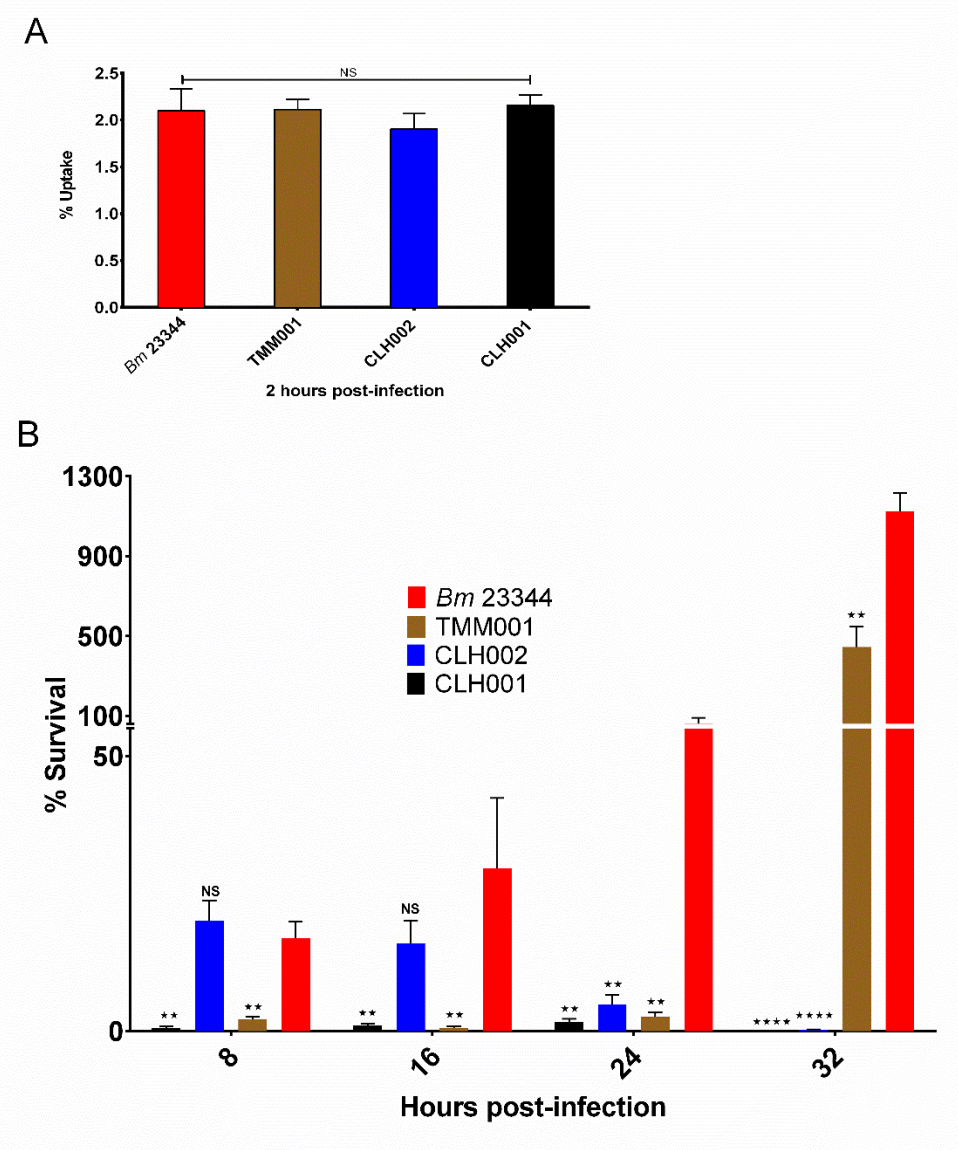


Figure 5: Macrophage uptake and survival assay. RAW 264.7 murine macrophages seeded at $5x10^6$ cells/well and infected with *B. mallei* 23344, TMM001, CLH002, or CLH001 at an MOI of 10 with 200 µg/ml of AG. After 1 h of incubation, the cells were treated with 50 µg/mL of gentamicin for extracellular bacterial killing. (A) Percent uptake was determined at 2 h post-infection. (B) At 8, 16, 24 and 32 h post-infection, bacteria were enumerated by plating cell lysate and percent survival was calculated. Error bars represent the SD of three independent experiments and significant difference at each individual time point was determined with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. NS = $p > 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

DISCUSSION

In addition to demonstrating that the CLH001 strain is viable in *in vitro*, these assays show that that CLH001 exhibits combined attenuated properties of both its *tonB* and *hcp1* deletions.

The fact that its growth kinetics are reduced in LBG but restored with the addition of $FeSO_4$ indicates that, like the *tonB* mutant TMM001, CLH001 is metabolically attenuated in low iron conditions. Meanwhile, CLH002 exhibits wild-type growth kinetics in both LBG and LBG with $FeSO_4$. This suggests that the growth deficiencies observed when CLH001 is grown in low iron conditions are attributed to the *tonB* deletion contained in the TMM001 backbone. Free iron is essential for bacterial metabolism, but is kept at very low levels in the host environment through a host defense process referred to as nutritional immunity (Weinberg, 1984). As a result of these low iron levels CLH001 should exhibit *in vivo* attenuation similar to that described in TMM001 (Mott *et al*, 2015).

Genes under the regulation of the VirAG regulon (including *hcp1*) have been shown to be upregulated under low iron and zinc conditions, such as those in M9G media (Burtnick & Brett, 2013). As such, the lack of Hcp1 expression in CLH001 and CLH002 further confirms the fact that the *hcp1* gene was successfully truncated in these mutants. Additionally, since Hcp1 is both a secreted protein and a structural protein required for the assembly of the T6SS-1 apparatus (Pukatzki *et al*, 2009; Silverman *et al*, 2012; Silverman *et al*, 2013), this assay demonstrates that we have successfully knocked out T6SS-1 function in these mutants.

MNGC formation has been described as an important mechanism of intra- and intercellular spread in *B. mallei* and *B. pseudomallei*, allowing the bacteria to disseminate

without being exposed to the host immune system (Kespichayawattana *et al*, 2000; Schwarz *et al*, 2014). It is hypothesized that MNGC formation is linked with granuloma formation and bacterial persistence (Boddey *et al*, 2007; Burtnick *et al*, 2010). The fact that both CLH002 and CLH001 are unable to form MNGCs in phagocytic and non-phagocytic cell lines *in vitro* suggests that these mutants may be deficient in dissemination and possibly persistence *in vivo*. Further, the percent of MNGC formation was roughly equivalent in RAW264.7 and HeLa cells infected with TMM001 and *B. mallei* 23344, suggesting the inability to form MNGCs can be attributed to the *hcp1* deletion.

A previous study by Burtnick et al. showed that *B. mallei* Δ*tssE*, another type six secretion system deficient mutant, showed decreased survival and replication kinetics in RAW 264.7 cells (Burtnick *et al*, 2010). Our results with RAW264.7 cells infected with CLH001 and CLH002 mirrored these findings, particularly at later time points (24 and 32 h). Interestingly, at 32 h the percent survival dropped to nearly undetectable levels, suggesting that the *hcp1* mutation has eliminated CLH001 and CLH002's ability to survive intracellularly for extended periods of time. In contrast, TMM001 exhibits poor early growth kinetics but later in infection (after 32 h) experiences a spike in growth and replication kinetics. This observation suggests that TMM001 has adapted to the macrophage environment. Since *B. mallei* are facultative intracellular organisms that use macrophage uptake as a vehicle for dissemination, CLH001's inability to survive in RAW264.7 macrophages provides a good indication that this mutant will exhibit *in vivo* attenuation.

Chapter 8. Evaluation of the attenuation, safety, and protective capacity of *B. mallei* CLH001 as a potential vaccine candidate against acute respiratory glanders

INTRODUCTION

There are currently no FDA approved vaccines for *B. mallei,* and the current treatment regimen, long-term aggressive antibiotic therapy, is only partially protective. The current study focuses on CLH001 and its suitability as a live attenuated vaccine to protect against acute respiratory glanders infection. In order to assess its *in vivo* attenuation and persistence and evaluate its ability to protect against acute lethal infection with *B. mallei*, we used a BALB/c mouse model of infection. This is a widely accepted model for the study of acute *B. mallei* and *B. pseudomallei* infections, as BALB/c mice are highly susceptible and succumb to infection at doses which are comparable to those observed in humans. Additionally, the course of infection in this model is said to mimic human infection in the course of infection, symptomology, and organ pathology (Leakey *et al*, 1998; Fritz *et al*, 2000; Lever *et al*, 2003).

Additionally, the NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ or NOD scid gamma (NSG) mice mouse model was used to assess the safety of CLH001 vaccination. The NSG mouse is considered an extremely immunocompromised mouse model, as it lacks B cells, T cells, NK cells, has poorly functional monocytes/macrophages and is deficient in multiple cytokine signaling pathways. There are a growing number of persons in the US who are immunocompromised at varying levels, and live attenuated vaccines are often contraindicated for these populations. Therefore, NSG mice provide an ideal model for

assessing the risk of adverse effects following administration of CLH001 into a fully susceptible host.

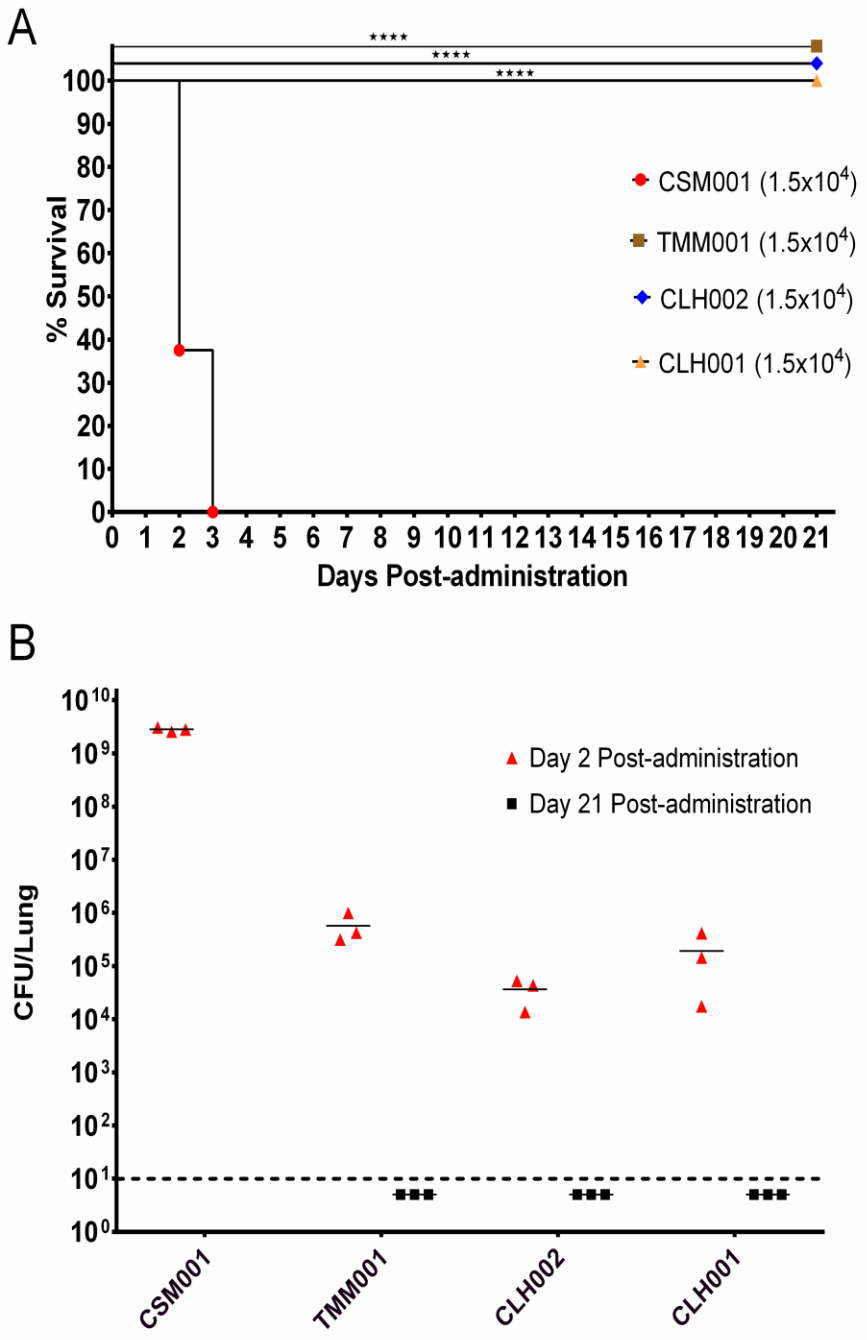
In some of our assays, the CSM001 strain was used in lieu of *B. mallei* 23344. Although more virulent based on $LD_{50,}$ CSM001's *in vivo* clinical presentation and dissemination characteristics are similar to those seen in *B. mallei* 23344 infection. As a result of transposon mutagenesis, the CSM001 strain contains not only the bioluminescent reporter gene, *lux* which can be used to monitor *in vivo* dissemination, but also a Km resistance gene (Massey *et al*, 2011). This Km resistance is not present in CLH001, and allows for distinction between vaccination and challenge strains. Because CSM001 exhibits high similarity to wild-type, both in lethality and disease progression, it remains an excellent challenge strain for evaluating vaccine-induced protection in a mouse model of glanders.

RESULTS

Survival Study

To assess the *in vivo* attenuation of mutant strains, BALB/c mice were administered 1.5 x 10^4 CFU of *B. mallei* Δ*tonB* (TMM001), Δ*hcp1* (CLH002), Δ*tonB* Δ*hcp1* (CLH001) or highly virulent *B. mallei lux* (CSM001). All the animals infected with the CSM001 succumbed to infection by day 3 post-administration. In contrast, all mice receiving TMM001, CLH002, or CLH001 survived to the end of the study (★★★★, $p < 0.0001$) (Figure 6A). At days 2 and 21 post-administration, the lungs, spleens and livers were removed, homogenized and plated for CFU enumeration. At day 2 post-administration, the CFU counts in organs from TMM001-, CLH002- and CLH001-infected mice were greatly reduced compared to those from CSM001-infected mice (Figure 6B-D). At day 21 post-

administration, bacteria were not recovered from the lungs and livers of TMM001-, CLH002- and CLH001-infected mice (Figure 6B-C). As previously observed, high CFU numbers were recovered from the spleens of TMM001-infected mice (Figure 6D and [Mott *et al*, 2015]). In contrast, lower numbers of bacteria were recovered from the spleens of CLH002-infected mice and no bacteria were recovered from the spleens of mice receiving CLH001.


A
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100
90
80
70
60
50
40
30
20
10
0
% Survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days Post-administration
CSM001 (1.5x10^4)
TMM001 (1.5x10^4)
CLH002 (1.5x10^4)
CLH001 (1.5x10^4)
B
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/Lung
Day 2 Post-administration
Day 21 Post-administration
CSM001
TMM001
CLH002
CLH001

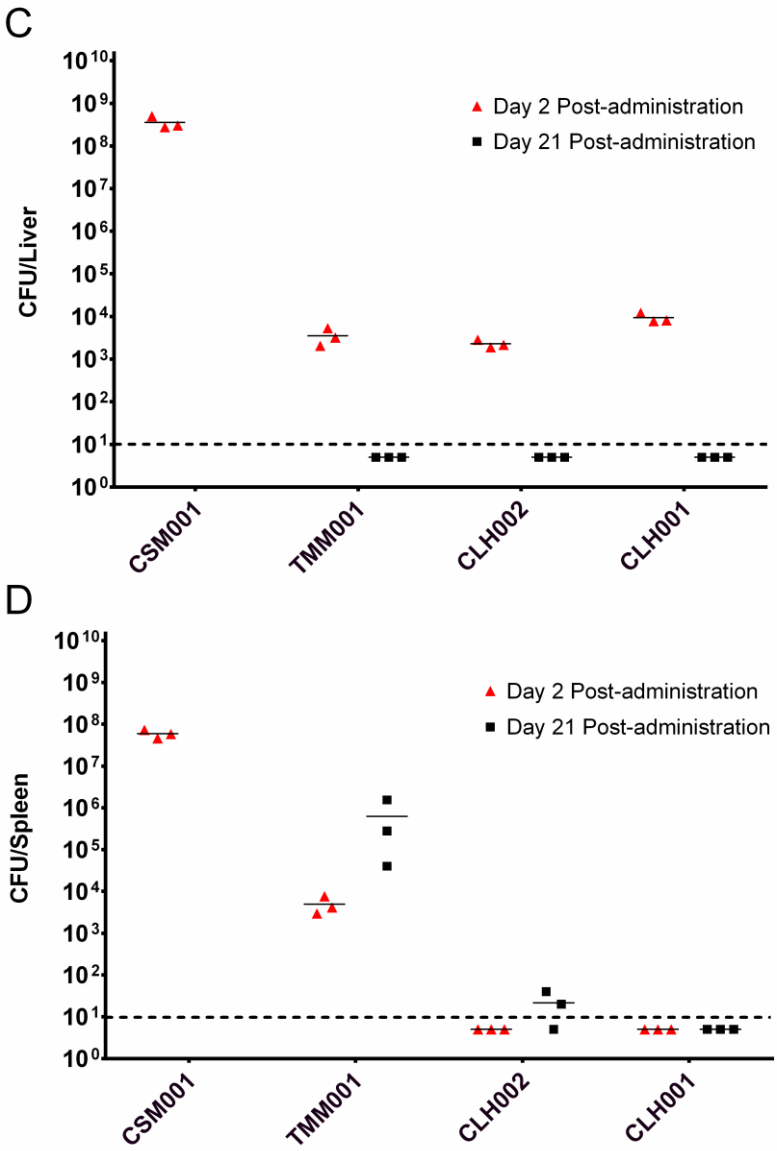


Figure 6: CLH001 is highly attenuated in BALB/c mice compared to CSM001 strain and exhibits increased safety over TMM001 and CLH002 strains at day 21 post-administration. (A) Percent survival of BALB/c mice (n = 8) following i.n. challenge with $1.5x10^4$ CFU of CSM001 (●), TMM001 (■), CLH002 (◆), or CLH001 (▲) at 21 days post-administration. Differences in survival times were determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. **** $p \leq 0.0001$. Colonization of mouse lungs (B), livers (C), and spleens (D) (n = 3) at day 2 (▲) and day 21 (■) post-administration with $1.5x10^4$ CFU of TMM001, CLH002, and CLH001. The limit of detection was 10 CFU/organ (horizontal dotted line).

Histopathological analysis of the tissues (lungs, livers, and spleens) of mice challenged with the different strains was compared to PBS-treated BALB/c mice (Figure 7, 8, and 9, respectively). The organs of TMM001-infected mice presented with minimal to moderate pathologic changes, including minimal perivascular and peribronchial inflammatory infiltrates in the lung sections (Figure 7B), foci of mild hepatocellular necrosis (Figure 8B), and mild to moderate necrosis of follicles were visible in the spleen (Figure 9B). While the TMM001-infected organs generally exhibited increased abnormal findings relative to similar organs from the other treatment groups, only their spleens exhibited significant changes in histopathology compared to spleens from PBS-treated mice (*, $p = 0.02$) (Figure 9E) and minimal pathologic changes were noted in lungs and livers (Figure 7E and 8E). Overall, reduced pathologic changes were noted in CLH002-challenged mice compared to TMM001-infected mice. The lungs of CLH002-challenged BALB/c mice were considered mostly unremarkable (Figure 7C), but the livers and spleens exhibited small foci of necrosis (Figure 8C and 9C). In contrast, organs of mice vaccinated with CLH001 mutant were unremarkable and resembled organs from PBS-treated mice. (Figure 7D, 8D, and 9D).

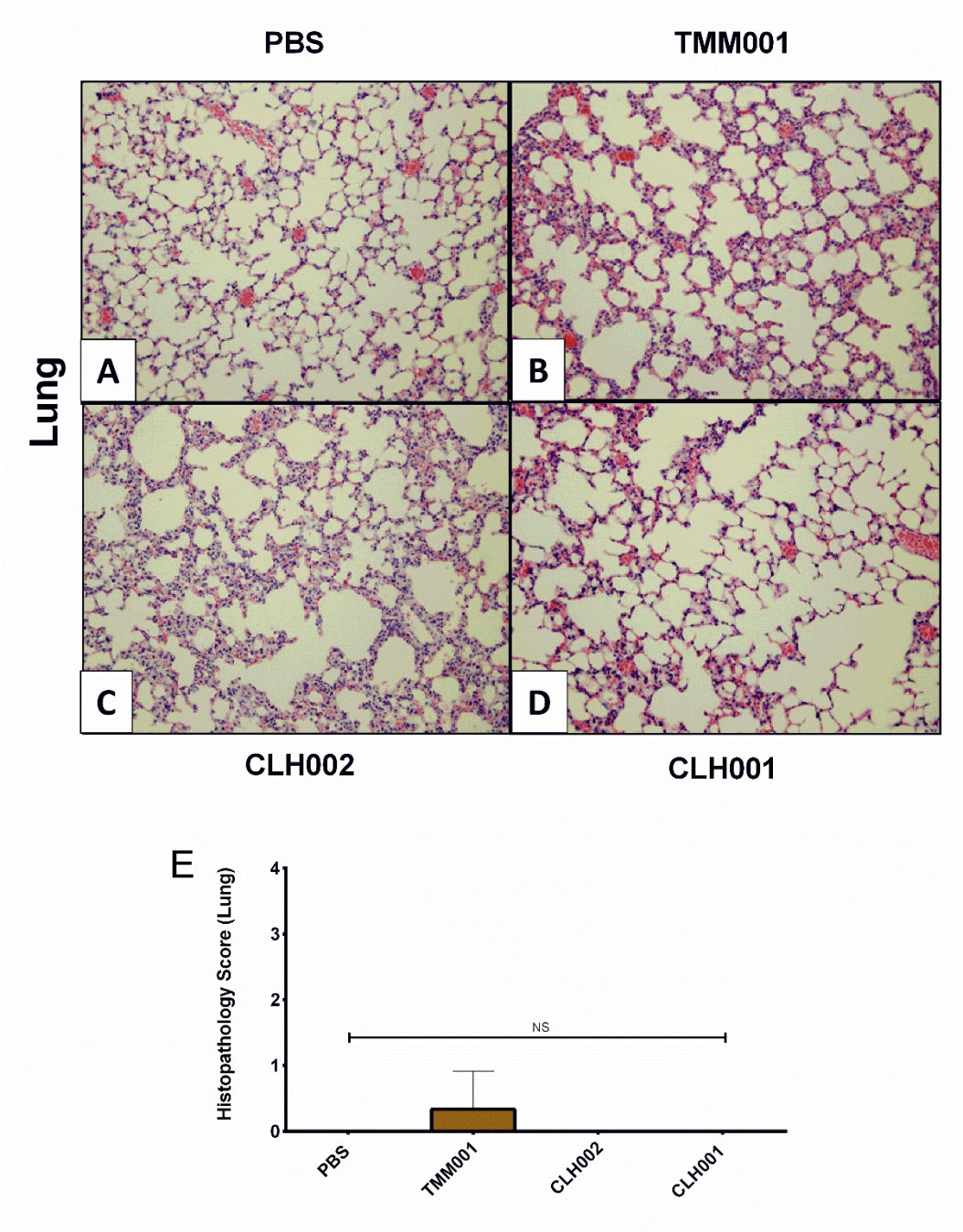


Figure 7: Lung histopathology and scoring. H&E stained lungs of mice at 21 days post-administration of PBS (A), or $1.5x10^4$ CFU of TMM001 (B), CLH002 (C), or CLH001 (D). Scores (E) were assigned for lung sections after microscopic examination. Bars plotted with SD are representative of the mean histopathology scores of three animals. Statistical significance was determined by the Mann-Whitney test. NS = $p > 0.05$.

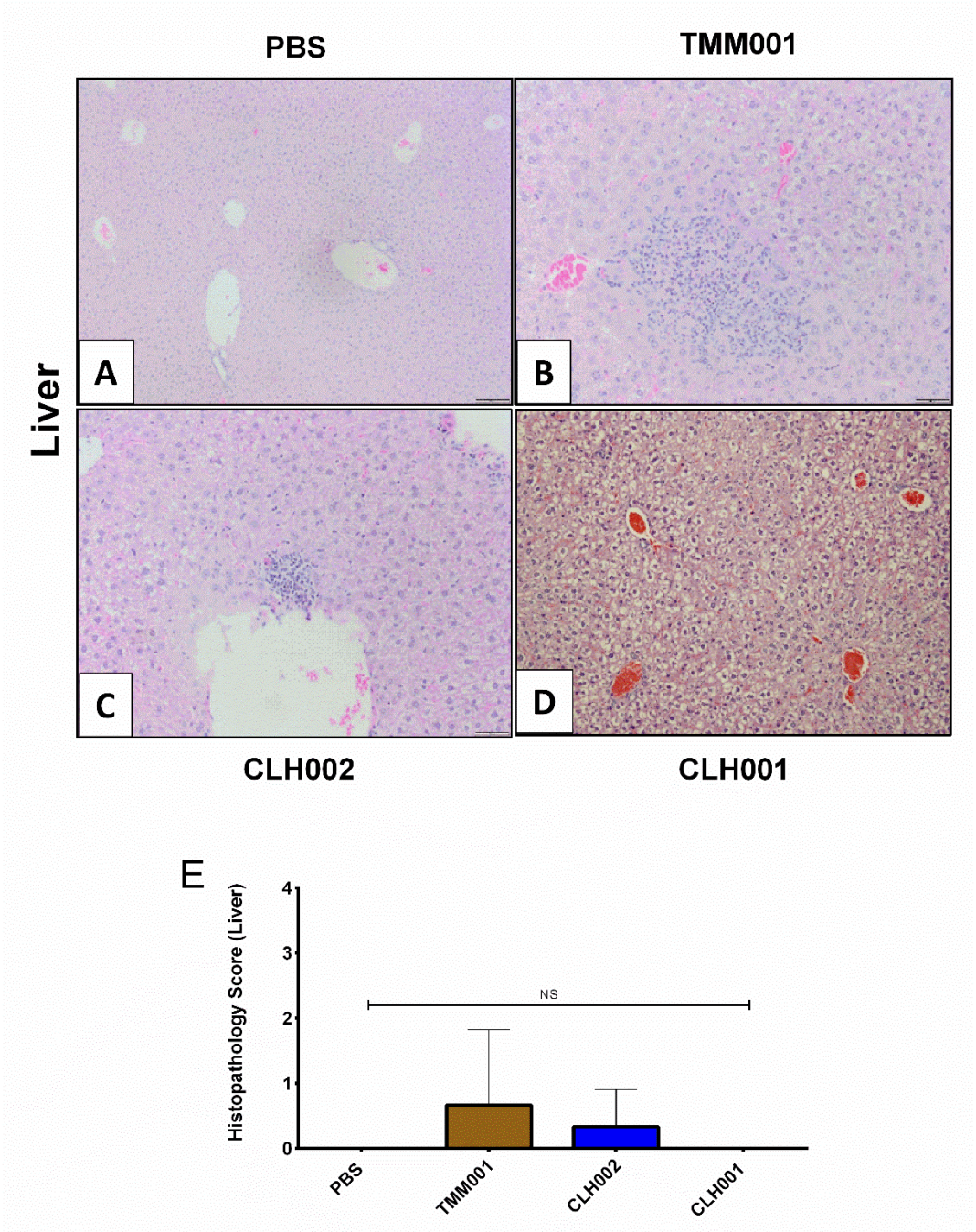


Figure: 8 Liver histopathology and scoring. H&E stained livers of mice at 21 days post-challenge with PBS (A), or $1.5x10^4$ CFU of TMM001 (B), CLH002 (C), or CLH001 (D). Scores (E) were assigned for liver sections after microscopic examination. Bars plotted with SD are representative of the mean histopathology scores of three animals. Statistical significance was determined by the Mann-Whitney test. NS = $p > 0.05$.

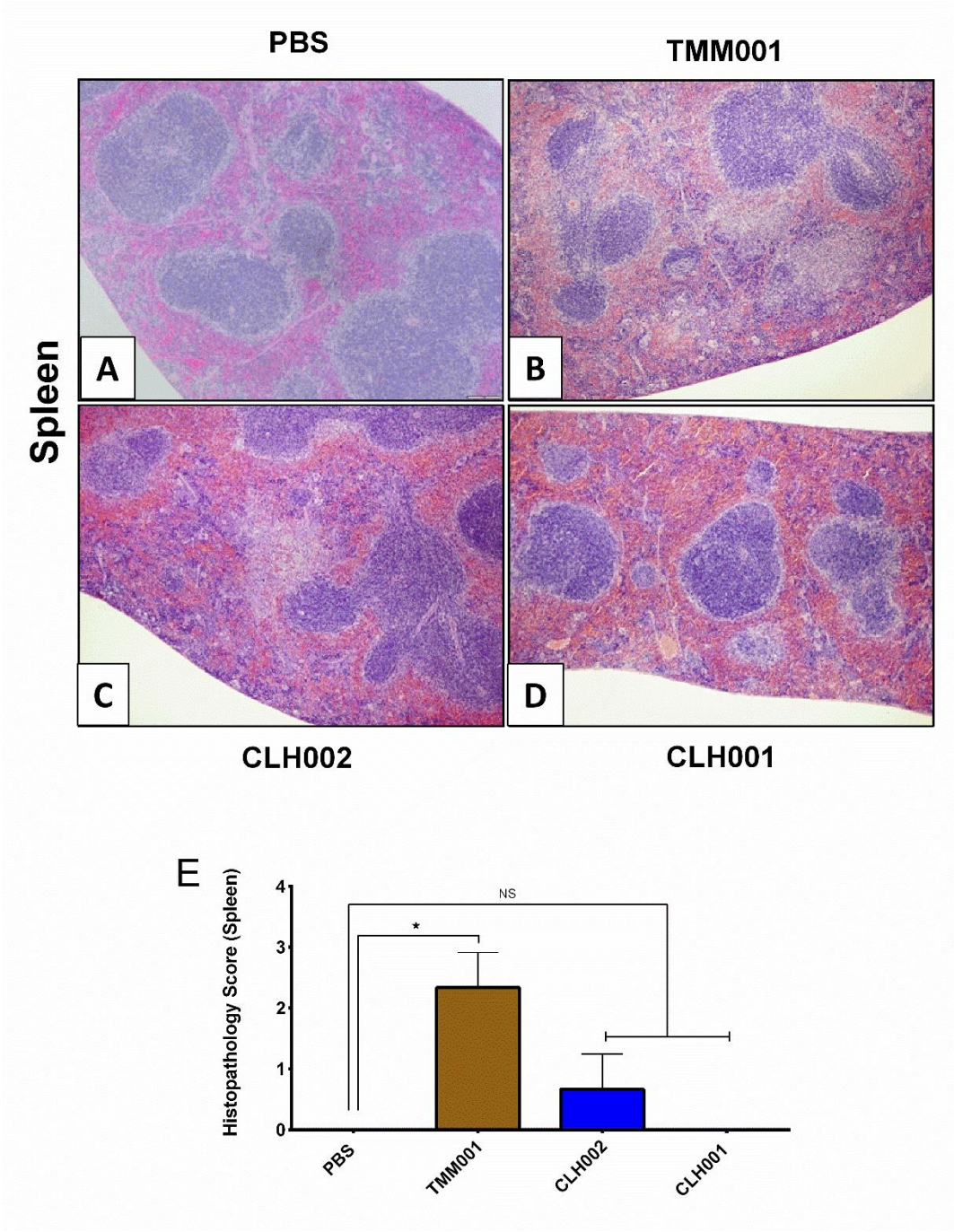
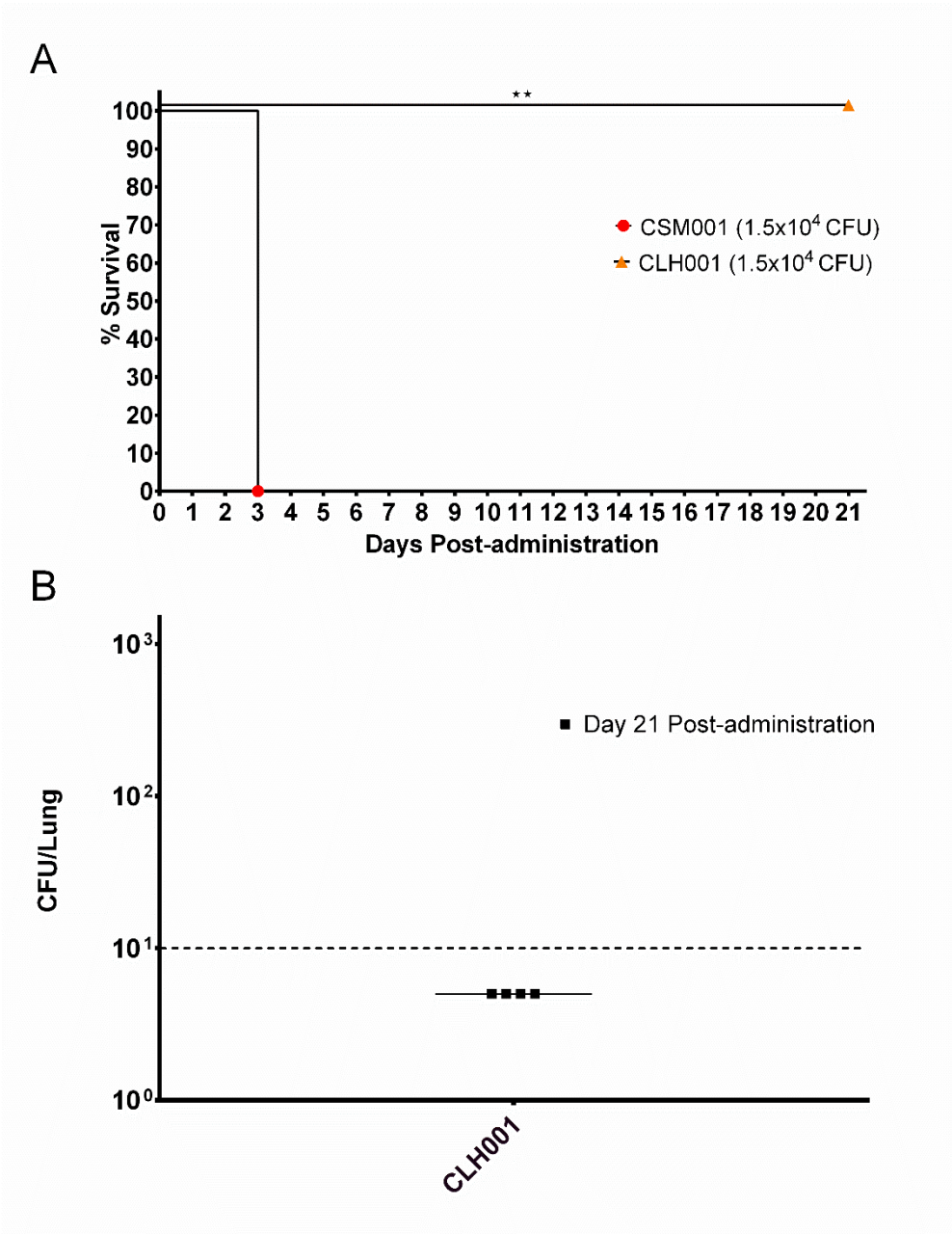


Figure: 9 Spleen histopathology and scoring. H&E stained spleens of mice at 21 days post-challenge with PBS (A), or $1.5x10^4$ CFU of TMM001 (B), CLH002 (C), or CLH001 (D). Scores (E) were assigned for spleen sections after microscopic examination. Bars plotted with SD are representative of the mean histopathology scores of three animals. Statistical significance was determined by the Mann-Whitney test. NS = $p > 0.05$, ⋆ $p \leq 0.05$.

Immunocompromised Mouse Survival Study

The safety of the CLH001 vaccine was further evaluated in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ or NOD scid gamma (NSG) mice. NSG mice are considered the most highly immunodeficient mouse available and such immunodeficiency provides a practical model to test vaccine-associated morbidity and mortality. NSG mice challenged i.n. with $1.5x10^4$ CFU of CSM001 or CLH001 were used to evaluate persistence and/or dissemination to target organs. All mice challenged with CSM001 (n = 4) succumbed to infection by day 3 post-challenge. In contrast, mice receiving CLH001 (n = 6) survived to the end of the study (★★, p = 0.0027) (Figure 10A). At 21 days post-challenge, the organs of surviving mice were evaluated for CFU and no bacteria were detected in the lungs, livers, or spleens (Figure 10B, C and D, respectively) of any of the mice. Gross pathology and histology analysis of the lungs and livers indicated that the architecture was unremarkable compared to organs from an uninfected NSG mouse (Figure 11) and that the spleens of CLH001-infected mice exhibited minimal architectural changes (Figure 12).


A
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100
90
80
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10
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% Survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days Post-administration
CSM001 (1.5x10^4 CFU)
CLH001 (1.5x10^4 CFU)
B
10^3
10^2
10^1
10^0
CFU/Lung
Day 21 Post-administration
CLH001

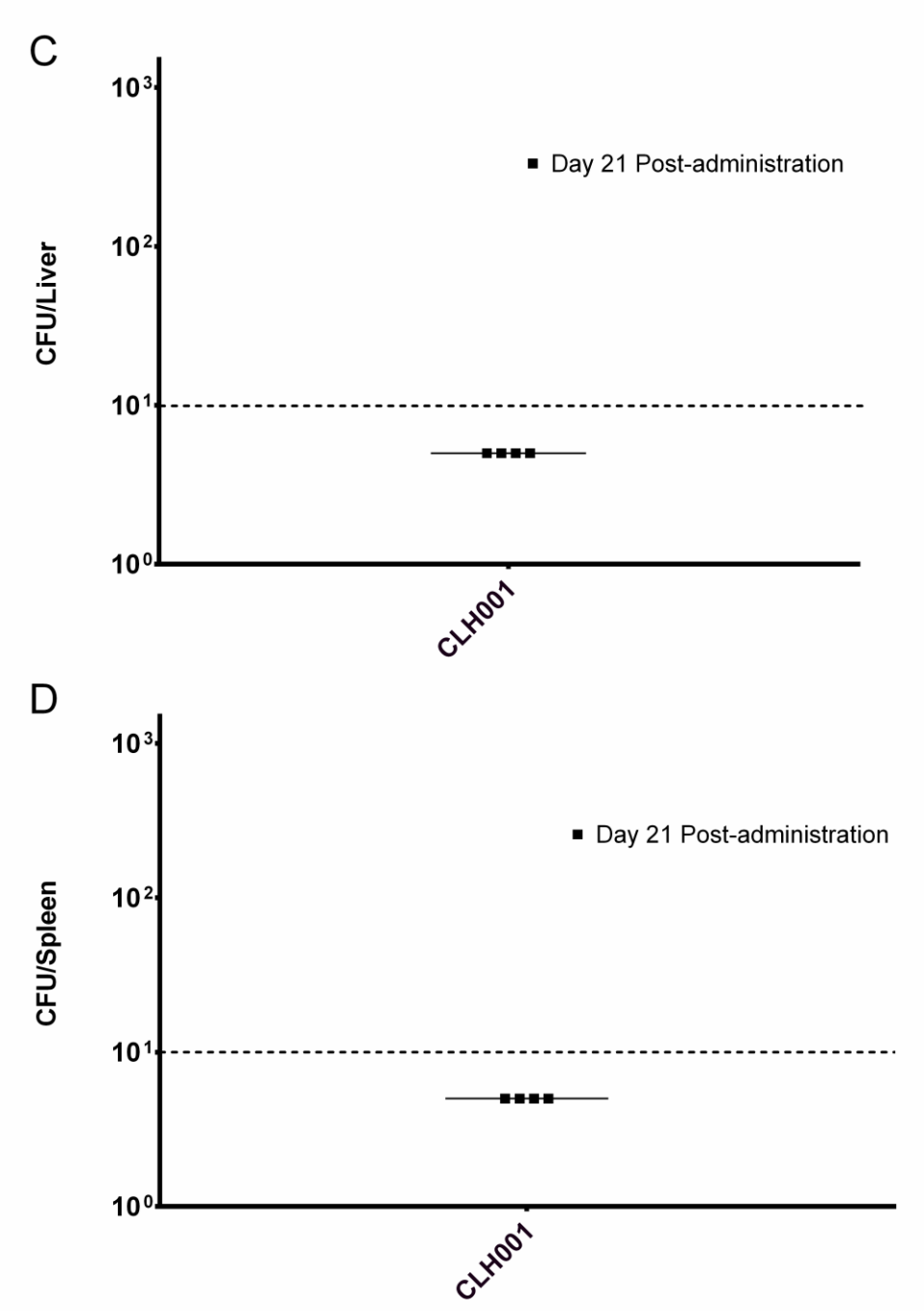


Figure: 10 NSG mice infected with CLH001 showed 100% survival and complete bacterial clearance. (A) Percent survival of NSG mice following i.n challenge with $1.5x10^4$ CFU of CSM001 (n = 4, ●), or CLH001 (n = 6, ▲) at 21 days post infection. The statistical significance was determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. ★★ $p \leq 0.001$. Colonization of mouse lungs (B), livers (C), and spleens (D) (n = 4) at day 21 post infection with $1.5x10^4$ CFU of CLH001 (■). The limit of detection was 10 CFU/organ (horizontal dotted line).

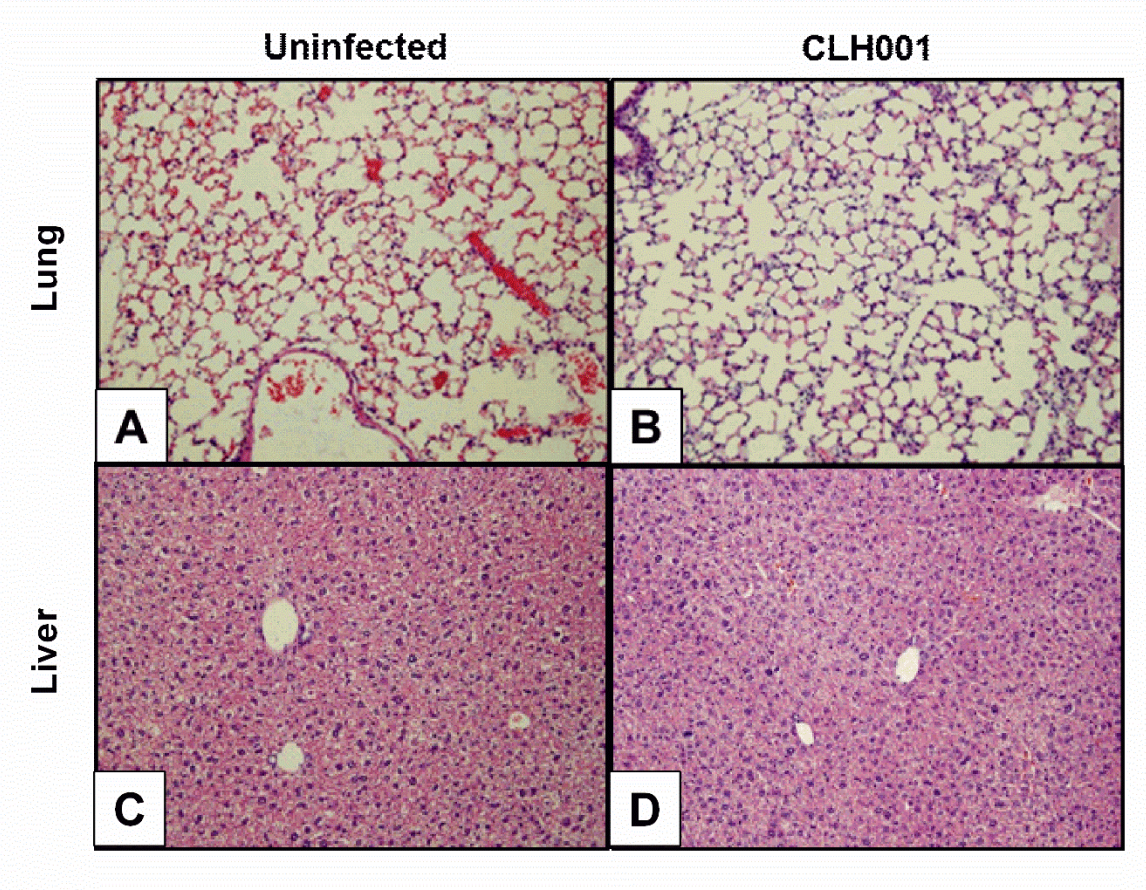


Figure: 11 Representative images of lung and liver pathology from infected and non-infected NSG mice. Displays the unremarkable histopathology seen in H&E stained lungs (A-B) and livers (C-D) from an uninfected mouse and mice challenged with $1.5x10^4$ CFU of CLH001 at 21 days post-infection.

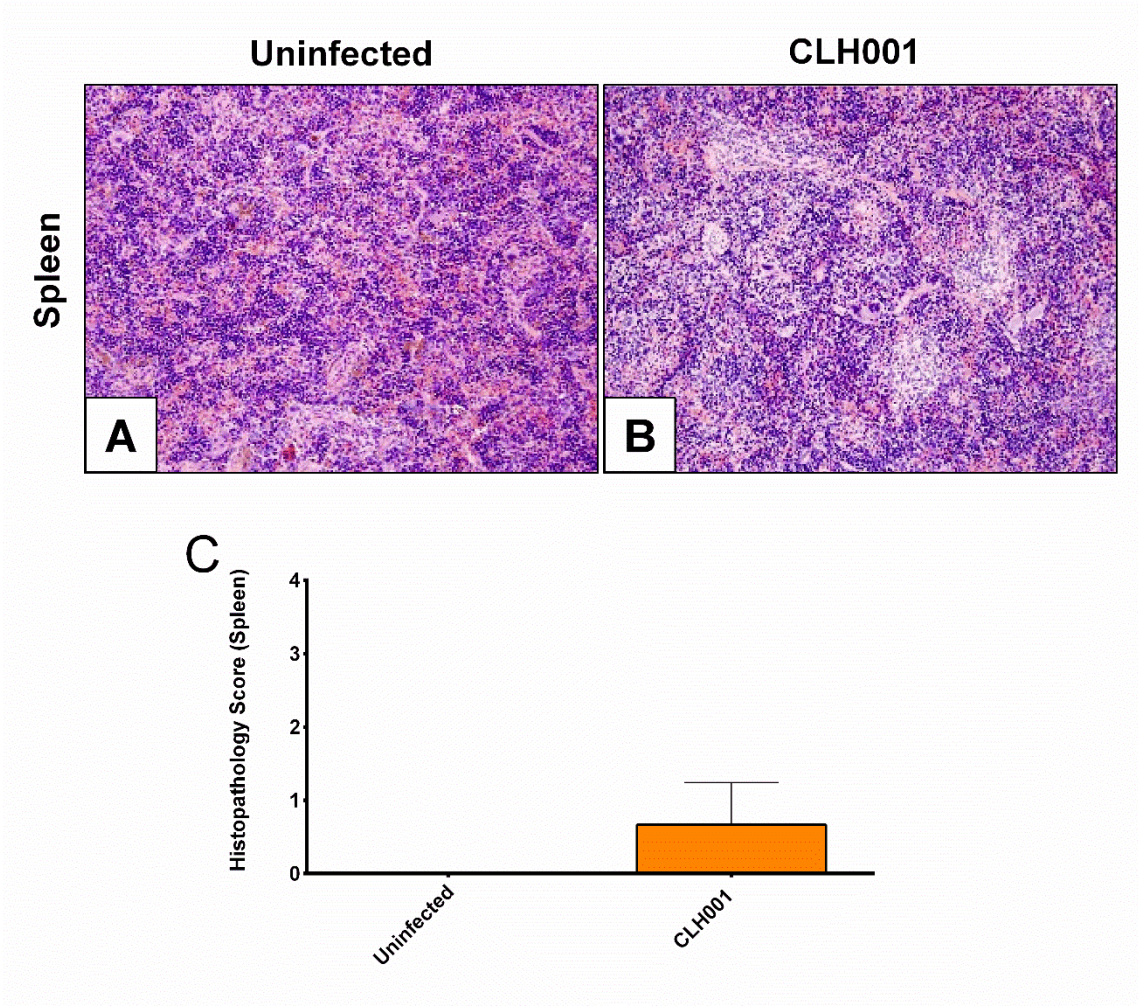


Figure: 12 NSG mouse spleen histopathology and scoring. H&E stained spleens of an uninfected mouse (A) and mice (n = 2) at 21 days post-challenge with $1.5x10^4$ CFU of CLH001 (B). Scores (C) were assigned for spleen sections after microscopic examination.

Single Dose Vaccination Study

Since the previous experiments had established the attenuation and safety of the CLH001 strain, a single dose vaccination study was performed to assess CLH001's ability to protect BALB/c mice against a lethal dose of CSM001. The TMM001 strain was included in this study for comparison. Mice received a single i.n. vaccination with PBS, or $1.5x10^4$ CFU of CLH001 or TMM001. At day 21 post-vaccination mice were i.n. challenged with $1.5\ x10^4$ CFU of CSM001 and monitored for survival. As had previously been shown, TMM001 provided significant protection against CSM001 challenge (Mott *et al*, 2015). At the 14 day experimental end point, animals vaccinated with TMM001 exhibited survival of 62.5% (***, $p < 0.001$) (Figure 13). However, Single dose vaccination with CLH001 was no more protective than vaccination with PBS as all mice administered PBS- and CLH001-vaccination succumbed to infection by day 3 and day 4 post infection, respectively and no difference in mean time to death was noted between the two groups (mean time to death = 3 days). Additionally, sera collected from mice (n=3) at day 21 post vaccination showed that while TMM001-vaccinated mice showed a robust *B. mallei*-specific IgG total (mean reciprocal endpoint titer = 51,200 ± 0) with a Th1-bias (IgG2a:IgG1 ratio = 4.4), but CLH001-vaccinated mice produced only a weak antibody response (mean reciprocal endpoint titer = 533 ± 189) (Table 3).

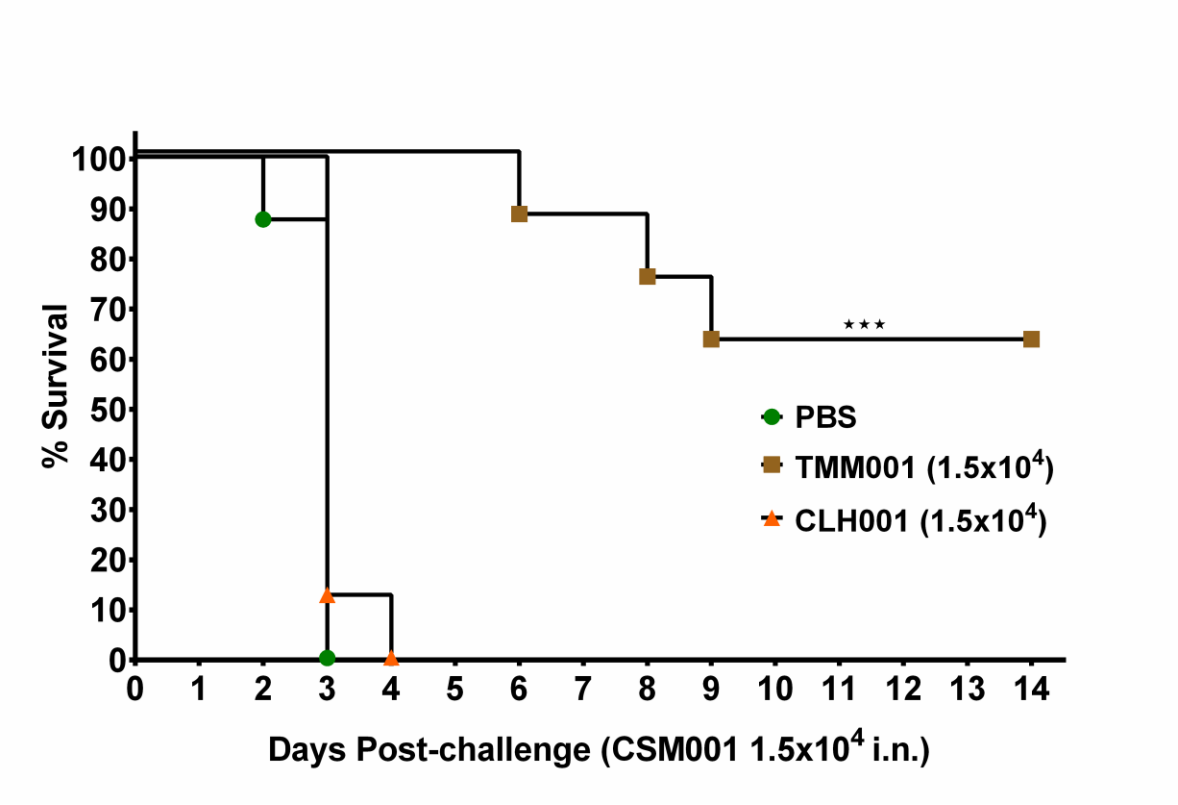


Figure: 13 Single dose vaccination with CLH001 is not protective against lethal dose challenge with CSM001. Mice were i.n. vaccinated with PBS (n = 11, ●), $1.5x10^4$ CFU of CLH001 (n = 11, ■), or $1.5x10^4$ CFU of CLH001 (n = 11, ▲). At 21 days post-vaccination, mice were i.n. challenged with $1.5x10^4$ CFU of CSM001. The statistical significance of differences in survival times was determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. ***, $P < 0.001$.

Table 3. Serum antibody responses of BALB/c mice receiving single i.n. vaccination

Vaccine/Dosage [a]	Serum titer[b]			Serum ratio IgG2a/IgG1
	IgG	IgG2a	IgG1	
PBS	ND[c]	ND	ND	--
TMM001 / 1.5x10^4 CFU	51,200 ± 0	136,533 ± 48,272	31,289± 14,504	4.4
CLH001 / 1.5x10^4 CFU	533 ± 189	3,733 ± 1,995	ND	--

[a] Antibody titers were determined at 3 weeks post vaccination. PBS control mice were vaccinated with 50 µl of PBS.

[b] To determine serum antibody titers, sera from 3 mice/group were tested by indirect ELISA with irradiated *B. mallei* ATCC 23344 whole cells used as the antigen. Titers of sera from 3 mice were performed in triplicate and reported as the mean reciprocal endpoint titer ±S.D.

[c] Not detected, because titers less than or equal to 100 were considered to be negative.

Prime and Boost Vaccination Study

Next, an experiment was performed to evaluate whether the protection garnered by CLH001 vaccination could be increased by implementing a prime and two boost vaccine regimen using 1.5x10^4 CFU of CLH001 or a prime and two boost using a tenfold higher dose of CLH001 (1.5 x10^5 CFU). Mice received a prime and two boost vaccination (14 days apart) with 1.5x10^4 or 1.5x10^5 of CLH001. Sera was collected from mice (n = 3) at two week intervals following each prime and boost dose, and compared to serum from TMM001-vaccinated mice. *B. mallei*-specific IgG, IgG2a, and IgG1 reciprocal endpoint titers were determined via indirect ELISA. As predicted, CLH001-vaccinated mice developed an anamnestic humoral response as indicated by test results of all IgG and IgG isotypes following each subsequent CLH001 vaccination at both doses. The CLH001 (1.5x10^5 CFU) prime and two boost sera had the highest *B. mallei*-specific IgG total mean reciprocal titers for this vaccine (42,667 ± 6,967) and gave similar values to those seen in

TMM001-vaccinated mice (51,200 ± 0). Further, the observed ratio of IgG2a:IgG1 ≥ 1.0 has also been described as being favorable for protection (Table 4). At 21 days post last vaccination, the prime and two boosts vaccinated BALB/c mice (PBS, $1.5x10^4$ or $1.5x10^5$ CFU CLH001) were challenged with 1.5 $x10^4$ CFU of CSM001. All PBS-treated mice succumbed to infection by day 5 post-challenge; however, animals vaccinated with both CLH001 doses exhibited survival of 62.5% (★★★, $p < 0.0002$) and 100% (★★★★, $p < 0.0001$), respectively, at the 35 day experimental end point (Figure 14A).

Organs were collected 21 days post-vaccination and 35 days post-challenge for CFU enumeration. Bacteria were not detected in the lungs, livers, or spleens (Figure 14B, C and D, respectively) of any of the mice tested. Additionally, gross pathology and histology analysis of these organs from CLH001 vaccinated animals at 35 days post infection indicated that the architecture was unremarkable compared to organs from PBS-treated uninfected mice (Figure 15).

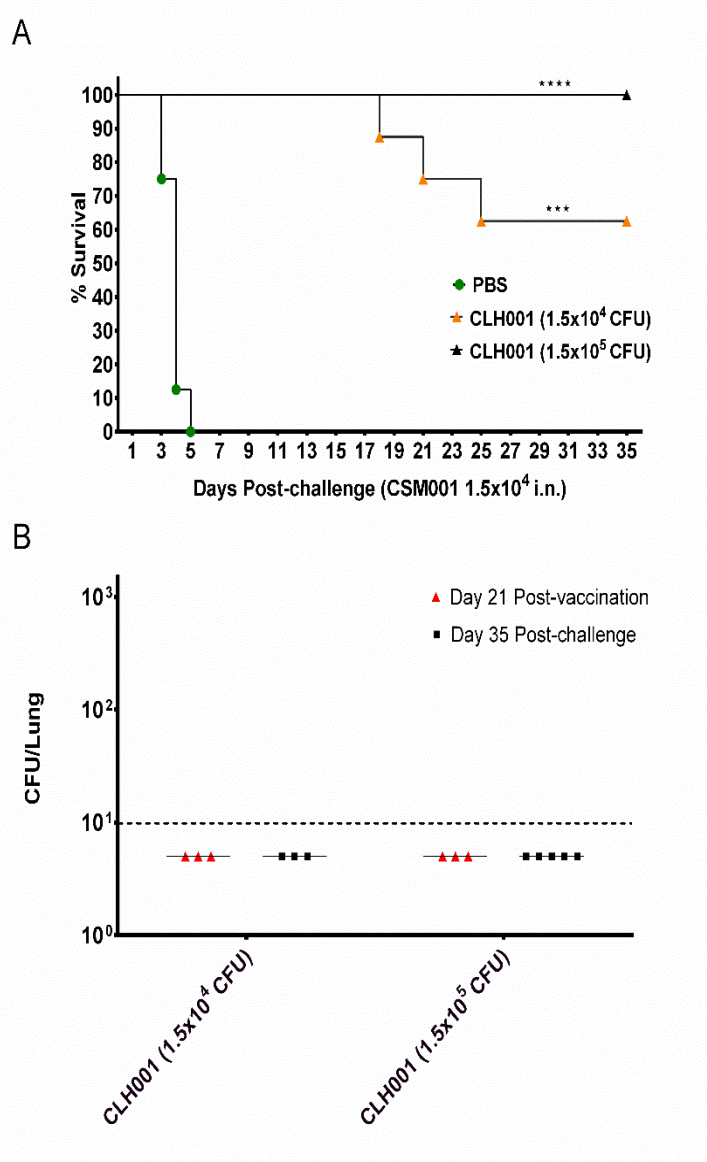
Table 4. Serum antibody response of BALB/c mice i.n. vaccinated with live attenuated *B. mallei* strains

Vaccine/Dosage (P, B, or 2B) [a]	Serum titer[b]			Serum ratio IgG2a/IgG1
	IgG	IgG2a	IgG1	
PBS	ND[c]	ND	ND	--
TMM001 / 1.5x10^4 CFU (P)	51,200 ± 0	136,533 ± 48,272	31,289± 14,504	4.4
CLH001 / 1.5x10^4 CFU (P)	400 ± 0	1,333 ± 377	ND	--
CLH001 / 1.5x10^5 CFU (P)	3,733 ± 2,325	944 ± 163	ND	--
CLH001 / 1.5x10^4 CFU (B)	4,267 ± 2,133	5,511 ± 473	1,422 ± 154	3.9
CLH001 / 1.5x10^5 CFU (B)	19,200 ± 7,692	31,259 ± 7,853	8,533 ± 1,742	3.7
CLH001 / 1.5x10^4 CFU (2B)	19,911 ± 5,321	18,489 ± 6,517	8,533 ± 3,695	2.2
CLH001 / 1.5x10^5 CFU (2B)	42,667 ± 6,967	96,711 ± 8,533	24,178 ± 2,011	4

[a] Antibody titers were determined at 2 weeks post primary vaccination (P), 2 weeks post boost (B), and 3 weeks post second boost (2B). PBS Control animals were vaccinated with 50 µl of PBS. Results of sera from TMM001 vaccinated animals (prime only) were included for comparison.

[b] To determine serum antibody titers, sera from 3 mice/group were tested by indirect ELISA with irradiated *B. mallei* ATCC 23344 whole cells used as the antigen. Titers of sera from 3 mice were performed in triplicate and reported as the mean reciprocal endpoint titer ±S.D.

[c] Not detected, because titers less than or equal to 100 were considered to be negative.


A
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% Survival
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PBS
CLH001 (1.5x10^4 CFU)
CLH001 (1.5x10^5 CFU)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Days Post-challenge (CSM001 1.5x10^4 i.n.)
B
10^3
10^2
10^1
10^0
CFU/Lung
Day 21 Post-vaccination
Day 35 Post-challenge
CLH001 (1.5x10^4 CFU)
CLH001 (1.5x10^5 CFU)

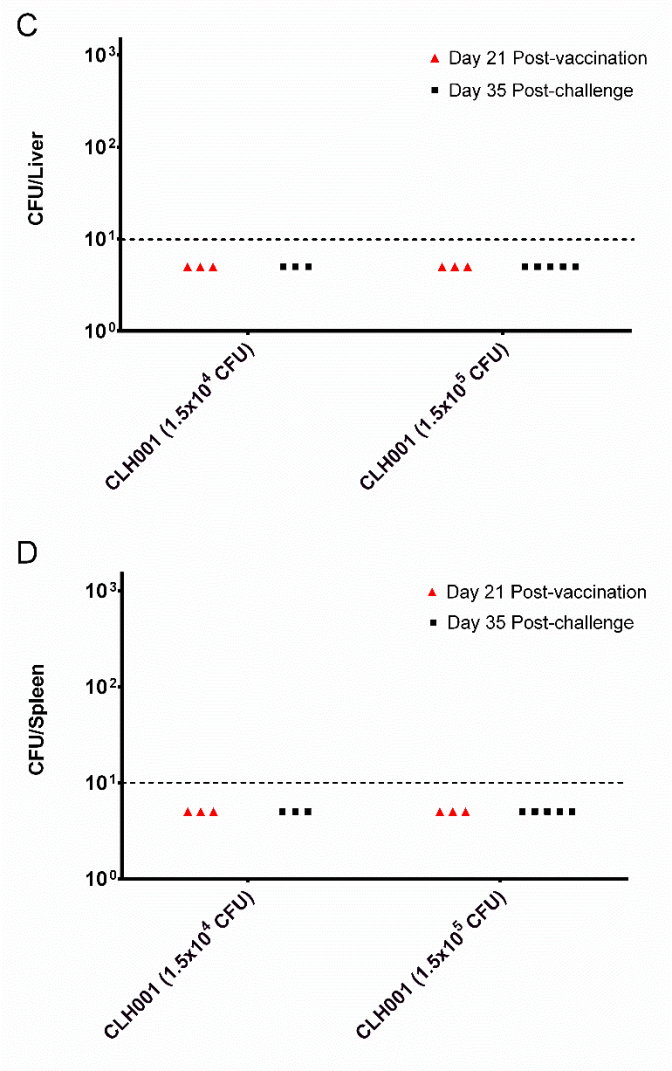


Figure: 14 Prime and boost vaccination with CLH001 ($1.5x10^5$ CFU) provides 100% protection with no discernable organ colonization following CSM001 challenge. (A) Mice were i.n. immunized with a prime and two boost of PBS (n = 8, ●), CLH001 at $1.5x10^4$ CFU of CLH001 (n = 11, ▲), or $1.5x10^5$ CFU (n = 11, ▲). Three weeks after receiving their second boost, mice were i.n. challenged with $1.5x10^4$ CFU of CSM001. The statistical significance of differences in survival times was determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. *** $p \leq 0.0002$, **** $p \leq 0.0001$. Colonization of mouse lungs (B), livers (C) and spleens (D) (n = 3/CLH001-vaccination group) at day 21 post-vaccination (▲) and day 35 post-challenge with CSM001 (n = 3 for CLH001 $1.5x10^4$ group and n = 5 for CLH0001 $1.5x10^5$ group; ■). The limit of detection was 10 CFU/organ (horizontal dotted line).

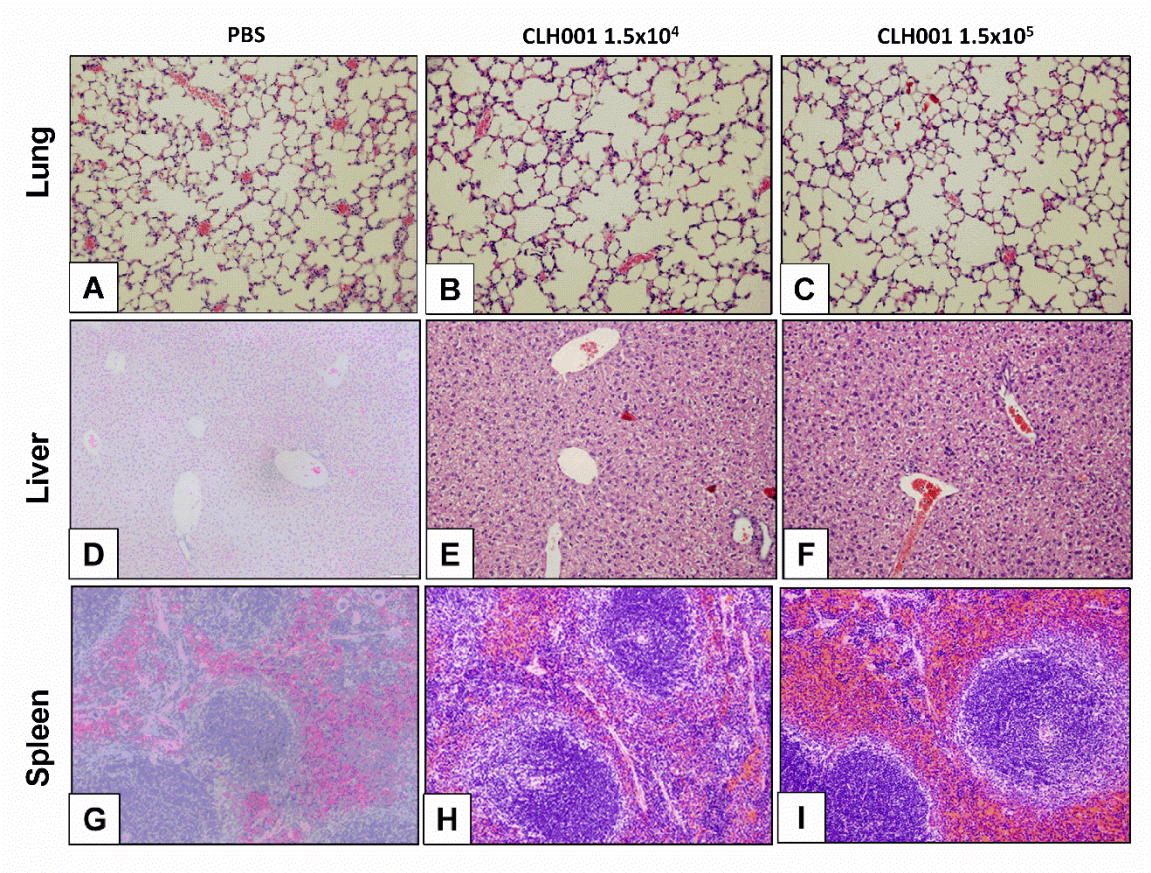


Figure: 15 Organ pathology from CLH001 prime and boosted vaccinated mice is unremarkable at 35 days post-infection with CSM001. Panels A-I display the unremarkable pathology seen in H&E stained lungs, liver and spleen of PBS vaccinated control mice (A, D, and G, respectively), CLH001 ($1.5x10^4$ CFU) prime and boost immunized mice at 35 days post-challenge with CSM001 (B, E, and H, respectively), and CLH001 ($1.5x10^5$ CFU) prime and boost immunized mice at 35 days post-challenge with CSM001 (C, F, and I, respectively).

High Dose *B. mallei* ATCC 23344 Challenge Prime and Boost Vaccination Study

Next, an experiment was performed to evaluate whether CLH001 was protective against high dose challenge with *B. mallei* wild-type strain ATCC 23344. BALB/c mice were prime and boosted twice (14 days apart) with PBS or $1.5x10^5$ CFU of CLH001, and challenged with $3.5x10^5$ CFU of *B. mallei* 23344 at 21 days after the last vaccine boost. *B. mallei*-specific IgG, IgG2a, and IgG1 reciprocal endpoint titers from sera collected two weeks following each vaccination closely mimicked those seen in the previous prime and boosts vaccination experiment (IgG mean reciprocal endpoint titer = 45,511 ± 9,583 and IgG2a:IgG1 ratio = 3.9)(Table 5). All PBS-treated mice succumbed to infection, while 87.5% of CLH001-vaccinated mice survived until the experimental endpoint (Figure 16A). Although bacteria were not detected from the lungs, livers, or spleens (Fig. 16B, C and D, respectively) of any of the mice at day 21 post-vaccination, or the lungs (Fig. 16B) at day 35 post-challenge, significant bacterial counts were recovered in two of the livers ($4.97x10^4$ and $1.28x10^5$ CFU/organ) (Fig. 16C) and all of the spleens (mean = $3.5x10^8$ CFU/organ) (Fig 16D).

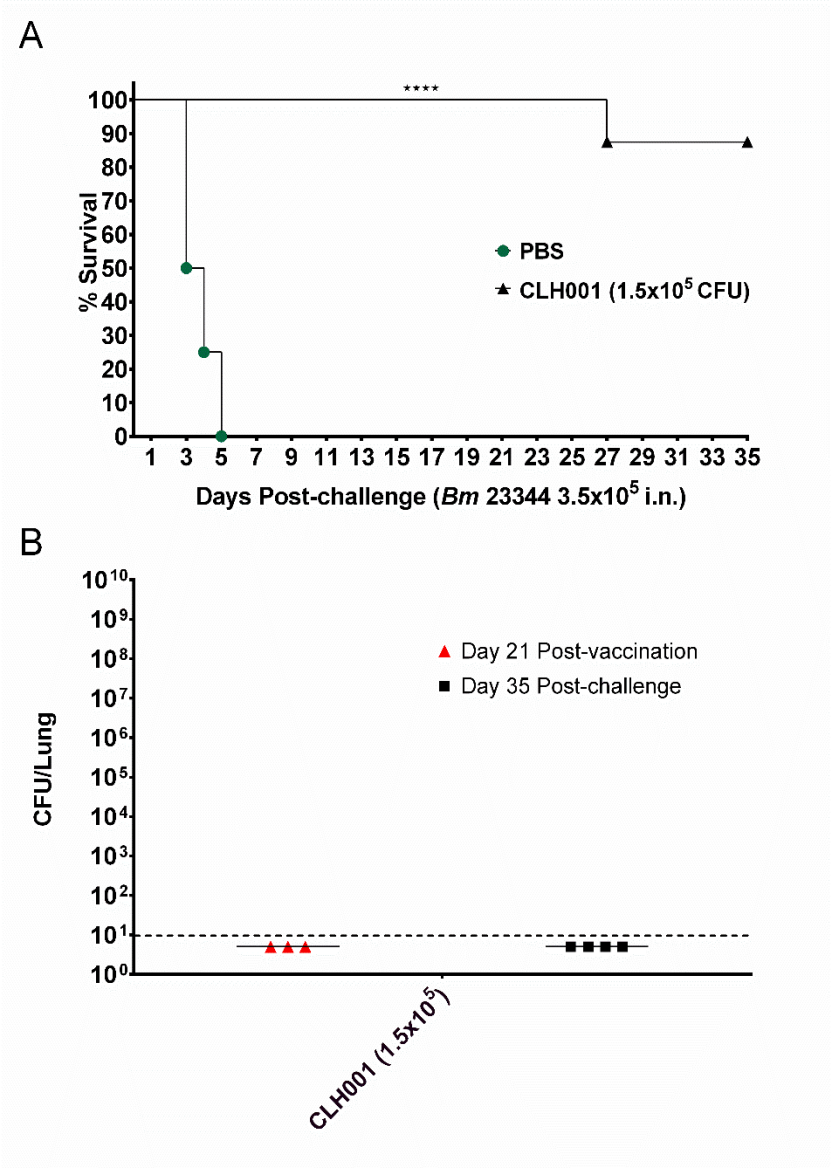
Table 5. Serum antibody responses of BALB/c mice i.n. vaccinated with live attenuated *B. mallei* strains (high dose *B. mallei* ATCC 23344 challenge study)

Vaccine/Dosage (P, B, or 2B) [a]	Serum titer[b]			Serum ratio IgG2a/IgG1
	IgG	IgG2a	IgG1	
PBS	ND[c]	ND	ND	--
TMM001 / $1.5x10^4$ CFU (P)	51,200 ± 0	136,533 ± 48,272	31,289± 14,504	4.4
CLH001 / $1.5x10^5$ CFU (P)	3,968 ± 1,304	978 ± 308	ND	--
CLH001 / $1.5x10^5$ CFU (B)	10,667 ± 3,695	42,667 ± 6,967	10,667 ± 3,017	4.4
CLH001 / $1.5x10^5$ CFU (2B)	45,511 ± 9,853	96,711 ± 9,853	24,844 ± 4,927	3.9

[a] Antibody titers were determined at 2 weeks post primary vaccination (P), 2 weeks post boost (B), and 3 weeks post second boost (2B). PBS Control animals were vaccinated with 50 µl of PBS. Results of sera from TMM001 vaccinated animals (prime only) were included for comparison.

[b] To determine serum antibody titers, sera from 3 mice/group were used tested by indirect ELISA with irradiation-killed *B. mallei* ATCC 23344 whole cells used as the antigen. Titers of sera from 3 mice were performed in triplicate and reported as the mean reciprocal endpoint titer ±S.D.

[c] Not detected, because titers less than or equal to 100 were considered to be negative.


A
****
100
90
80
70
60
50
40
30
20
10
0
% Survival
PBS
CLH001 (1.5x10^5 CFU)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Days Post-challenge (Bm 23344 3.5x10^5 i.n.)
B
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/Lung
Day 21 Post-vaccination
Day 35 Post-challenge
CLH001 (1.5x10^5)

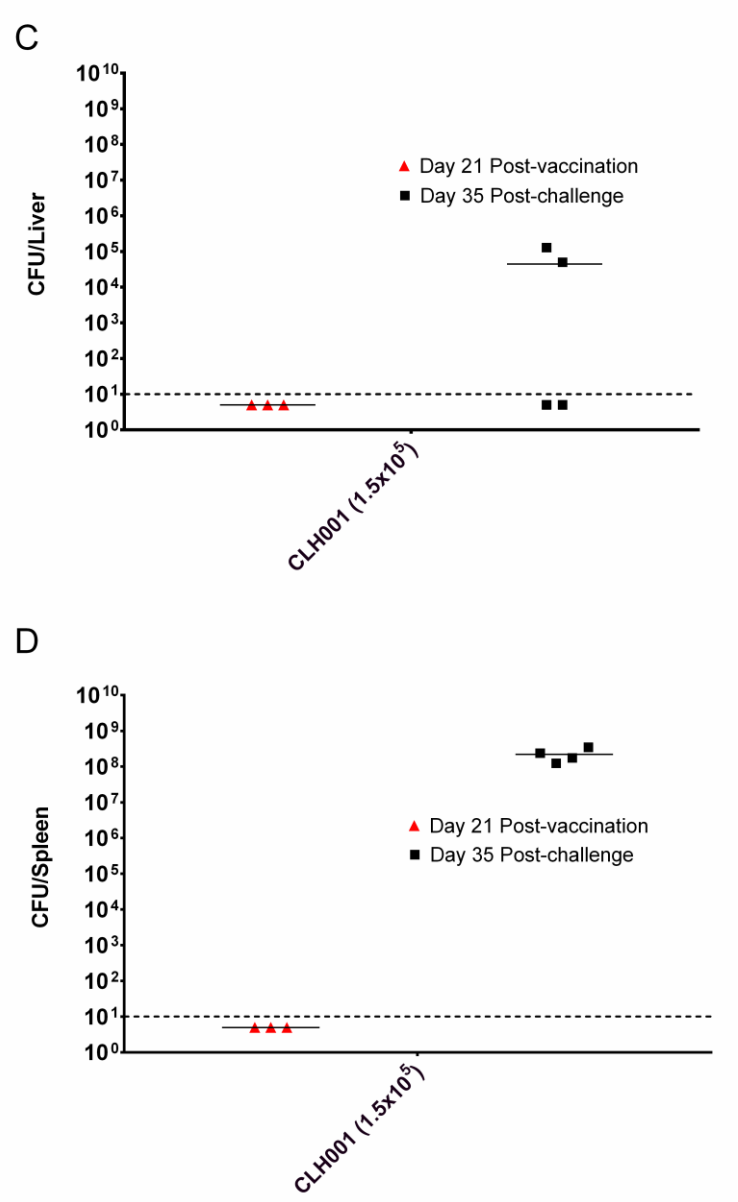


Figure: 16 Vaccination with CLH001 (1.5x105 CFU) provides significant protection following *B. mallei* 23344 high dose challenge, but bacterial organ colonization was observed. (A) Mice were i.n. immunized with a prime and two boosts regimen of PBS (n = 8, ●), or 1.5x105 CFU of CLH001 (n = 11, ▲). Three weeks after their last boost, mice were i.n. challenged with 3.5x105 CFU of B. mallei 23344. The statistical significance of differences in survival times was determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. ***, P < 0.0002. Colonization of mouse lungs (B), livers (C) and spleens (D) of CLH001-vaccinated mice at day 21 post-vaccination (n = 3, ▲) and day 35 post-challenge (n = 4, ■). The limit of detection was 10 CFU/organ (horizontal dotted line).

Serum Bactericidal Assay

The correlation between higher *B. mallei*-specific antibodies in animals vaccinated with CLH001 at $1.5x10^5$ CFU and increased survival rates led to the hypothesis that antibodies likely play an important role in protection. As such, a serum bactericidal assay was performed to evaluate whether antibodies from CLH001-vaccinated mice were able to reduce bacterial burden. *B. mallei* ATCC 23344 was incubated in LBG media containing guinea pig complement and one of the following: heat-inactivated naïve sera, heat-inactivated CLH001 sera, or anti-*B. mallei* LPS monoclonal antibody (anti-LPS mAb). The number of bacteria grown in the presence of naïve serum indicated nearly a six-fold increase over the initial bacterial concentration; meanwhile, the number of bacteria grown in the presence of CLH001 sera or anti-LPS mAb decreased below the initial bacterial concentration and contained significantly less bacteria than the naïve sera culture ($p=0.0062$ and $p=0.0063$ for these groups respectively) (Figure 17).

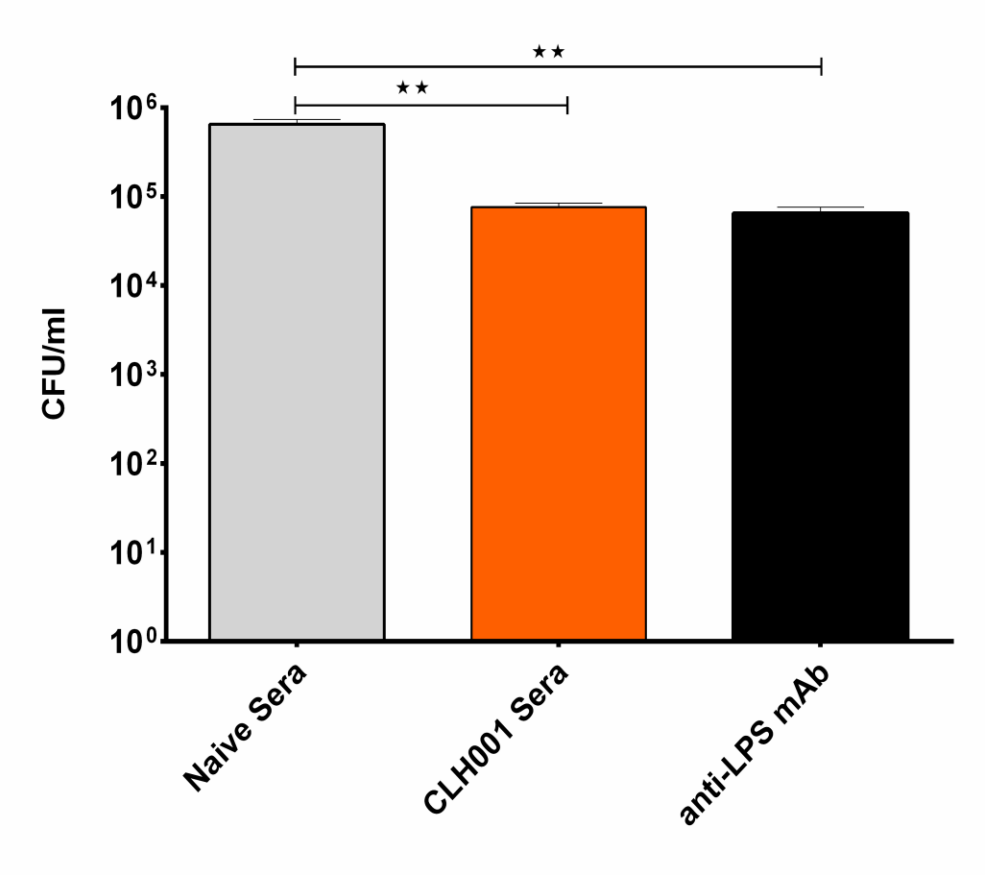


Figure: 17 CLH001 serum promotes killing of *B. mallei* 23344 *in vitro*. Serum bactericidal assays were performed by incubating 1.0 x10^5 CFU of *B. mallei* 23344 with guinea pig complement plus heat-inactivated naïve sera, heat-inactivated CLH001 sera, or anti-*B. mallei* LPS monoclonal antibody at 37ºC, with gentle agitation. At 6 h after incubation, samples were serially diluted and plated on LBG agar to determine viable CFU/ml. Experiments were performed in triplicate. The statistical significance of differences in bacterial CFU/ml was determined by one-way ANOVA. ★★ $p \leq 0.01$.

Passive Vaccination Study

Based on the evidence that humoral response played a role in the protection, an experiment was performed to evaluate whether passive vaccination with sera from CLH001-vaccinated mice could protect against lethal infection with CSM001. BALB/c mice (n = 3) received a single i.p. vaccination with 250 µl of pooled sera from naïve mice, PBS prime and boosted mice, or $1.5x10^5$ CFU of CLH001 prime and boost mice. At 24 h post-vaccination, mice were i.n. challenged with 2.1 $x10^3$ CFU of CSM001 and monitored for survival. Although analysis of CLH001-vaccinated sera demonstrated moderate *B. mallei*-specific IgG titers (reciprocal mean titer = 38,400 ± 0) (data not shown), passive vaccination with CLH001-vaccinated sera was not protective as mice in all three groups succumbed to infection by day 3 (Figure 18).

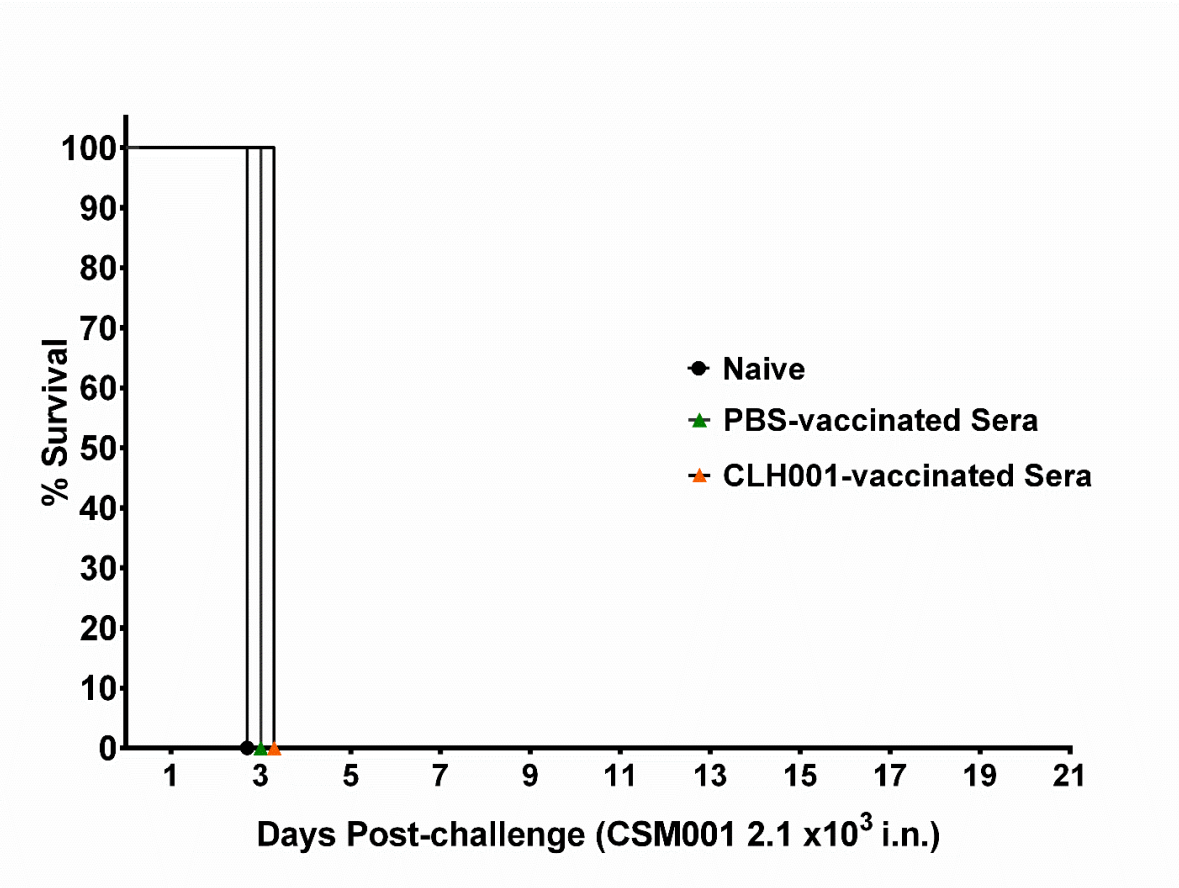


Figure: 18 Passive transfer of CLH001-vaccinated serum is not protective against lethal dose challenge with CSM001. BALB/c mice were i.p. immunized with 250 µl of pooled sera from naïve mice (n = 3, ●), mice prime and two boost vaccinated with PBS (n = 3, ▲), or mice prime and two boost vaccinated with 1.5x10^5 CFU of CLH001 (n = 3, ▲). Twenty-four hours later, mice were i.n. challenged with 2.1x10^3 CFU of CSM001. Survival times were plotted on a Kaplan-Meier curve.

IFNγ Analysis

Next, an ELISA was performed to measure and compare the IFNγ levels in sera (A) and lung lysates (B) of PBS prime and two boost vaccinated mice and CLH001 prime and boost vaccinated mice at day 0 (prior to challenge) and day 2 (post-challenge). Prior to challenge, no IFNγ was detected from the sera (Figure 19A) or lung lysate (Figure 19B) of either PBS- or CLH001-vaccinated mice. By day 2 post-challenge IFNγ was detected in both the serum (Figure 19A) and lungs (Figure 19B) of both groups and although not significant, the mean IFNγ levels were about 1.5 times higher in both CLH001-vaccinated sera and lung lysates compared to mean IFNγ levels in PBS-vaccinated mice (1,069.5 pg/ml vs. 701.5 pg/ml and 5,265.1 pg/g vs. 3,489.2 pg/g, respectively).

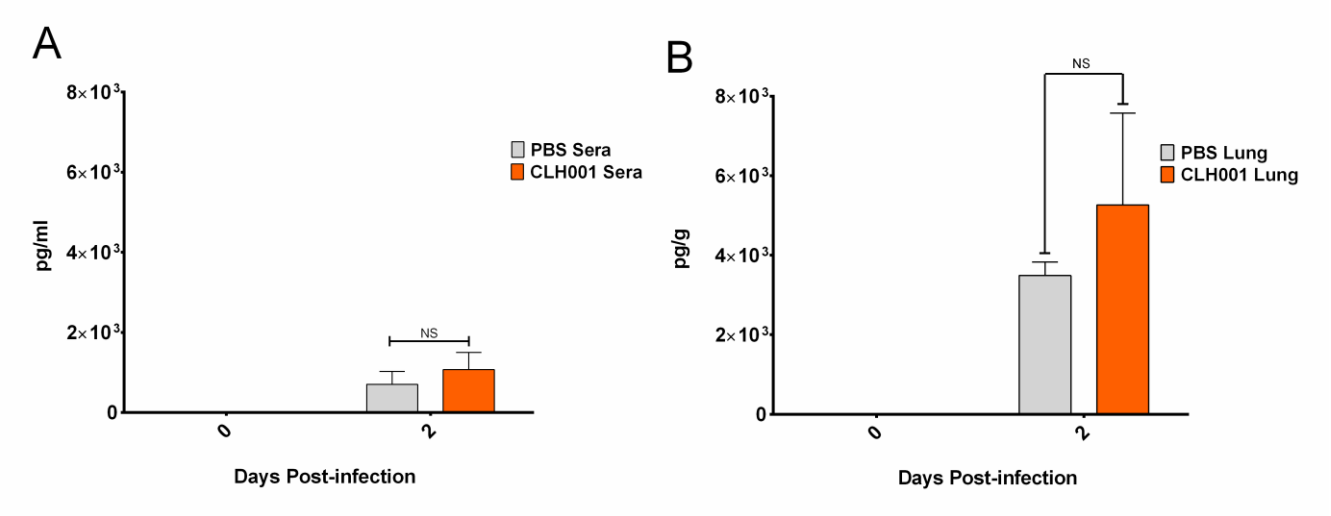
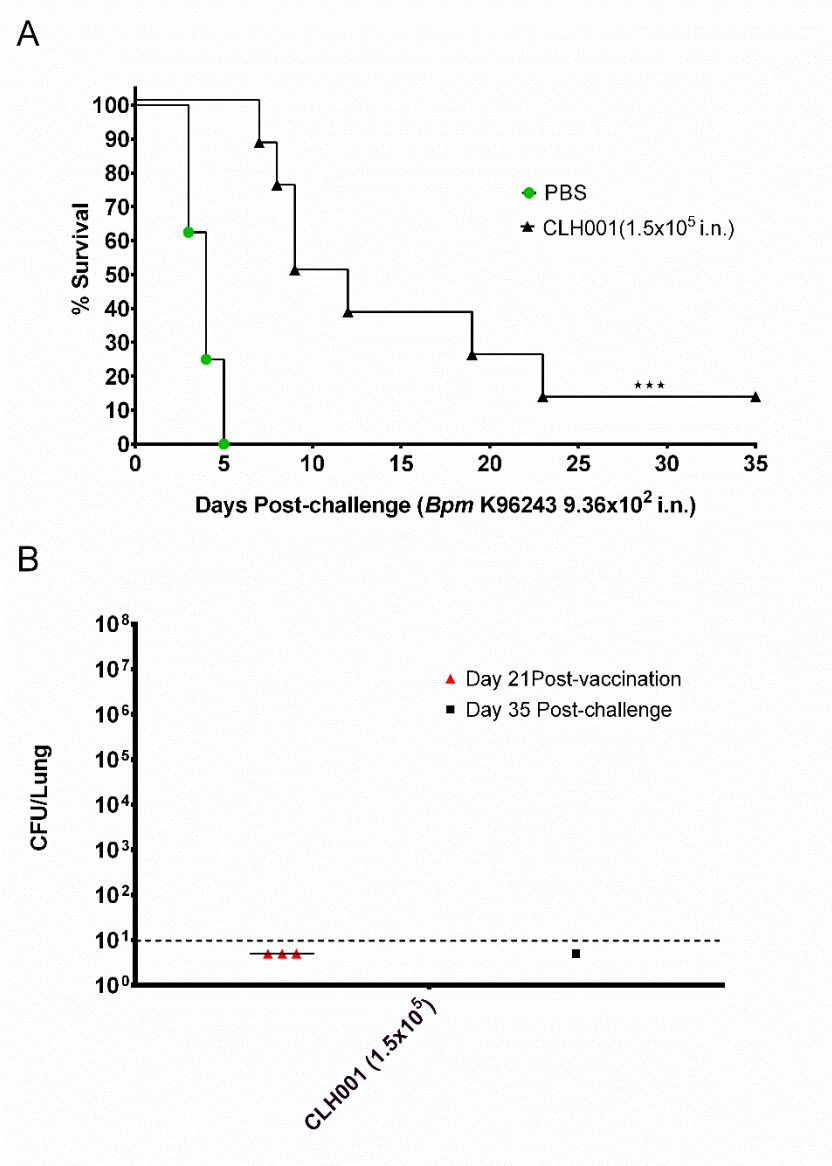


Figure: 19 CLH001 vaccinated mice exhibit elevated IFNγ lung and serum levels at day 2 post-challenge with *B. mallei* 23344. IFNγ ELISA results of sera (A) and lung lysates (B) from PBS- and CLH001-vaccinated mice at days 0 and 2 post-infection with *B. mallei* 23344 at $3.5x10^5$ CFU. Data represents the results from three mice. The statistical significance of differences in IFNγ production between treatment groups was determined by one-way ANOVA. NS = not significant ($p > 0.05$).

B. pseudomallei Cross-protection Study

Finally, because of the high degree of genetic homology between *B. mallei* and *B. pseudomallei*, an experiment was performed to evaluate whether CLH001 would provide cross-protection against lethal infection with *B. pseudomallei* K96243. BALB/c mice were prime and boosted twice (14 days apart) with PBS or $1.5x10^5$ CFU of CLH001, and challenged with $9.36x10^2$ CFU of *B. pseudomallei* K96243 at 21 days after the last vaccine boost. While all PBS-vaccinated mice succumbed to infection by day 5, one mouse in the CLH001-vaccinated group survived to the 35 day experimental endpoint (12.5% survival). Additionally, there was an increase in mean time to death in CLH001-vaccinated animals that succumbed to infection (10.5 days vs. 4 days), accounting for the statistical significance between groups (***, $p < 0.001$) (Figure 20A). Although bacteria were not detected from the lungs, livers, or spleens (Figure 20B, C and D, respectively) of any of the mice at day 21 post-vaccination, or the lung of the mice surviving to day 35 (Fig. 20B), bacterial counts were recovered from the liver (180 CFU) (Figure 20C) and spleen ($6.76x10^4$ CFU) (Figure 20D) of the surviving mouse. Interestingly, when pooled serum from CLH001 prime and two boost vaccinated mice was analyzed for *B. mallei* and *B. pseudomallei*-specific IgG totals, it was found that CLH001 generated a strong antibody response against *B. mallei* (reciprocal mean titer = 38,400 ± 0), but a weak antibody response to *B. pseudomallei* (reciprocal mean titer = 9,600 ± 0) (data not shown).


A
100
90
80
70
60
50
40
30
20
10
0
% Survival
0
5
10
15
20
25
30
35
Days Post-challenge (Bpm K96243 9.36x10^2 i.n.)
PBS
CLH001(1.5x10^5 i.n.)
***
B
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/Lung
Day 21Post-vaccination
Day 35 Post-challenge
CLH001 (1.5x10^5)

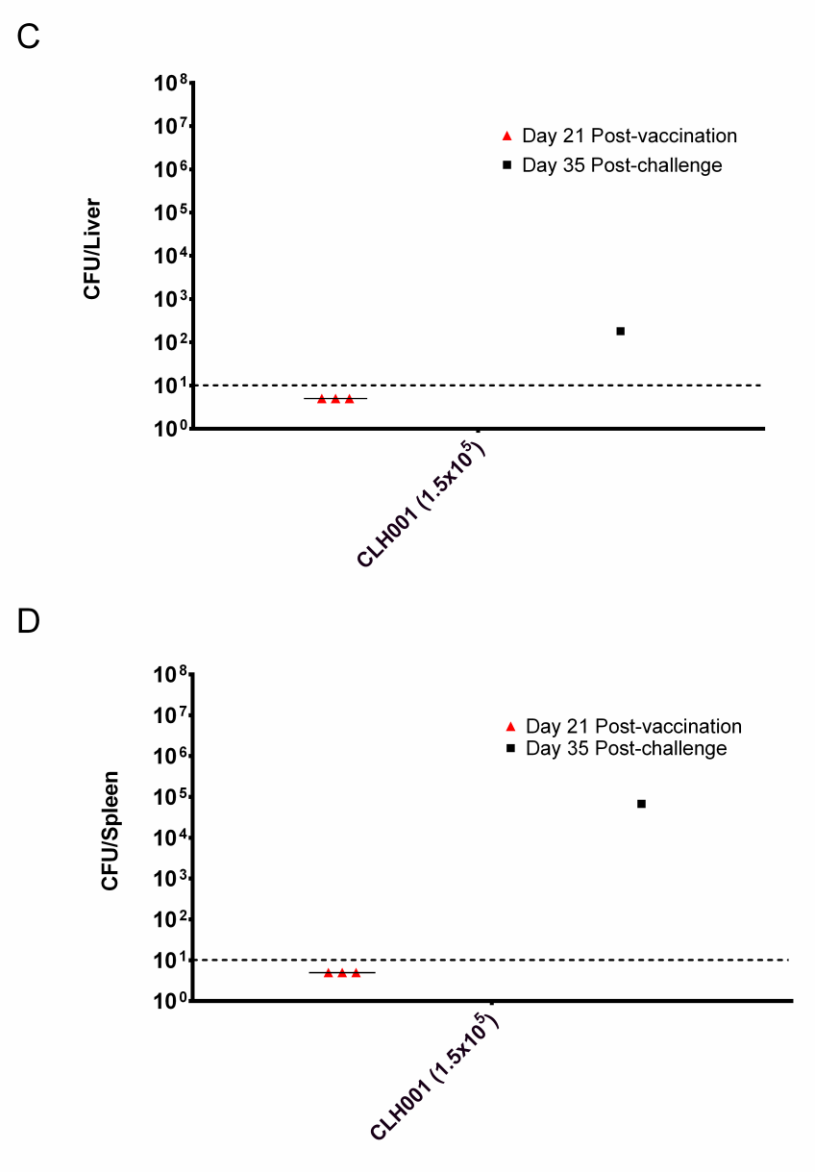


Figure: 20 Vaccination with CLH001 ($1.5x10^5$ CFU) provides partial protection against *B. pseudomallei* K96234 lethal challenge. (A) Mice were i.n. immunized with a prime and two boosts regimen of PBS (n = 11, ●), or $1.5x10^5$ CFU of CLH001 (n = 11, ▲). Three weeks after their last boost, mice were i.n. challenged with $9.36x10^2$ CFU of *B. pseudomallei* K96234. The statistical significance of differences in survival times was determined by plotting Kaplan-Meier curves, followed by a log-rank (Mantel-Cox) test. ***, P < 0.001. Colonization of mouse lungs (B), livers (C) and spleens (D) of CLH001-vaccinated mice at day 21 post-vaccination (n = 3, ▲) and day 35 post-challenge (n = 1, ■). The limit of detection was 10 CFU/organ (horizontal dotted line).

DISCUSSION

This study represents the evaluation of a *B. mallei* double deletion mutant as a live-attenuated vaccine candidate. Overall, our data indicates that the addition of the Δ*hcp1* deletion in the TMM001 strain is successful in addressing the persistence issue associated with the TMM001 backbone strain (Mott *et al*, 2015). In all survival and vaccination studies performed (including the NSG mouse study), the vaccine strain was cleared from all target organs by 21 days post-administration. Additionally, histopathology analysis of target organs from animals receiving this vaccine strain showed unremarkable tissue sections. In this study, we have demonstrated that CLH001 is attenuated *in vivo*. The additional gene deletion in the CLH001 strain provides increased safety and added protection against wild-type reversion, and as a result, CLH001 has become the first *B. mallei* strain to be excluded from the US Federal Select Agent Program (Appendix A). This exclusion provides an obvious advantage by allowing further vaccine characterization and optimization work to be performed more cost effectively and expeditiously in biosafety level 2 laboratories.

Another advantage of this double mutant is that, unlike the TMM001 backbone strain, its attenuation is not solely dependent on the organism's inability to uptake bound iron sources. Virulence of the TMM001 has been shown to be partially restored when free iron is supplied (Mott *et al*, 2015). Approximately 1% of the Caucasian population suffers from hemochromatosis, an inherited genetic defect resulting in excess free iron (Bacon *et al*, 2011). Administration of an iron-deficient strain like TMM001 to this population could potentially result in adverse effects. In fact, this risk was highlighted in another Gram-negative pathogen, *Yersinia pestis* UC91309, in which a live attenuated strain deficient in

host iron scavenging was responsible for a fatal case of laboratory acquired septicemic plague in a researcher with hemochromatosis (Frank *et al*, 2011). However, the additional gene deletion of CLH001 eliminates this safety concern. Although not tested for protection against *B. mallei* challenge, mice given $1.5x10^4$ CFU of CLH002 strain (Δ*hcp1* mutant) showed 100% survival, complete clearance of the lungs and liver, minimal splenic colonization, and minimal liver and spleen histopathology. Taken together, these results indicate that CLH001 may be sufficiently attenuated to be tolerated if inadvertently administered to a person with hemochromatosis.

The inability of a single dose vaccination of CLH001 to protect against CSM001 challenge was likely due to the fact that the bacteria is cleared too quickly to generate an adequate immune response. This assertion is based on survival study data showing that CLH001 exhibits decreased dissemination compared to CSM001 and TMM001 strains and is not able to be detected in the spleen (Figure 6D). Additionally, in support of this conclusion, CLH001 was completely cleared from target organs by day 21 in all studies, including the NSG survival study.

A number of vaccine studies have examined the correlation between a vaccine's ability to generate high *B. mallei*-specific IgG titers and a Th1-driven immune response (IgG2a:IgG1 ratio ≥1) with its ability to provide protection against *B. mallei* infection (Amemiya *et al*, 2002; Ulrich *et al*, 2005; Amemiya *et al*, 2006; Bandara *et al*, 2008; Nieves *et al*, 2011). This study supports this assertion, with the greatest protection observed in vaccinated mice ($1.5x10^5$ CFU CLH001) with the highest IgG total titers and IgG2a: IgG1 ratios. This correlation, along with the fact that CLH001-vaccinated serum was able to reduce viable bacterial counts when co-cultured with *B. mallei*, provides evidence that this

live attenuated vaccine stimulates a strong humoral response that is at least partially responsible for protection. The results of our passive vaccination study seem to go against our assertion regarding the protective role of humoral immunity in CLH001, as we found that i.p. immunization with serum from CLH001-prime and 2 boost vaccinated animals was not able to protect against lethal respiratory challenge with CSM001. However, a review of passive transfer studies in *Burkholderia* showed that there was a great deal of variation in methodologies with regard to the amount of serum used to vaccinate (50 to 400 µl), time between vaccination and challenge (1-24 h) and degree of protection they provided (Amemiya *et al*, 2006; Fernandes *et al*, 2007; Nieves *et al*, 2011; Silva *et al*, 2013). As such, it is quite possible that the failure of CLH001- prime and two boosts vaccinated serum to protect against challenge was due to the fact that our methodology requires further optimization.

Numerous studies have demonstrated that production of IFNγ early in infection is essential to the control of *B. mallei* infection. Evidence supporting this claim include the fact that IFNγ knockout mice are completely susceptible to lethal and sublethal infection (Rowland *et al*, 2010), as well as BALB/c mice in which IFNγ has been antibody neutralized (Whitlock *et al*, 2008). Additionally, it has been shown that treatment of mouse models, which are deficient in IFNγ production (including MYD88 -/-, MCP-1 -/-, and CCR2 -/-) with recombinant IFNγ, provided increased survival and reduced organ colonization following lethal challenge with *B. mallei* (Goodyear *et al*, 2010; Goodyear *et al*, 2012). Although not significant, at 2 days post-infection, mice prime and boosted with CLH001 showed elevated levels of circulating and localized IFNγ production in the sera and lung lysate samples, respectively. IFNγ is known to be involved in the classical

activation of macrophages. Because alveolar macrophages are the first cells encountered in a respiratory infection with *B. mallei*, this increased IFNγ production may have played a role in the protection garnered by CLH001 vaccination.

CLH001 represents the first *Burkholderia* vaccine that approaches sterile immunity against high doses of the *B. mallei* CSM001 strain. As such, its inability to provide complete protection and prevent colonization with *B. mallei* ATCC 23344 was unexpected. It is likely that the high challenge dose was able to overwhelm the immune response generated by CLH001 using our current vaccination regimen. The challenge dose given in this experiment represents a twenty-fold increase over CSM001 bacteria used to challenge in the initial experiment. Although it is evident that further vaccine optimization is required, CLH001 exhibits superior safety and protection as compared to other previously tested vaccines (Sarkar-Tyson & Titball, 2010; Mott *et al*, 2011; Choh *et al*, 2013; Silva & Dow, 2013; Mott *et al*, 2015). Future optimization will focus on reducing the number of boosts and determining the ideal vaccine dose that will protect against higher dosages of *B. mallei* 23344 and other *B. mallei* strains. The high antibody titers and significant protection achieved in this study provides rationale for vaccine optimization, including increasing the CLH001 vaccine dosage, testing alternative vaccination routes, and/or adding an adjuvant to maximize immune responses. Silva *et al*., demonstrated that in the closely related organism *B. pseudomallei*, administration of a live attenuated vaccine via a subcutaneous route resulted in vigorous recruitment of professional antigen presenting cells (APCs) and stimulated a robust humoral response capable of providing partial protection against lethal i.n. dose challenge with *B. pseudomallei* (Silva *et al*, 2013). Although not clear whether superior to i.n. vaccination in terms of protection, this route of

vaccination represents a more conventional and palatable vaccination method that warrants exploration with CLH001. Adjuvants are commonly incorporated into vaccine formulations to increase and/or tailor innate, adaptive and humoral responses. Although our vaccine was not fully protective in the second trial, it is possible that the cellular response elicited by CLH001 was insufficient. Inclusion of the appropriate adjuvant in our vaccination formulation may increase the magnitude of the cellular response generated by CLH001 vaccination alone. One such adjuvant that has shown promise in *B. mallei* and *B. pseudomallei* vaccine formulations and prophylactic therapy is CpG oligodeoxynucleotide (CpG ODN) (Whitlock *et al*, 2010; Judy *et al*, 2012). The CpG ODN is a Toll-like receptor 9 (TLR9) agonist that has been shown to activate B and NK cells, stimulate the antibody production, and drive Th1 cell development (Vollmer *et al*, 2004). Incorporating an adjuvant like CpG into our vaccine formulation has the potential to increase protection and reduce the number of required vaccine dosages by stimulating a more robust Th1 biased humoral and cellular response.

Another possible method of increasing our vaccine's protective capacity would be the addition of recombinant Hcp1 or other protective antigen to the vaccine formulation. Hcp1 has been shown to be an important antigen, as Hcp1 antibodies have been detected in sera from multiple glanders infected mammals, including horses, humans and mice (Srinivasan *et al*, 2001; Scholz *et al*, 2006; Schell *et al*, 2007). Additionally, Hcp1 vaccination has been shown to be partially protective against lethal challenge with *B. mallei* (Whitlock *et al*, 2010). Therefore, it is plausible to propose that supplementation of the CLH001 live attenuated vaccine with recombinant Hcp1 could increase the antibody response garnered by CLH001. Exploring ways to increase antibody or cellular responses

may provide increased protection and reduce the number of dosages necessary to induce a protective response. We are confident that continuing the optimization of this CLH001 strain will result in a live attenuated strain that can be advanced into pre-clinical studies.

Given the weak *B. pseudomallei*-specific IgG response to CLH001-vaccination, it is not surprising that it was only partially protective against a lethal dose of *B. pseudomallei* K96243. It is likely that this weak antibody response is due to the fact that CLH001 is missing two strongly immunogenic antigens, Hcp1 and FliC. The loss of Hcp1 is the direct result of mutagenesis, whereas FliC is missing because, unlike *B. pseudomallei*, *B. mallei* does not produce flagella. Although CLH001 doesn't seem to be able to provide cross protection, we are interested to see if this *tonB hcp1* double mutation can be adapted to other Burkhholderia species. As such, we are currently in the process of constructing a *tonB hcp1* mutant in *B. pseudomallei* K96243 that can be evaluated for protection against respiratory melioidosis.

Although this current study has only explored the role of humoral immunity in response to CLH001-vaccination, it is widely accepted that the generation of a robust, albeit appropriate, cellular response is also important for protection (Bondi & Goldberg, 2008; Mott *et al*, 2011; Choh *et al*, 2013; Silva & Dow, 2013). As such, we have begun a series of experiments to characterize the cellular responses to the vaccine, including adoptive transfer, T cell recall, and T cell proliferation studies.

Conclusions

The findings from this study demonstrate that *B. mallei* CLH001 exhibits combined attenuation properties of each of the single gene deletions, which contribute to its *in vitro* and *in vivo* attenuation. Like the *B. mallei tonB* mutant strain (TMM001), CLH001 exhibits decreased growth kinetics when free iron is in limited concentrations. Additionally, like the *B. mallei hcp1* mutant (CLH002) it is deficient in Hcp1 secretion and unable to cause MNGC formation in HeLa cells or RAW 264.7 macrophages. It is likely that the additive effects of the *tonB* and *hcp1* gene deletions contained in CLH001 are responsible for its decreased intracellular replication and eventual clearance from macrophages as well as its lack of virulence in BALB/c and NSG mice when administered intranasally at bacterial CFU concentrations equivalent to 22 LD_{50} of wt *B. mallei* CSM001.

CLH001's clearance from organs of both BALB/c and NSG mice by 21 days post-administration suggest that this strain addresses the *in vivo* persistence that was reported in the TMM001. Further, the complete survival and minimal organ histopathology observed in NSG mice attests to the safety of this strain. The additional protection from wild-type reversion afforded by the double gene deletion along with its demonstrated *in vivo* attenuation allowed CLH001 to be the first *B. mallei* strain to obtain Select Agent exclusion status (Appendix A).

Although a single intranasal vaccination with CLH001 ($1.5x10^4$ CFU) was not protective against lethal challenge, a prime and two boost i.n. vaccination regimen with $1.5 x10^5$ CFU was fully protective against i.n. challenge with $1.5x10^4$ CFU (22 LD_{50}) of CSM001. More importantly, the fact that no vaccine or challenge strain bacteria were

able to be recovered from the lungs, livers, or spleens of surviving mice and that their organs were free of pathology make CLH001 the first vaccine candidate that provide protection which appears to have achieved sterilizing immunity.

When the same vaccination strategy was applied against a higher dose of *B. mallei* 23344 ($3.5x10^5$ CFU), the results were not quite as impressive (87.5% survival). The incomplete protection and organ colonization seen in response to higher challenge doses suggest that further optimization to include increasing vaccine dosage, changing route of administration, adjuvantation, or supplementation with recombinant protective antigens may be required to increase the protection afforded by CLH001. Providing these optimization strategies can increase the protection garnered by CLH001, this vaccine candidate shows great promise for advancement into preclinical testing.

CLH001 vaccination in mice generates a vigorous Th1 biased humoral response as evidenced by *B. mallei*-specific IgG, IgG1 and IgG2a serum titers. This along with the results of the serum bactericidal assay suggest that humoral immunity plays a role in the protection provided by CLH001. However, further testing is required to gain a complete understanding of the immune response elicited by CLH001 vaccination, including the contributions of innate and cell mediated immunity.

Appendix A Exclusion of a Select Agent Notification

AUG. 24. 2015 11:31AM NO. 6824 P. 1

DEPARTMENT OF HEALTH AND HUMAN SERVICES — Public Health Service, Centers for Disease Control and Prevention (CDC)

August 20, 2015

Michael Shriner, Responsible Official
University of Texas Medical Branch
301 University Blvd. Admin Bldg.
Galveston, TX 77555
Fax: (409) 772-8138

SUBJECT: Exclusion of a Select Agent, 42 CFR part 73.3 and 9 CFR part 121.3

Dear Mr. Shriner:

This letter is in response to your request to exclude an attenuated mutant strain of *Burkholderia mallei* strain CHL001 *(ΔtonB Δhcp1)*, submitted by Principal Investigator Alfredo Torres. The request has been reviewed by the Federal Select Agent Program and the following decision has been determined:

- *Burkholderia mallei* strain CHL001 *(ΔtonB Δhcp1)* was determined to be attenuated compared to wild-type *B. mallei*, such that it does not pose a severe threat to public and animal health and safety. Therefore, *B. mallei* strain CHL001 *(ΔtonB Δhcp1)* is **excluded** from the requirements of the select agent regulations effective August 20, 2015.

Please note that if the attenuated strain is subjected to any manipulation that restores or enhances its virulence, the resulting select agent will be subject to the requirements of the select agent regulations [42 CFR part 73.3 (e) (2) and 9 CFR. part 121.3 (e) (2)]. The exclusion will be listed on the select agent website [42 CFR part 73.3 (e) (1) and 9 CFR part 121.3 (e) (1)].

The determination provided in this letter applies only to issues regarding select agent regulations (42 CFR part 73 and 9 CFR part 121). We also note that the NIH Guidelines remain applicable to this and other research involving recombinant DNA, including review and approval by an Institutional Biosafety Committee. The NIH Guidelines can be accessed at the following web address: http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm.

Please direct any questions or comments regarding this letter to Dr. Denise Gangadharan. She can be reached by telephone at 404-718-2018 or via email at DGangadharan@cdc.gov.

for Robbin S. Weyant, PhD, RBP (ABSA)
Captain, USPHS (Ret.)
Director, Division of Select Agents and Toxins
Department of Health and Human Services
Centers for Disease Control and Prevention

Freeda E. Isaac, DVM
Director, Agriculture Select Agent Program
United States Department of Agriculture
Animal and Plant Health Inspection Service

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