THESIS

CHARACTERIZING THE ROLE OF THE HSPX PROTEIN FROM MYCOBACTERIUM

TUBERCULOSIS AS A SUBUNIT VACCINE CANDIDATE IN THE SMALL ANIMAL MODEL OF

TUBERCULOSIS

Submitted by

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ABSTRACT

CHARACTERIZING THE ROLE OF THE SMALL HEAT SHOCK-PROTEIN AND

MOLECULAR CHAPERONE, HSPX, FROM *MYCOBACTERIUM TUBERCULOSIS* AS A

POSSIBLE VACCINE CANDIDATE

Tuberculosis (TB) is a bacterial disease of the lung, caused by *Mycobacterium tuberculosis*, and currently remains an important human pathogen. The only vaccine against tuberculosis licensed for human use is a live, attenuated strain of the closely related *Mycobacterium bovis* Bacille-Calmette Guerin (BCG), which offers little protection against pulmonary disease in adults, particularly against latent infection. Current vaccine strategies against TB include the development of subunit vaccines, which contain one to a few antigens. Subunit vaccines are delivered with an adjuvant formulation to mount an appropriate T cell response against the pathogen. One such antigen for vaccine design is the 16kDa small heat shock protein and molecular chaperone from *Mtb*, HspX. This protein has been implicated as a latency-associated antigen due its late expression and ability to re-stimulate T cells from latently infected patients. A previous study in our laboratory revealed that native HspX purified from *Mtb* was protective in mice against pulmonary TB when given as a vaccine. HspX expressed and purified from *E coli* in its recombinant form was not able to protect. We hypothesize that because HspX functions as a molecular chaperone, it requires its binding partners to remain biologically active as a vaccine. In this study, we test this hypothesis and we also tested the capacity of native HspX to protect guinea pigs experimentally infected with TB. Our results illustrate that, while native HspX protects in the mouse model, it does not confer protection in guinea pigs, suggesting differences in the establishment of *Mtb* physiologically in the lungs.

ACKNOWLEDGMENTS

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1 Introduction; Literature review

1.1 Microbiology of Tuberculosis

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), a bacterial disease of the lungs; it is a gram variable, acid-fast bacilli with a thick cell wall structure that includes unique mycolic acids and lipids that give it its characteristic waxy structure [1]. *Mtb* is a slow growing organism [1], making it challenging to culture, with some species of mycobacteria that cannot be cultured outside the host. The waxy cell wall provides the organism with protection; it can live in harsh and stressful environments and evade immune killing for extended periods of time by remaining in a state of dormancy by forming granulomas in the lung. It is an intracellular pathogen that preferentially resides inside alveolar macrophages of the lung. There are historical documents of TB as a disease dating as far back as ancient Greece and Rome, and there is also evidence of granulomas found in the lungs of ancient Egyptian mummies [1]; yet in our modern era we are still struggling to gain a complete understanding of the organism and thus how we can subsequently control it.

1.1.1 Epidemiology

Tuberculosis is responsible for approximately 3 million deaths every year, making it one of the deadliest pathogens in the world [2]. Tuberculosis is a worldwide problem but is endemic primarily in Africa and Asia where inappropriate surveillance and lack of access to affordable treatment greatly hinder communities where HIV is also a significant burden, Figure 1-1.

Estimated TB Incidence Rate, 2006
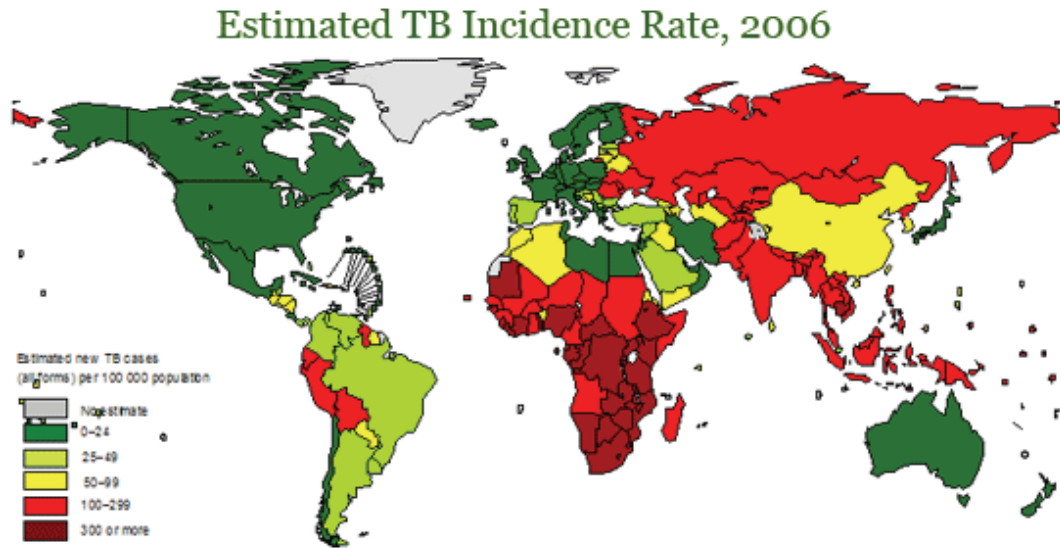
Figure 1-1

The ability of the bacillus to live intracellularly within macrophages allows for protection from the host's immune system, and also makes treatment difficult once infection has been established.

1.2   Disease course of *Mtb*: active and latent infection

1.2.1   Active infection:

Upon exposure to, and subsequent infection with *Mtb*, a small percentage (~10%) of people will develop active disease presenting with physical symptoms and will be contagious through active coughing via aerosol droplets [3]. Most patients (~90%) however, are able to contain the infection in a latent state (they are not presenting with physical symptoms and are not

contagious) that can be characterized by the formation of granulomatous legions in the lung [4], with ~10% chance of reversion within their lifetimes, while HIV co-infected patients have a 10% chance per year of developing active tuberculosis [3].

1.2.2   *Latent TB infection:*

Latent TB infection (LTBI) can be characterized by the formation of many granulomas dispersed in the lung, while remaining asymptomatic and not contagious [5]. The legions segregate infected macrophages thus walling off the bacilli from outer influences within the host. Infected macrophages are contained within a structure of lymphocytes (both CD4+ and CD8+ T cells), foamy macrophages, other mononuclear phagocytes, with a fibrous collagen cuff forming around the entire structure with blood vessels eventually making their way into the legion [6]. During late, chronic infection a central core of necrosis forms in the granuloma leading to cavitary TB that can be visualized in chest X-rays. It is thought that both the bacilli and host contribute to the formation of the granuloma and if this homeostasis within the microenvironment is broken, the bacilli are released from the granuloma and disseminate to active disease [7].

The microenvironment in the granuloma is very harsh with reactive nitrogen intermediates (RNIs), nitric oxide (NO), nutrient starvation and hypoxia, to which the bacteria must adapt [8]. Mycobacteria present within the granulomas are thought to be in a metabolically latent state since there is no active replication, but viable organisms have been isolated [4]. In humans, the granuloma takes a few weeks to develop which is well into the adaptive immune response. Over time, the granuloma will lose its structural integrity and the ability to contain the bacilli, and they will quickly disseminate leading to active TB in the patient, allowing again for transmission.

1.3   Prevention, Diagnoses, and treatments

1.3.1   Prevention

The only vaccine against TB licensed for use in humans is an attenuated strain of the closely related *Mycobacterium bovis,* Bacille Calmette-Guérin (BCG) and its efficacy is questionable [9]. However, it is currently the only available vaccine for the prevention of tuberculosis, and although it does offer protection in children from severe childhood forms of TB, it offers very little protection from pulmonary tuberculosis in adults [9].

BCG is the most widely given vaccine at birth, with almost 3 billion doses given since its introduction in the 1920s [10], yet many still get infected and many die as a result. Because of the failure of BCG to establish long-term protection in the host [11], there has been a strong emphasis on the development of second-generation vaccines that are either a subunit (containing only an antigen delivered with an adjuvant) or a recombinant BCG strain over-expressing known immunogenic antigens [3, 12]. A full review of tuberculosis vaccine strategies will be discussed in section 2.6.

1.3.2   Diagnosis

The only quick diagnostic test available for tuberculosis is by measuring a delayed-type hypersensitivity (DTH) reaction by injecting purified protein derivative (PPD), a sterile extract of *Mtb* proteins, directly under the skin [9], and if a person has been exposed to tuberculosis a DTH reaction will occur leading to a measurable induration in the skin [7]. A DTH response is characterized as a localized site of inflammation due to the recruitment of cytokine secreting T cells to the site of injury; a granuloma in the lung due to TB infection can be classified as a localized DTH response by the immune system [7].

Because PPD is a relatively crude extract, the response is not specific to TB, and patients who have been previously exposed to other species of mycobacteria in the environment, or

previously vaccinated with BCG will give a positive response [9]. The only way to differentiate a true infection with *M tuberculosis* from other mycobacteria and/or BCG vaccination is through a sputum smear or a chest X-ray, which in most cases is not a feasible option.

1.3.3   Treatment

Treatment options for tuberculosis are limited to a few number of antibiotics and require a long treatment course, for about 6-9 months most commonly with isonaizid (INH) and rifampin (rif) [13]. Due to the long treatment time required, compliance and costs are major issues. Therefore proper prevention and diagnosis are required for TB control, especially for latently infected persons. Since so many that are infected with TB are in a latent, asymptomatic state, a vaccine is a more realistic answer both in terms of delivery and cost to administer once established.

1.4   Immunity to tuberculosis

1.4.1   Immune response to TB

It has been widely accepted that control of tuberculosis in the host is dependent on a T-helper type 1 (Th1) response, requiring CD4+ T cells and is largely pro-inflammatory [14-16]. Upon activation from infected macrophages, CD4+ T cells secrete the Th1 cytokine gamma-interferon (IFN-γ), which is a down-regulator of the Th2 response; therefore driving the polarity to a Th1-type response when the cytokine is present (Figure 1-2). Cytokines interleukin-4 and -10 (IL-4 and IL-10, respectively) are Th2 cytokines that act to inhibit cytokine secretion from Th1 cells. Th2 responses are largely anti-inflammatory and are primarily activated during infection with helminthes and allergies, and are not believed to play a major role in TB immunity [14];

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although they have been implicated in tissue destruction late in infection in the lung with the presence of Th2 type cytokines and T regulatory cells in chronic legions [17]. T regulatory cells are believed to down-regulate the Th1 response, perhaps as a control mechanism; the precise role of these cells and Th2 cytokines in TB disease has yet to be determined and for the purposes of this review, will only focus on the Th1 response.

Figure 1-2
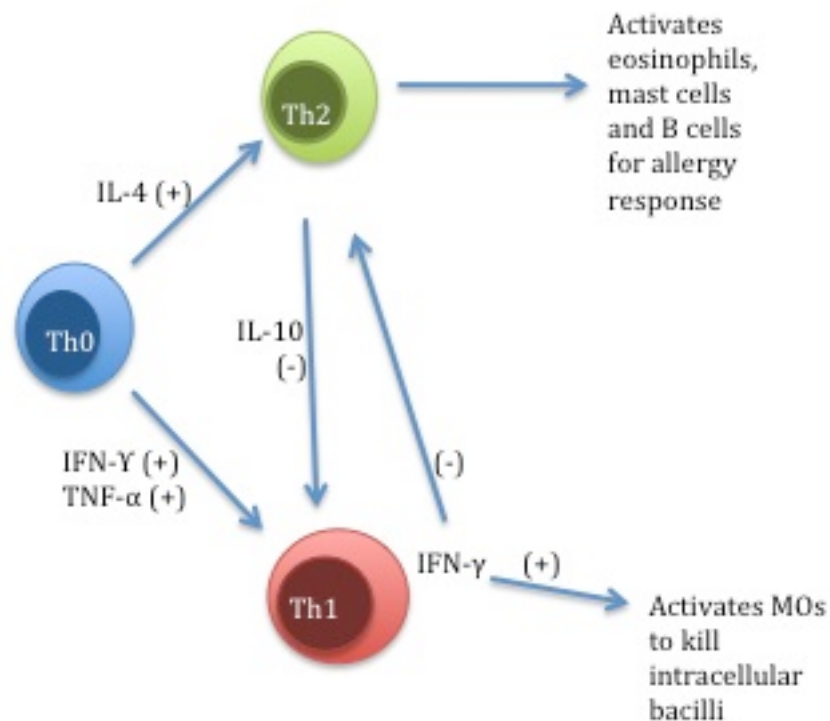
Simplified representative of Th cell differentiation. Naïve CD4+ Th cells (Th0) will differentiate into Th1 type cells or Th2 type cells depending on cytokine stimulation. Cytokines IFN-Y and TNF-α will give rise to a Th1 type response. Cytokines IL-10 and IL-4 will give rise to a Th2 type response.

Mycobacterium tuberculosis uptake into the macrophage is mediated through the binding of several receptors such as the complement receptor, mannose receptors and toll-like receptors

(TLRs) [18, 19]. Toll-like receptors are membrane spanning receptors, termed pathogen recognition receptors (PRRs), on the cell surface of various immune cells that recognize and bind ligands on pathogens, thus leading to cell activation via transcription factors to drive cytokine secretion [19]. TLRs 2, 4 and 9 have been implicated in tuberculosis infection and mice that harbor mutations in these genes are unable to control bacterial growth [20]. Prolonged TLR-2 engagement, however, can lead to decreased MHC-II peptide loading and presentation, thereby down-regulating the immune response and avoiding immune activation [19].

Upon phagocytosis, *Mtb* activated macrophages secrete IL-12, which in turn activates IFN-γ producing T cells [16]. IFN-γ then acts on macrophages as a positive response regulator to induce intracellular killing through the activation of phagolysosome fusion and production of nitric oxide and reactive nitrogen intermediates [16]. Mycobacteria however, have developed ways around these attempts from the host by inhibiting phagolysosome fusion, producing nitrate reductases to deactivate RNIs, and production of ammonia to neutralize the acidic phagosome [6].

It is believed that IL-12 secreted from macrophages is required for T cell activation; it is responsible for the activation and expansion of IFN-γ secreting Th1-type cells and has also been implicated in formation and maintenance of the granuloma [21]. Humans and mice with mutations that lead to insufficient IFN-γ or IL-12 production develop much more serious disease to TB and are unable to form proper granulomas [14]. IFN-γ is arguably one of the most important cytokines produced during tuberculosis infection, as mice and humans with mutations in this gene are very susceptible to tuberculosis, and it can always be detected early in the sera infected individuals [14]. IFN-γ is commonly used as a marker for tuberculosis infection but as we uncover more about how the host responds to infection, IFN-γ should not be the sole marker used because it is a general pro-inflammatory cytokine and does not necessarily correspond directly to TB infection, per se [14].

Tumor necrosis factor alpha (TNF-α) is secreted by activated macrophages, dendritic cells and T cells, and together with IFN-γ synergize to induce intracellular killing of bacilli [14]. This

cytokine, however, has a history with TB because it can be damaging to the host, and it is believed that both the host and the bacilli play active roles in tissue necrosis and damage during infection with the thought that both timing of TNF-α secretion and bacterial control can play a role in the damaging effects seen from TNF-α [20].

A cell mediated Th1-type response is critical in controlling TB infection; therefore new generation TB vaccines are focusing on activating this arm of the immune system [22]. Tuberculosis infected macrophages present antigen that has been processed in the phagosome in the context of MHC-II via the endocytic pathway [23] making the CD4+ T cell the primary cell for TB immunity. The primary function of these cells is believed to be for the secretion of IFN-γ, which as mentioned previously, induces intracellular killing of the pathogen and are believed to be essential in granuloma formation and this becomes readily apparent in animals that are lacking CD4+ T cells since they exhibit more severe forms of disease [24].

There is not as much evidence regarding the involvement of CD8+ T cells, although it has been suggested that these cells are recruited later in disease in the lung and therefore may be important in latent immunity to TB [15, 25]. They are also more abundant during early infection with a high dose of bacteria [25]. Current vaccine strategies are focusing on generating CD4+ T cell immunity [9] as it has been shown that protection cannot happen without these cells. Further investigations regarding the involvement of CD8+ T cells will be required for a complete understanding of these cells function.

Because of the complexity of the disease course and the immune response, designing a vaccine against TB is a daunting task for many reasons. *Mtb* does not have one virulence factor such as a protein or toxin that can be neutralized or targeted; *Mtb*'s virulence determinants seem to be its ability to survive within macrophages under harsh conditions for a long period of time [1]. A majority of exposed people are able to control the infection with the innate immune responses and either eliminate the pathogen or enter latency [26]. There is a 5-7 day delay for the cellular response to appear, thus making a "window" between innate and adaptive immunity

where infection could be established in host's that are unable to control the infection via the innate response [26]. Thus the goal of a new TB vaccine is to induce this immunity to be long-term and more protective against infection.

1.5 Current vaccine strategies and vaccine development with TB immunity in mind

1.5.1 History of tuberculosis vaccination

The first successful vaccine, developed by Edward Jenner and Louis Pasteur, was the *vaccinia* (cowpox) vaccine and was given for the prevention of small pox and thus the name "vaccine" was coined [27]. At the time, vaccine development was based largely on trial and error, with tragic side effects often leading to death. The first vaccines generally contained a complex mixture of secreted filtrates that was injected directly into the patient [27]. The approach for vaccine design in general has shifted over time to gain a more complete understanding of the host's immune response to the disease so it can be properly manipulated to offer long-term protection [27]. Logically, the simpler the vaccine construct (such as a subunit vaccine), the more specific and controllable the immune response will be.

BCG was developed in the early 1900's by serial passage of *M bovis* on potato agar to make the less virulent vaccine strain [22]. It has since been used as a TB vaccine worldwide, with nearly 3 billion doses given [22]. A majority of the Western world does not vaccinate against TB, due to a potential false-positive PPD reading in BCG vaccinated individuals making proper diagnostics challenging, and infection rates are low in this part of the world [10].

1.5.2 Current TB vaccine strategies

It has been well established that BCG does not offer enough protection to achieve sterile immunity in the adult population, with efficacy rates from 0-80%, depending on the study [28].

The precise mechanisms as to why BCG fails are still unknown, but it is known that *M. bovis*, along with non-pathogenic environmental mycobacteria (EM) share many antigens that can cross react with *Mtb* thereby limiting the protection of BCG vaccination against tuberculosis [9, 22, 29]. This pre-sensitization; whether it'd be due to *Mtb* or EM exposure, or previous vaccination with BCG, means that it is unable to act as an adequate booster vaccine; and because BCG is live, it requires replication in the host [10]. Therefore, EM are seen as a "vaccine" that sensitizes the host, and BCG is then seen as the "infection" in the host [9].

Although some consider BCG ineffective, completely eliminating it as a vaccine could be seen as unethical and impractical since it does confer protection in infants and children up until about the age of puberty, and it is already given to millions each year [10]. Thus new vaccine development for TB can be divided into two major categories: development of a recombinant BCG (rBCG) strain to over-express known immunogenic antigens that are found in *Mtb*; or the development of subunit vaccines which contain only an antigen based on recombinant DNA and protein technologies, and is delivered with an adjuvant formulation and given as a booster to BCG later in life [9]. BCG has an amazing track record for safety in infants and the immunocompromised, thus rBCG strains may prove to be successful during safety evaluations. The option of boosting with multiple doses of BCG has proven to be detrimental in the guinea pig model so this idea has not been further evaluated [30].

Subunit vaccines contain a limited number of antigens, which are usually proteins or polyproteins that are known to induce an immune response in humans against TB. Particular focus has been on the early secreted antigen ESAT-6, secreted proteins Ag85A and B, which are fibronectin binding proteins, and TB10.4, among others [31, 32]. (A more comprehensive review about each subunit vaccine in clinical trials is given below in section 2.7). ESAT-6 is an early-secreted protein that is unique to TB (it is not expressed in BCG), is a known virulence factor and is recognized early in infection by most TB patients [33].

Antigenic proteins are carefully being chosen based on previous experiments that have

demonstrated the ability of the antigen's to induce an immune response in the host; protection is then studied later. It has been demonstrated through various studies that *Mtb* can express different proteins throughout the course of infection [34] and that these antigens can be stage specific [33]; in other words, patients with active TB can recognize and respond to different antigens than patients who are latently infected [35]. ESAT-6 and CFP-10, for example, are strongly recognized by active TB patients and not as much in latently infected patients when comparing against a later secreted antigen [33].

Targeting latent vs. acute antigens for TB vaccine design is therefore a rational approach for vaccine design. Given that the majority of people who are infected with TB are latently infected, it is logical to consider latency-associated antigens. Latency antigens can serve two purposes for vaccines: either given prophylactically as normal, or given as a post exposure vaccine to prevent reactivation [17]. Andersen and colleagues have developed a "multi-stage" subunit vaccine that contains both latent and early antigens and was able to protect before and after exposure in the small animal model [36, 37]. However, developing post exposure vaccines has proven to be difficult for many reasons mainly regarding safety [38]. Post-exposure vaccines would essentially be boosting the immune response to someone who has already been exposed and therefore already has a strong established T cell response, thus the post-exposure vaccine would need to further amplify this response [17]. So far there has not been much success with post-exposure vaccines and their use is still controversial [38].

The best model of studying latent TB is in the Cornell mouse model that involves infecting animals then treating them with anti-mycobacterial drugs to reduce the bacterial burden to level where the mice enter latency [39]. The animal either naturally succumbs to re-activation, or it is induced with steroids. This model however, has its limitations and latent TB disease still needs to be better studied to properly make a vaccine that targets this stage of disease [39]. At this time, we are probably closer to implementing a multi-stage TB vaccine rather than a post-exposure vaccine due to the difficulties faced with post-exposure vaccine testing, particularly in clinical

trials involving humans, since latently infected people will be required in large enough numbers.

1.5.3   *Developing a successful vaccine: animal models and testing*

For a vaccine to be considered "successful" in regards to pre-clinical trials, the following traits are tested in animals [40]: 1) The ability of the vaccine to induce an antigen specific cellular response. Re-stimulating PBMCs and/or splenocytes ex vivo with the specific antigen after vaccination will give a specific cytokine response that can be measured. 2) The vaccine must be able to induce and enhance proliferation of antigen specific T -cells and mount a CD4+ T cell mediated response. This can be verified via flow cytometry by gating of cells via surface markers to distinguish cell types such as memory cells, regulatory cells, etc to verify that the immune response is the correct type. 3) The ability of the vaccine to protect in the small animal model from disease when challenged, which is tested by enumerating CFU from infected tissue homogenates and performing histopathology by a certified veterinarian. CFU counts in the lung reveal the levels of circulating T cells that are able to focus at the site of inflammation, and spleen data reveal the levels of sensitized T cells that have entered circulation via the thoracic duct from draining lymph nodes [40]. Generally, a 1-log reduction in bacterial numbers compared to BCG is considered an initial successful vaccine.

Once testing for these traits in animals is complete, testing in humans begins on a small group of TB negative adults, and the same immunogenicity that was seen in animal models must be mirrored in humans [41]. Most importantly, the vaccine needs to be safe and a vaccine generally goes through multiple animal models before moving into humans.

1.5.3.1   *The mouse model*

The most common model for TB is the mouse due to associated costs, availability of immunological reagents, and a full annotation of the mouse genome, making it a great model to

study the immune response to TB infection and/or vaccination [42]. It was in fact established in the mouse through adoptive transfer that CD4+ T cells are required for protection against TB infection [43]. The T cell response in mice is very similar to humans in that infected macrophages are activated in similar ways to humans, and the T cell response is similar [44]. Much like humans, the adaptive immune response takes a finite amount of time to appear, which is probably due to the time it takes for the bacillus to sensitize T cells and for the cells to arrive at the site of infection [44].

In the field, mice are usually vaccinated and challenged about a month later, when immunity is peaking, thus mice model effector immunity and not memory [40]. Therefore vaccine testing in mice measures an early protective response and is generally not carried out to survival. The limitation of this model is that the pathology exhibited in the lung tissue is not as damaging as is seen in humans and the guinea pig, and do not give DTH responses thus limiting protection data to earlier stages of infection [45].

1.5.3.2   The guinea pig model
Whereas the mouse models the immune response in humans more accurately, guinea pigs model disease pathology more accurately in term of granuloma formation and tissue necrosis in the lung [46]. Mice are unable to form the classical necrotic center that can be seen in humans and guinea pigs granulomas, however guinea pigs do not form cavitary TB, instead the central core begins to mineralize [44]. Upon challenge, there is rapid bacterial growth and bacterial dissemination for about 2 weeks before a stationary growth phase is reached and granulomas form and eventually mineralize. Guinea pigs that are not BCG vaccinated will succumb to disease, with a classical granuloma forming by 30 days post infection and by 100 days post infection, the mineralized, heavily infiltrated granuloma is apparent [47]. BCG protects these animals for up to 70 weeks, thus they can model long-term protection beyond 30 days with

survival studies, however in most studies guinea pigs are still challenged 30 days after the final vaccination [11]. Guinea pigs are more susceptible to tuberculosis than mice so they have somewhat become the gold standard for TB vaccine testing and any vaccines screened in the guinea pig must perform at least as well as BCG [40]

1.5.3.3 *Beyond the small animal model*

Other animals are used for TB vaccine testing, but much more rarely usually due to costs. Other rodents used have been the rabbit and rat, but to a much lesser degree. Rabbits are good animals models for toxicology [48] and mimics disease pathology more closely, however they are susceptible to *M bovis* (and not *Mtb*) and will eventually die from disease, but are usually too expensive [44]. As a final animal model for vaccine testing, the non-human primates model disease very well and respond to BCG vaccination, although recently it was found that some species may not respond the same to BCG as others [44]. Due to expense, these animals are usually the last to be tested before human trials begin.

There is not just one animal that should be relied upon for TB vaccine testing. Whereas the mouse will provide a wealth of immunological data, the guinea pig will provide good survival and protection data. If vaccines are able to succeed in these animals, then the more closely related primates are used. Various animal models will be required for any vaccine to make it to clinical trials.

1.6   TB vaccines in or on their way to clinical trials

1.6.1   *Recombinant BCG and live/modified Mycobacteria*

Despite the lack of efficacy of BCG, it has proven itself to be very safe, which is a big hurdle that new vaccines need to clear. Any new tuberculosis vaccines need to be extremely safe

in children and the immunocompromised, as there is a high population of HIV/TB co-infected individuals in the TB endemic areas. Phase I and phase II clinical trials in humans are solely safety and immunogenicity studies to test the vaccine's tolerability in humans, and trials start in healthy, TB negative adults. Live, attenuated vaccines do not require the use of an adjuvant since they are self-adjuvanting and can stimulate strong enough T cell responses on their own.

M bovis is part of the *Mtb* complex and therefore shares many of the immunogenic antigens as TB, thus acts as a good Th1 immune stimulator for TB [11]. The goal with BCG vaccination is to induce a strong cell mediated response without an adjuvant, and then trigger specific immunity against antigens. Generally, intracellular organisms such as viruses and certain bacteria are presented via MHC class I because their antigens are "leaked" into the cytosol from the phagosome as a result of fusion with the lysosome [27]. *Mtb,* although an intracellular organism, is primarily presented via MHC class II through the endogenous pathway, meaning that the bacilli remain in the phagosome and thus require presentation via MHC class II to CD4+ restricted T cells [19].

The fear with live-modified vaccines is that they may be able to cause disease in the immunocompromised since they are not able to destroy the organism in the vaccines and the organism takes advantage of the weekend immune system to establish an infection. "Koch" reactions are also a concern, whereby disease occurs after vaccination in healthy adults due to an "over" response of the immune system [27]. Although there have been very few reported cases of disseminated *M bovis* infection after vaccination [49], it still remains as one of the safest vaccines today since it is given to a large population of HIV co-infected people and neonates and any new live vaccines will have to meet this standard of safety.

1.6.1.1   *Recombinant BCG: rBCG30*

This vaccine is a recombinant BCG vaccine that expresses the 30kDa major secretory

protein Ag85B on the pMTB30 plasmid. Hoft, Horwitz, et al have shown that rBCG30 was protective in guinea pigs and reduced the bacterial burden by at least 0.5 logs in their lungs [50]. This group tested several BCG vaccines and found, on average, a statistically significant difference in the survival of the animals. When assayed for self-transmissibility, it was found the plasmid was not able to be transferred to other bacteria, thus the vaccine was deemed safe in this regard [32].

Upon entering human safety trials, rBCG30 was well tolerated in the human patients and revealed specific Ag85B responses in CD4+ and CD8+ T cells and these differences were significant when compared with the current BCG vaccine [51]. This study also showed evidence of a memory response by measuring IFN-γ secretion from *ex vivo* PBMCs from vaccinated individuals after antigenic stimulation. These results demonstrated the ability of rBCG30 to be safe and properly immunogenic in humans.

1.6.1.2   *Recombinant BCG with endosome escape: AERAS 422, AERAS-403*

These rBCG vaccines were produced to over-express the cytolysin gene *Lysteriolysin-O* from *Listeria monocytogenes* (rBCGΔUre:CHly) [52] or perfringolysin-O (PfoA) from *Clostridium perfringens* [53], along with a deletion of a urease gene to allow the bacteria and antigens to escape the endosome in a pH independent fashion. Endosome escape allows these *Mtb* antigens to be presented to CD8+ T cells in the context of MHC class I (as opposed to CD4+ T cells) since they are able to escape into the cytosol of APCs [22]. AERAS 422 over-expresses Ag85A, Ag85B and TB10.4, which were all incorporated into an expression plasmid. The final protect, BCG expressing *PfoA* and over-expressing Ag85A, Ag85B and TB10.4, enhanced survival and protection in the mouse and guinea pig [53]. Specific activity was measured and each individual antigen contained in the vaccine strain gave a significantly better response when compared to the standard BCG vaccinated animals and controls. It was also found to be safe and

well tolerated in SCID mice (mice that are devoid of T cells and B cells). Currently ongoing phase I clinical trials in healthy adults in the USA [12].

1.6.1.3   *Inactivated Mycobacterium tuberculosis*

Since BCG has proven to be safe, there has also been some research into a live, mutated *M. tuberculosis* or other non-virulent strains of mycobacteria such as *M microti* or *M vaccae*; in which these strains would have known immunogenic gene from *Mtb* knocked-in to the genome [40]. The RD1 region is a region of genes that is found in *Mtb* but not present in BCG and other mycobacteria, and contain 9 open reading frames for known immunogens such as ESAT-6 and CFP-10, therefore this region is of interest in development of live vaccines [54]. Initial studies in human T-cells lines, human blood monocytes and murine infection models indicated that H37Rv:ΔRD1 was less virulent than H37Rv and was similar in pathogenesis to BCG in infected tissue [54]. However, when this study was carried out to long-term murine infection, it was found that the RD1 knock out strain was significantly more virulent than BCG by evaluating histopathology of the lungs and CFU enumeration of infected tissues [55]. Thus the development of these vaccines has been slower than others (see below).

1.6.1.4   *Heat inactivated Mycobacterium vaccae*

The protection efficacies of whole, heat killed *M vaccae* was tested in 3 different trials in Africa in BCG vaccinated, HIV+ and HIV- infected adults. A decreased treatment time for TB and sputum conversion from positive to negative was observed, and it was reported to decrease TB cases overall [3]. However, it was found when injected, the vaccine left permanent, visible and sometimes painful scars, thus it was evaluated in an oral formulation in a phase I setting with similar results as the injectable vaccine, but sensitized T cells were unable to produce TNF-α [56]. Between these results and the lack of public results clearly indicating significant protection

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and the fear of Koch reactions, focus is not on live vaccines that are not rBCG formulations and until more data appears, further clinical studies will be recovered.

1.6.1.5   RUTI

RUTI is an experimental therapeutic vaccine of fragmented *M tuberculosis* delivered in liposomes to target treatment of latently infected persons. LTBI takes 6-9 months treat with conventional antibiotics, yet the highest bactericidal activity of isoniazid (INH) occurs within two days of treatment [57]. This makes sense when we think about latent TB infection: most of the bacteria are not actively replicating and therefore the drugs do not work against latent or dormant bacilli. RUTI will be given alongside chemotherapy to reduce the treatment time by triggering an immune response against latent antigens to target dormant cells for killing. RUTI is made from fragmented *Mtb* that was grown under the stressful conditions of hypoxia and acidic pH to enhance expression of latency antigens, then was fragmented and delivered in liposomes. A problem with chemotherapy is that it is not always 100% effective, and bacteria can still remain because they are hidden away while in latency. The goal of RUTI is to bridge this gap; in conjunction with chemotherapy killing actively growing bacilli and curing active disease, administration of RUTI soon after chemotherapy has began will boost the immune response that is already there to latent antigens [57]. This, in theory would cover the whole spectrum of bacilli that are present during infection.

The exact mechanisms of how RUTI provides long term protection and treatment is still somewhat unclear, but it was able to increase strong Th1 response to 13 known TB antigens and expanded a large population of IFN-γ secreting CD8+ T cells in the lungs [57], which is consistent with latent infection responses [25]. The current hypothesis is that RUTI will not be a replacement for chemotherapy since it targets latent antigens, which are presumably not as abundant during chronic infection. Shortly after chemotherapy begins, there is an increase in Th1

effector cells in peripheral blood within 4 weeks [57], and RUTI tags along by taking advantage this immune response and at the same time, is presenting latent antigens to these effector cells to more efficiently kill the bacilli.

1.6.2   *Protein, polyprotein and subunit vaccines*

The basis for these vaccines is the use of recombinant gene and protein technology to make constructs of antigens delivered with adjuvants, as opposed to whole organisms. The goal of these vaccines is to induce an antigen specific T cell response to TB that can offer long-term protection. The approach with administering subunit vaccines is that they will be given as boosters to the already administered BCG; thus this is how they are being tested in animals. Since these constructs are quite simple, they require delivery with an adjuvant to launch a strong Th1 response. Adjuvants are formulated to bind to cell surface receptors that kick-start immunity, such as liposomes, lipid A derived adjuvants and cationic compounds [40]. The responses they elicit through their binding are non-specific and are designed to help the vaccine establish Th1 immunity.

Subunit vaccines are composed of immunogenic antigens from tuberculosis including Ag85, ESAT-6, TB10.4 and others. These antigens were previously shown in animal models to be immunogenic and protective. As I will discuss later in the section, all of the subunit vaccines in clinical trials contain antigens that are secreted early in infection and not necessarily able to mount enough of a response against latency antigens. As mentioned previously, Andersen and colleagues have developed a multi-stage subunit containing early and late antigens with initial promising results [37], but none are in clinical trials in humans. One such latency antigen is HspX, which is highly expressed during latency and during stationary growth [58] and latently infected patients preferentially recognize this antigen over non-infected or active TB patients [59]. HspX therefore serves as a potential vaccine target and will be discussed in more detail

further in this section.

1.6.2.1 Mtb72F/AS02A

Currently in phase II clinical trials [12], this vaccine performed well in four animal models and was well tolerated in initial human trials. It contains two antigens Mtb32 plus Mtb39 arranged in the linear order of Mtb32C-Mtb39-Mtb32N formulated in GlaxoSmithKline (GSK) AS02 adjuvant. In monkeys, it was found to protect better than BCG alone when looking disease pathology and survival and there was almost total clearance of the bacilli from the Mtb72F vaccinated monkeys [48]. In humans, it was shown that upon *in vitro* re-stimulation with PPD or Mtb72F the classic Th1 associated cytokines IFN-γ, TNF-α and IL-2 were produced [60]. IL-2 is known to enhance proliferation of antigen specific cell and is an indicator of memory T cells [48].

A previous study with Mtb72F demonstrated that when given to guinea pigs, it increased survival and decreased disease burden, however in the mouse it did not increase survival, but it did significantly induce a Th1 response [61]. Moreover the same study revealed that when Mtb72f was given as a DNA vaccine instead of a subunit design to guinea pigs, it was found to not significantly increase survival when compared to BCG alone, but there was evidence of tissue healing and clearance in the pathology of the lung. Nevertheless, these results are promising based on the differences in pathological evidence between controls and vaccinated animals; and the fact that it succeeded as a subunit vaccine in the more rigorous guinea pig model of TB and has gone through three animals models (mouse, guinea pig and non-human primate) all demonstrating immunogenicity and protection [48]. Phase II clinical trials in adolescents with previous BCG vaccination has been completed and another phase II trial is ongoing in infants in the Gambia [12].

1.6.2.2   SSI H4:IC31 (AERAS 404)

Developed at the Statens Serum Institute this construct contains the recombinant proteins Ag85B-TB10.4 as a fusion delivered in the IC31 adjuvant from Intercell [62]. Initial safety and protection studies in animal models revealed that this vaccine protected better than BCG alone; it was only protective with the two proteins fused together, rather than each one alone, and induced specific TB10.4 CD4+ T cell responses [62, 63]. Originally developed with Ag85B and ESAT-6 (H1), but ESAT-6 was later replaced with TB10.4 (H4) to avoid discrepancies with diagnostic tests. Completed three phase I trials in Europe and South Africa in BCG vaccinated adults, and ongoing phase I trial in Europe in BCG vaccinated adults [12].

1.6.2.3   SSI H56:IC31

Another vaccine under development lead by Andersen and colleagues at the Statens Serum Institute is a formulation of the H1 vaccine (Ag85B, ESAT-6) with the addition Rv2660 (a latency associated antigen) delivered with the IC31 adjuvant [37]. Rv2660 and Rv2559 were previously shown to be recognized by PBMCs of latently infected patients, as opposed to early antigens ESAT-6 and CFP-10 and thus were incorporated into the H1 vaccine [36]. Unlike the rest of the subunit vaccines in clinical trials, this is the first to contain both early and late antigens. Indeed, when given to mice, H1 was able to protect but H56 (with the additional latency antigen) was able to protect mice for much longer (up to 25 weeks), suggesting Rv2660 is responsible for the long-term protection [37]. Interestingly, Rv2660 was unable to protect against disease alone, suggesting that T cell responses to this antigen are low, but when complemented with early antigens, T cell responses are higher to the antigen. When evaluated as a post-exposure vaccine using a modified Cornell model in the mouse, almost all the T cell responses were poly-functional expressing IFN-γ, TNF-α and IL-2; this experiment was repeated four different times in different laboratories with similar results. Clinical studies have been initiated.

1.6.3   DNA and Live-vector delivered vaccines.

1.6.3.1   1.7.3.1 MVA85A

A replication deficient Vaccinia virus that over-expresses Ag85A is the furthest advanced vaccine in clinical trials, with phase IIb still on-going and phase III beginning in Africa [12]. Clinical trials conducted in South Africa revealed the vaccine was deemed safe in adolescents and children and induced multiple CD4+ T cell subsets that were specific to Ag85A, along with polyfunctional T cells [64]. There was also evidence of a memory response based on the presence of IL-2. However it may be worth noting that different clinical isolates of *Mtb* express different proteins and antigens that can vary from each other which could contribute to their characteristic virulence and how they would respond to a vaccine; and it was shown that the more virulent Beijing strain of *Mtb* expresses significantly less Ag85A [65]. These antigenic differences should be considered during vaccine design and using multiple antigens in vaccine constructs could potentially avoid this problem.

1.6.3.2   AERAS-402 (Ad35)

Currently Ad35 is the only TB vaccine that is utilizing a live, replication deficient Adenovirus. The serotype 35 expressing TB antigens Ag85A, Ag85B and TB10.4 are contained as a single fusion peptide. Replication deficient adenoviruses serve as good vectors and are strong inducers of CD8+ T cell immunity and are therefore being considered for vaccines for intracellular pathogens [3]. However there is a high prevalence of individuals that have neutralizing antibody to wild-type adenoviruses, primarily Ad5; thus serotype Ad35 was chosen, because there is a low prevalence of anti-Ad35 in the general population in African communities [66].

In mice, the vaccine was found to induce antigen specific CD4+ and CD8+ T cells, and there was evidence of disease clearance in the lungs of mice vaccinated with Ad35 when

compared to BCG and controls [66]. Is worth noting that two different mice strains were used for this study, and they gave slightly different cellular responses to vaccination. BALB/c mice had a predominant CD8+ T cells response; whereas C57B/6 mice had a more balanced CD4+/CD8+ response, with a more pronounced CD4+ T cell response in the lungs. These data should be considered when studying T cell responses to TB, and recently it has been suggested that CD8+ T cells may play more a protective role than originally thought in TB protection, particularly later in infection [25]. Also, the strain of mouse should be considered, as it evident by the various results this group saw.

Magalhaes, et al have shown promising results in non-human primates when using Ad35 as a booster to BCG, although their sample size was not large enough for statistical significance [67]. IFN-γ responses to Ag85A and Ag85B were detected one week after vaccination and were stronger when compared to BCG only vaccinated animals and also induced poly-functional T cells co-expressing IFN-γ, TNF-α and IL-2.

Currently in phase IIb clinical trials throughout Africa, phase I was successfully completed in adults with or withput previous BCG vaccination with no adverse effects or safety issues and a full immunological response was achieved [68]. The underlying issue is the prevalence of Ad5 serotype antibodies, it is believed that in sub-Saharan Africa, >5% of people carry the neutralizing antibodies to Ad35 so there should in theory be enough herd immunity achieved to protect a majority of the population being vaccinated [68].

1.6.4   DNA vaccines

To date, there have not been any DNA vaccines against tuberculosis in humans to reach clinical trials, but they are still being looked at as boosters to BCG vaccination. DNA vaccines are self-adjuvanting by inducing strong cellular responses and can stimulate both CD4+ and CD8+ T cell responses [69]. DNA vaccines are constructed in a plasmid with an antigen(s) from

tuberculosis that is then expressed inside the host. Several studies with DNA vaccines have been done in animal models with variable results [11, 70]. Derrick, et al show evidence of protection in the mouse when given a DNA plasmid containing an ESAT-6/Ag85B fusion [69]; however, when mice were vaccinated with hsp65 DNA post challenge, there was severe pulmonary destruction reminiscent of a Koch reaction and no protection was observed [68, 70]. Even with variable results, it is very early in the pipeline for tuberculosis DNA vaccines and still deserves further investigations.

1.6.5   *Vaccinating with HspX: Hypothesis*

Several groups have utilized the HspX antigen from tuberculosis as a possible vaccine candidate [71-75]. The 16kDa heat shock protein from *Mtb* is considered a latent antigen since its expression is up-regulated during hypoxia and is required for growth inside macrophages *in vitro* [58]. It has been established that during the growth course of *Mtb,* it expresses certain antigens that can be attributed to a specific growth phase. For example, there are antigens unique to early, log-phase growth (such as ESAT-6 and CFP-10) and antigens unique to stationary, or slowed growth, such as HspX [76]. It is also presumed that growth of latent bacilli within the granuloma of infected lungs are in a state of metabolic latency and are in a harsh microenvironment that contains low pH, hypoxia and free radicals, among others. Thus, slowed, stationary growth in culture is similar to what the bacilli are experiencing in the granuloma.

HspX is in the family of small heat-shock proteins with an α-crystallin (acr) domain, and functions as a molecular chaperone [77]. The chaperoning activity is conserved among all α-crystallins, and mutations on the N-terminal side of these proteins ablates chaperoning activity [78]. Mammals have an acr homologue which is found in the eye lens to maintain transparency by prevented aggregates of other proteins [77]. In *Mtb*, HspX along with about 50 other genes, are under transcriptional control of the two-component response regulon, DosR, which is

activated during stress and hypoxia [79]. DosR binds to a DNA motif just upstream of these latency associated genes, and disruptions in the motif sequence leads to much lower binding of DosR, and thus less transcription of these genes [80].

HspX therefore serves as a good vaccine target for latency and to prevent re-activation of TB. The protein is also recognized by the sera of latently infected individuals as opposed to actively infected patients, and does not seem to interfere with PPD testing of TB [59]. A previous experiment of ours showed that the native HspX purified from *Mtb* was able to protect mice from pulmonary TB, but the recombinant form of the protein expressed and purified from *E coli,* was not protective (unpublished data).

The results of initial study suggested that there are differences between the native and recombinant purified protein. With the knowledge that HspX functions as a molecular chaperone, we hypothesize that when HspX is being purified in its native form, it is being co-purified with any binding partners that it would be chaperoning. When the protein is expressed in and purified from *E coli* in its recombinant form, it no longer contains these chaperoned molecules, because we believe they are mycobacteria specific (figure 1-3). We thus hypothesize that HspX requires its binding partners in order to remain biologically active *in vivo* as a vaccine.
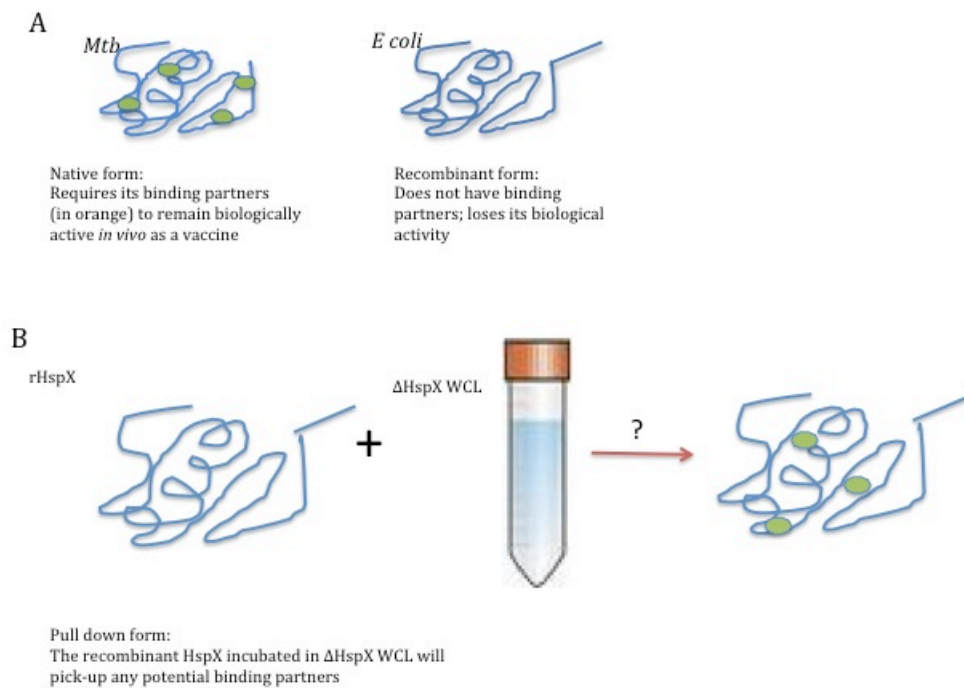
Figure 1-3

To test our hypotheses that HspX requires its bindings partners to remain biologically active *in vivo*, we have formulated three different purification schemes for HspX and we will test all of these constructs in the mouse and guinea pig model of TB. We will then vaccinate animals with native HspX expressed and purified from *Mtb* (nHspX), recombinant HspX expressed and purified from *E coli* (recHspX), and recHspX that was allowed to allowed to incubate in the whole cell lysate (WCL) of an HspX knock-out strain (ΔHspX), termed recHspX-PD, for "recombinant HspX pull-down". The intention of this experiment was to allow recHspX to "pick-up" and subsequently bind any molecules that it could be chaperoning that are present in the lysate. An HspX knock-out lysate was used to avoid the native HspX from binding to itself, thus if any protection is seen, it will not be do to the native HspX.

We aim to test these three HspX vaccine constructs in the mouse and guinea pig model of TB, and monitor for survival. The following three chapters detail experiments in the mouse and guinea pig model of tuberculosis in which we tested HspX as a vaccine and its ability to induce a proper immune response and protect against challenge with tuberculosis. Here we show our data supports the hypothesis that HspX functions as a molecular chaperone and, additionally, that its binding partners are required for HspX to function when given as a vaccine in the small animal model of tuberculosis.

2 HspX in the mouse model of tuberculosis

(Adapted from Taylor and Wieczorek, et. al. (2011) Nature Cell Biology and Immunology, in revisions)

2.1 Introduction

The only vaccine against TB licensed for human use is Bacille Calmette-Guerin (BCG), which is a live, attenuated strain of the closely related *Mycobacterium bovis*. It is the most widely given vaccine at birth in the world [3] yet is clearly unable to prevent tuberculosis. A majority of people that are infected with TB are latently infected; they are in an asymptomatic state and are unable to spread the disease to others, but are carriers nevertheless. Indeed, most people are able to fight off infection with tuberculosis via the innate immune response, but some are not able to and progress to either active disease or latent disease [5]. Thus to design an effective vaccine against tuberculosis, we must have an understanding of the immune response and how the bacilli respond to this response, particularly during latency.

Latent infection can be characterized by the formation of the hallmark granuloma in the lung where the infected macrophages are walled off in a legion surrounded by foamy macrophages, mononuclear phagocytes, and CD4+ and CD8+ lymphocytes, thereby allowing for immune evasion with a physical barrier [9]. The microenvironment inside the granuloma is harsh with the presence of reactive nitrogen intermediates (RNIs), free radicals, nutrient starvation and acidic pH to which the bacilli must adapt [9]. The *DosR* (Rv3133c/Rv3132c) two-component response regulon present in *Mtb* controls upwards of 50 genes that have been implicated to be activated during hypoxic growth or stressful conditions, thus leading to the expression of so-called latency-associated genes [34, 80]. One such gene under control of the *DosR* regulon is the small 16 kiloDalton (16kDa) heat shock protein HspX (α-crystallin, acr, Rv2031c) and is believed to function as a molecular chaperone [81]. It is up-regulated during hypoxia [82], is required for growth within macrophages *in vitro* [58]*, and is preferentially recognized by latently infected TB

patients [59]. There is a clear metabolic down-shift that occurs in the bacilli during nutrient and oxygen starvation and it is clear that BCG is unable protect from latency given the high number of people still infected, yet have been vaccinated.

HspX is preferentially recognized by peripheral blood mononuclear cells (PBMCs) recovered from patients who are in latency, suggesting that it is able to stimulate T cells [59]. In that same study, PBMCs from TB negative, BCG vaccinated patients were unable to mount a response to HspX when re-stimulated with the antigen *in vitro*, suggesting that BCG is unable recognize and mount a response against this latency antigen [28]. Several studies utilizing a recombinant BCG (rBCG) over-expressing HspX [83], and a DNA vaccine comprising of a plasmid with the fusion of Ag85B-ESAT6-HspX [74] were shown to be at least as immunogenic and as protective as BCG in mice in terms of T cell responses, pathology and CFU recovery from infected tissues.

HspX was purified in three different forms and these three constructs were used to vaccinate animals prior to a low-dose aerosol challenge. HspX was purified in its native form from *Mycobacterium tuberculosis,* and two forms of the recombinant HspX were expressed and purified from *E coli.* Upon purification of the recombinant protein, we incubated the protein with whole cell lysate (WCL) from an HspX knock-out strain. The purpose of this assay was to allow the recombinant HspX to bind to any chaperoned partners that could be present in the lysate, thus upon re-purification, the recombinant HspX has pulled-down any binding partners. If this pull-down construct is able to protect animals against pulmonary tuberculosis, then this is evidence that HspX requires its binding partners to protect.

The general consensus is that immunity against tuberculosis requires a strong cell-mediated response for control [14, 15, 84] and a complete understanding of this response is required for a successful vaccine. Upon phagocytosis, the macrophage is activated to secrete interleukin -12 (IL-12) which in turn activates and drives T cells into a Th1 type response secreting IFN-γ [14]. IL-12 is a crucial cytokine in tuberculosis immunity as it is required for T cell activation; mice

and humans with mutations in the IL-12 receptor show increased susceptibility to disease [14]. Thus the response mounted against tuberculosis is strongly pro-inflammatory and is dependent upon IFN-γ secreting CD4+ T cells [85]. Tumor necrosis factor (TNF-α) is another cytokine implicated during infection with TB and is secreted from activated phagocytes and together with IFN-γ, synergize to induce production of RNIs and NO within macrophages, both of which have been well documented as a killing mechanisms against tuberculosis [24]. IFN-γ, TNF-α, and IL-12 are key cytokines in TB immunity and multi-functional T cells co-expressing these cytokines, along with IL-2 which is a cytokine associated with memory T cells [24], are believed to be an indicator of vaccine protection and can discriminate between active and latently infected persons [86].

Therefore new vaccine strategies against TB are targeting activation of the cellular immune response with the use of TB specific antigens in subunit vaccines or recombinant BCG strains over-expressing these antigens [10]. Subunit vaccines currently being developed are generally focused on antigens secreted early during infection with tuberculosis [17, 53, 62, 66]. Due to the high number of latent TB carriers, it is important that this population be targeted for tuberculosis control, thus targeting latency-associated antigens should also be considered for vaccine candidates.

For this study, we have chosen to evaluate the latency-associated antigen HspX as a vaccine in the mouse model of tuberculosis. We have previously shown that when given as a vaccine in the mouse, the native form of the protein purified from *Mtb* was able to protect against TB, while the recombinant form of the protein purified from *E coli* was not able to (unpublished data). Given the knowledge that HspX can function as a molecular chaperone, we hypothesize that the native protein is able to confer protection either from its ability to chaperone another molecule found in *Mtb,* or there is another unique attribute to it that is absent in the recombinant form.

We demonstrate here that native and pull-down HspX are able to protect mice from

pulmonary TB, supporting our hypothesis that differences that are seen in protection with HspX

may be due to the presence, or absence of, any chaperoned molecules bound to HspX.

2.2   Materials and Methods

2.2.1   Generation of ΔHspX

Mycobacterium tuberculosis (Mtb) HspX knock-out (ΔHspX) was made by allelic

exchange using a modified protocol of Pelicic and colleagues [87]. Briefly, *hspX*-flanking

regions from 825 bp to 107 bp upstream of *hspX* and from the second last codon of *hspX* to 644

bp downstream were amplified from genomic DNA of *Mtb* strain H37Rv with primer pairs:

hspXupF1[*Spe*I]:GGACTAGTTCGGCATGATACGACGA   and

hspXupR1[*Pac*I]:ACCTTAATTAACCATTGACCCTGTTGTCTG;   hspXdownF1[*Pac*I]:

ACCTTAATTAACTGACCACTGGGTCCGT   and   hspXdownR1[*Bam*HI]:

CGGGATCCTCCTCGTCGGTGACCT, respectively, and inserted into *Spe*I/*Bam*HI-digested

plasmid pPR27 [87]. The cloned regions in the resulting plasmid (pPR27ΔhspX) were determined

to be free of mutations by DNA sequencing. Plasmid pPR27ΔhspX was digested with *Pac*I,

blunted, and ligated with a ~1300 bp blunted *Bsp*HI/*Sma*I hygromycin resistance gene from

plasmid p16R1 [88]. Resulting plasmid, pPR27ΔhspXhyg1, which contains the hygromycin

resistance gene in the same orientation as the original *hspX* gene, was electroporated into *Mtb*

strain H37Rv and the cells were plated on Middlebrook 7H11 agar supplemented with OADC,

0.05% Tween-80, and 50 μg/ml hygromycin (7H11OADCTH) and incubated at 30°C. Three

transformants were cultured in 5 ml Dubos broth supplemented with OADC, 0.05% Tween-80,

and 50 μg/ml hygromycin for 6 weeks at 32°C. To obtain candidates that integrated the plasmid

into the chromosome and lost the plasmid-encoded *sacB* gene, 100 μl of each culture was spread

onto 7H11OADCTH supplemented with 2% sucrose and the plates incubated at 32°C for 6

weeks. To confirm plasmid loss by screening of the plasmid-encoded gentamycin resistance gene,

twenty colonies from each plate were patched onto 7H11OADCTH+2% sucrose plates containing or lacking 5 µg/ml gentamycin and incubated for six weeks at 32°C. Two of the 60 candidates were sensitive to gentamycin. Genomic DNA from both candidates was purified and screened by PCR with primers hspXoutF1: GCACCCGATCCTTGTCGAG and hspXoutR1: CACGACGTCGTCATTGACC. Results demonstrated that only one of the strains contained a single band corresponding to replacement of *hspX* with the hygromycin resistance gene. This strain (X4-19) was renamed ΔHspX.

2.2.2   Growth of Organisms

2.2.2.1   Preparation of seed stocks

One milliliter each of WT and ΔHspX glycerol stocks was transferred into a glass tube containing 9ml of Proskauer-Beck (PB) medium [89] and static cultures incubated at 37°C for 3 weeks, or until pellicle formation was visible on top of the media. Pellicles were harvested and used for sequential inoculation into 25 ml and 100 ml of PB media and incubated as before. Pellicles of *Mtb* from the final passage were transferred to 20 ml of PB media containing 20% glycerol (v/v); cells were mixed by agitation, and suspensions bath sonicated at 4°C for 10 min. One mL seed stock vials were prepared and stored at -80°C.

2.2.2.2   Preparation of Infectivity (working) stocks

One vial of each strain of seed stock was added to 9 ml of 7H9+OADC medium containing 0.1% Tween-80 and incubated at 37°C with shaking for 14 days. Cultures were inoculated into 45ml of 7H9+OADC+0.1% Tween-80 and incubated at 37°C with shaking, harvested, and 1 mL working stock vials were prepared and stored at -80°C. Three vials of each strain were randomly recovered, thawed, serially diluted in 7H9+OADC medium and plated in triplicate for determination of colony forming units (CFU).

2.2.3   Protein purification.

For purification of native HspX (nHspX) protein, whole γ-irradiated *Mtb* cells were broken using a French Press as described previously [90] and the resultant whole cell lysate was centrifuged at 40,000 x g. The supernatant was discarded and the pellet was resuspended in PBS with 1% (v/v) n-Octyl-β-D-thioglucopyranoside, and centrifuged at 27,000 x g. The supernatant was collected, exchanged into 10 mM ammonium bicarbonate and lyophilized. The dried material was suspended in 7.25M urea, 0.4% pH 3-10 ampholytes, 1.6% pH 4-7 ampholytes, 1% n-Octyl-β-D-thioglucopyranoside and 2mM DTT. The protein concentration was determined by bicichoninic acid assay (BCA; Pierce Chemical Company, Rockford, IL). A sample of 100 mg of soluble protein was applied to a preparative Rotorfor apparatus (BioRad, Herecules, CA), and proteins separated per manufacturer's instructions. Individual fractions (representing a pH range of 3 to 10) were harvested and resolved by SDS-PAGE using 4-12% gradient gels and transferred to nitrocellulose for analysis by Western blot using CS-49 (anti-HspX antibody). Membranes were developed with 4-bromo-3-chloro-2-indoyl-1-phospate following incubation with secondary antibody. Fractions containing HspX were pooled, exchanged into 3M urea, 20mM tris-HCl, 0.15M NaCl, and 0.1% n-Octyl-β-D-thioglucopyranoside, and the protein was purified by size exclusion chromatography using Sephadex S-75 resin (GE Life Sciences; Piscataway, NJ) with isocratic elution. Fractions were resolved by SDS-PAGE and analyzed by Western blot as described above. Fractions containing purified HspX were pooled, exchanged into 10 mM ammonium bicarbonate, protein quantified by BCA assay, and the purity was confirmed by SDS-PAGE and staining with silver nitrate.

Recombinant HspX (rHspX) was expressed and purified from *E coli*. The gene fragment encoding HspX was PCR amplified with primers CATATGGCCACCACCCTTCC-[forward] and CTCGAGTCAGTTGGTGGACCGGATCT-[reverse]. The amplified gene product was ligated into the multiple cloning site for pET15B (EMD Biosciences; Gibbstown, NJ) following digestion with Nde1 and Xho1 and the recombinant plasmid was transformed into *E coli*

BL21(DE3)pLysS expression srain (Invitrogen; Carlsbad, CA). The transformed *E coli* was plated onto LB media containing ampicillin and chloramphenicol, followed by sequential inoculation into 30mL , then 2L of LB broth. Cultures were grown at 37°C with shaking to an OD600 of 1.0, and then induced with 0.5mM IPTG. Cultures were harvested after 16 hours of growth at 37°C with shaking and broken by probe sonication. The final lysate containing soluble rHspX was purified by our standard methods as described previously [91]. Endotoxin levels were assessed with a Limulus Amebocyte Lysate (LAL) assay as described previously [91] and samples were found to contain less than 10 ng EU/mg protein.

For purification of the recombinant HspX pull-down (rHspX-PD) protein, purified recombinant HspX was incubated with ΔHspX WCL (10:1; lysate:recHspX ratio) in 10mM ammonium bicarbonate at 4°C overnight with gentle agitation. The mixture was applied to Ni2+ resin and recHspX was purified with any binding partners as described above.

2.2.4   Mice

All mice were purchased from Charles River Laboratories (Wilmington, MA). For the vaccine challenge studies, specific pathogen free female, 6-8 week-old C57BL/6 were held bio-safety level 3 barrier conditions. For the immunological assays, B6.B10(-/-)TLR4/JthJ and B6.B10(-/-)TLR2/JthJ mice were purchased and held under pathogen-free barrier conditions. All animals had unlimited access to sterile mouse chow and water, and all experimental procedures were approved by the Colorado State University Animal Care and Use Committee.

2.2.5   Vaccination procedure.

Mice were vaccinated subcutaneously with the HspX protein preparations, three times at two-week intervals with each HspX protein at 10μg each. BCG Pasteur was given at the time of

the third and final vaccination at a dose of 1 x 10^5 CFU, as a positive control. Each HspX vaccine was emulsified in dioctadecylammonium bromide (DDA @ 250µg/dose) and monophosphoryl lipid A (MPL @ 25µg/dose) as previously described [92]. Negative controls included one group of just saline and another group with just the adjuvant formulation alone.

2.2.6 Aerosol infection

Thirty days following the final vaccination, mice were infected using procedures previously described [93]. Briefly, bacterial stocks were diluted in 5 mL of sterile water to 2 x 10^6 CFU/mL and placed in a nebulizer attached to an airborne infection system (Glass-Col; Terre Haute, IN) and were exposed to 40 minutes of aerosol with ~50-100 bacilli delivered to the lungs of each animal. Bacterial load was determined by plating whole-organ homogenates onto 7H11+OADC agar and CFU was enumerated following incubation for 21 days at 37°C.

2.2.7 TNF-α and IL-12 ELISA assays

Wild-type C57BL/6, (-/-)TLR2 and (-/-)TLR4 mice were euthanized by CO_2 asphyxiation and bone marrow derived macrophages (BMMO) were harvested from the femur and tibia bones aseptically. The cells were counted, plated at 10^5 cells/well and cultured in complete RPMI 1640 (10% fetal bovine serum, penicillin-streptomycin, and L-glutamine) in 96-well IP sterile plates (Millipore; Bedford, MA). Following incubation at 37°C, 5% CO_2 for 7 days, cells were stimulated with 2µg/well, 1µg/well, 0.5µg/well, 0.25µg/well and 0.125µg/well of HspX protein preparations (native, recombinant and pull-down) in triplicate. LPS was used a positive control in the same concentration and media blanks were used as a negative control. Following 24 hours incubation at 37°C, 5% CO_2, cell supernatants were assayed for tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12) p40 subunit cytokines by enzyme-linked-immunosorbent-assay (ELISA; eBiosciences, San Diego, CA) as per manufacturers instructions. Plates were

developed for HRP reactivity with the provided substrate (30% H_2O_2 and 3-amino-9-ethylcarbazole) and read at 450 nanometers with a Biorad (Hercules, CA) plate reader and analyzed using the accompanying software. Responses are given in pg/mL of secreted cytokine and were averaged for each antigen.

2.2.8   Statistical analysis

All statistical tests were performed using the student two-tailed *t*-test and a $p<0.05$ was considered significant.

2.3   Results

2.3.1   ΔHspX mutant conformation

The *hspX* knockout strain was verified by southern blot and confirmed that a hyg resistance cassette had replaced HspX (figure 2-1). Figure 2-1 A is a representative diagram of the local HspX gene region showing the hyg replacement cassette. Figure 2-1 B shows the southern blot results hybridized to a probe specific for the local HspX genetic region in figure 2-1 A; our mutant strain was verified to not contain Rv2031c (HspX), and was replaced by a hygromycin resistance cassette.
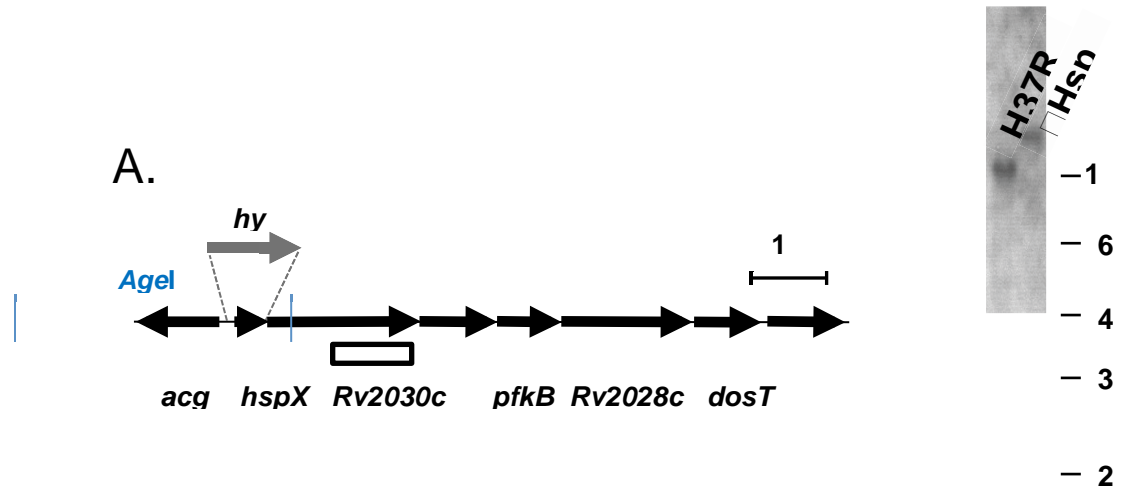
Drawing of the *M. tuberculosis hspX* genomic region and confirmation of the *hspX* mutant. A) Drawn are genes near or constituting the *hspX* operon and their relative orientations (arrows). The *hyg* region replaced with a hygromycin resistance gene (*hyg*) in the mutant is indicated (dashed lines). Also shown are the *Age*I sites nearest *hspX* and the region (open box) to which a Southern probe was generated. B) Southern blot result of *Age*I-digested DNA of *hspX* mutant (ΔHspX) and parent strain (H37Rv) hybridized with a probe to the region indicated in panel A. Locations of DNA molecular weight standards (in kb) are shown on the right side. As expected, digestion with *Age*I results in a 3670 bp *hspX* band in strain H37Rv. The corresponding band in ΔHspX is increased to 4244 bp due to replacement of *hspX* with *hyg*.

Figure 2-1

2.3.2   Conformation of protein purity

All HspX preparations were purified as described above. The native form of HspX was purified from *M tuberculosis.* The recombinant form of HspX was expressed and purified from an *E coli* expression strain (BL21 pLysS). The recombinant pull-down HspX was expressed and purified from the same *E coli* strain, and then the purified protein was incubated with whole cell lysate from our HspX mutant (ΔHspX).

Final purity was validated by performing SDS-PAGE and visualizing with silver nitrate and Western blots with anti-HspX and anti-penta-His antibody to verify the presence of HspX and the His tag on the recombinant forms. Recombinant HspX runs higher (~18kDa) on the gel than native HspX (~14kDa) due to the presence of this tag (figure 2-2). All proteins passed quality control based on purity from gels and western blots and all were assayed for endotoxin and were found to contain less than 10ng EU/mg. The appearance of dimers in the recombinant proteins is a common trait seen with TB proteins.

Figure 2-2
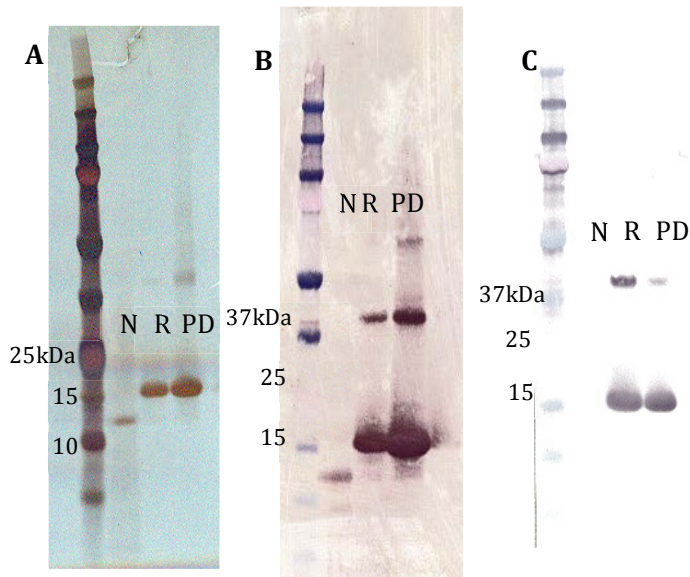
A) Silver stain of native, recombinant and pull-down HspX. The recombinant forms of the protein run higher on the gel due to the His-tag mass difference R and PD. B) Western blot with anti-HspX (CS-40) antibody for the three preparations. The recombinant forms of the protein appear to form a dimer (around 37kDa). C) Western blot with anti-His antibody. Notice the native HspX (N) did not react with the antibody, as expected. All proteins were at least 95% pure, free of endotoxin and passed quality control for vaccination.

2.3.3   HspX protection

HspX is an α-crystallin (acr) [94], functions a molecular chaperone [81], and is not believed to be post-translationally modified [95]. We therefore speculate that the differences seen in protection could be due to the fact that HspX is chaperoning something in *Mtb* that is absent in *E coli,* and during purification, rHspX may not contain any potential binding partners that are in *Mtb.* This binding partner may be required for HspX to function biologically *in vivo*, at least in terms of a vaccine.

We developed a recombinant HspX vaccine by performing a pull-down assay in which the already purified rHspX (from *E coli*) was incubated it with whole cell lysate from our ΔHspX strain with the intention that HspX would "pick-up" any binding partners in the lysate. The recombinant protein was then re-purified as described above with any potential binding partners.

In figure 2-3 we show, again, that the native HspX was able to significantly protect in terms of CFU counts when compared to BCG, and rHspX was not protective; however rHspX-PD was. Figure 2-4B illustrates that the PD vaccines was protective against ΔHspX challenge. These animals were not exposed to the HspX antigen and thus were not sensitized to it. This indicates that HspX alone may not be the immunogen and this is support of our hypothesis that HspX mediated protection is dependent on its ability to carry another molecule.

Figure 2-3
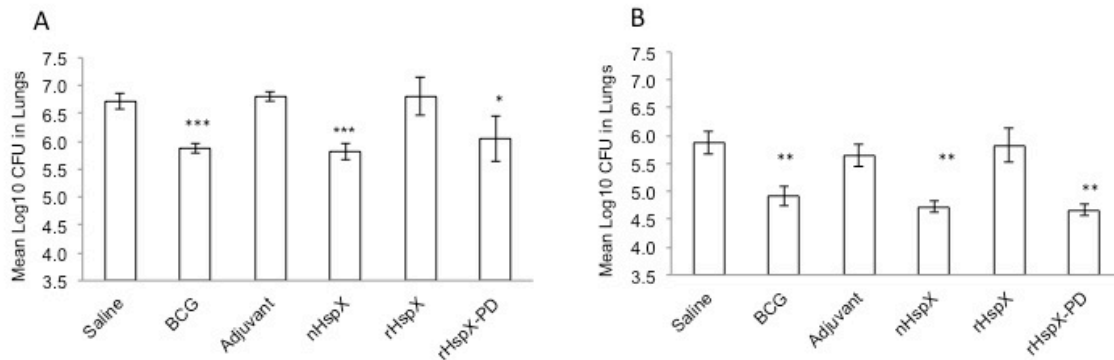
A) Mean log CFU in the lungs of wild type H37Rv infected mice. Native HspX protected as well as BCG (p<0.001) and HspX pull-down was also able to protect significantly (p<0.05). In terms of TB vaccine testing, a vaccine must protect as well as BCG to be considered successful. B) Mean long CFU in the lungs of ΔHspX infected mice. In this case, both native and pull-down were able to protect significantly, supporting our hypothesis. We have again verified that the recombinant form of HspX purified from *E coli* is not protective against pulmonary TB.

*** p<0.001, ** p<0.01, * p<0.05 and is significant when compared with saline injected animals.

2.3.4 Lung pathology of nHspX vaccinated mice

Mouse lungs were harvested 30 days post infection, fixed and processed for histopathology assessment as described by a Veterinary Pathologist blinded to the groups (HBO). Although nHspX was able to reduce CFU burden, the lung pathology from saline, adjuvant and nHspX vaccinated mice does not appear to significantly differ (Figure 2-4). The cellular infiltrate, however, was similar between BCG and nHspX-vaccinated animals suggesting that the cellular response is similar between the two groups. BCG vaccinated and nHspX vaccinated animals showed similar cellular infiltrate in that the legions contained lymphocytes and activated macrophages, suggesting that HspX was able to stimulate macrophages of the lung at the site of infection.

Figure 2-4
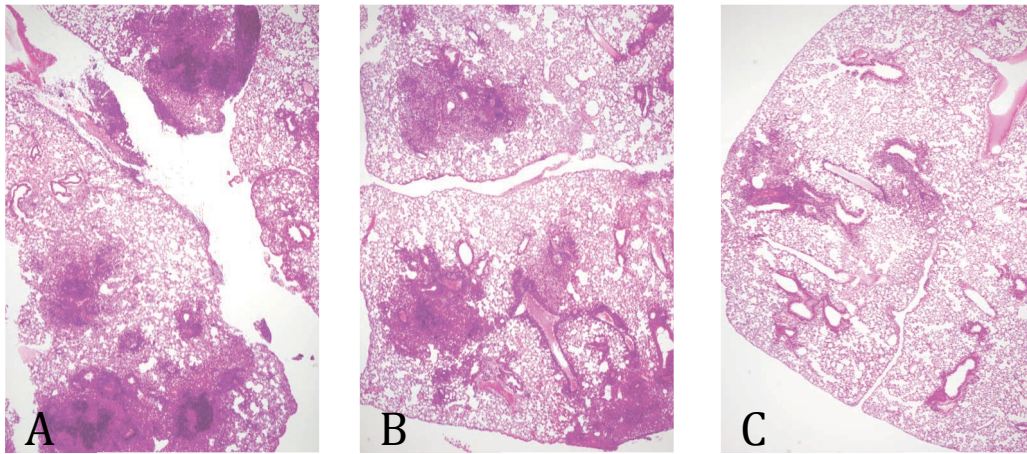
Mice were immunized with 10μg native HspX (nHspX) three times, two weeks apart, and then infected with a low dose aerosol of H37Rv *M. tuberculosis* 30 days after the last immunization. Mice were sacrificed 30 days post-infection and the lungs were processed as described. While inflammation in the nHspX-vaccinated animals (A) did not differ quantitatively from that seen in the saline control animals (B), the lesions were notably dominated by lymphocytes and a rim of activated macrophages. Neutrophils were absent. BCG-vaccinated animals (C) had quantitatively milder lung inflammation, but the composition of the infiltrates were similar to that of nHspX-vaccinated animals. H&E; 40X.

2.3.5   TNF-α and IL-12 ELISA Responses to murine macrophages

Bone marrow derived macrophages were harvested from C57BL/6 wild type mice, TLR 2 (-/-) and TLR4 (-/-) mice, and then stimulated with either of the HspX vaccine preparations and assayed for TNF-α or IL-12 secretion as described. Multiple tuberculosis ligands have been shown to interact and bind with toll-like receptors (TLRs) on the surface of macrophages [20]. Toll-like receptors 2, 4, and 9 have been implicated in TB immunity and responsible for cell signaling pathways to activate TB infected macrophages through cytokine secretion and increased expression of MHC class II on the cell surface [19].

We therefore looked at the ability of HspX to activate macrophages through TLRs 2 and 4 by measuring early cytokine secretion and our results suggest that HspX-pull-down may be signaling through TLR-4 (Figure 2-5). None of the other vaccine preparations were able to induce

IL-12 secretion (data not shown) and the responses to TNF-α were not as strong for the other HspX vaccines. We have shown elsewhere with flow cytometry that native HspX is able to strongly induce IFN-γ secretion from splenocytes from vaccinated animals with the ELISPOT assay and induced poly-functional T cells, which co-express cytokines IFN-Y, TNF-α and IL-2 (Taylor and Wieczorek et al. Nature Cell and Immunology, in revisions 2011).

Taken together, these data suggest that HspX is able to induce a Th1 type response, however it requires an adjuvant to appropriately mount a strong response, as is the case with all subunit vaccines [11]. The macrophages that were harvested for ELISA described in this report were naïve and stimulated *in vitro* after they were allowed to grow; whereas the IFN-γ secretion that was measured in our previous study was taken from splenocytes harvested from vaccinated and challenged mice. These results suggest that splenocytes harvested from vaccinated and challenged mice were sensitized (unlike the bone marrow derived macrophages, which were naïve) and were able to mount a specific Th1 type response when stimulated with the antigen. None of the vaccines were able to induce IL-12 responses from macrophages (data not shown).

Figure 2-5
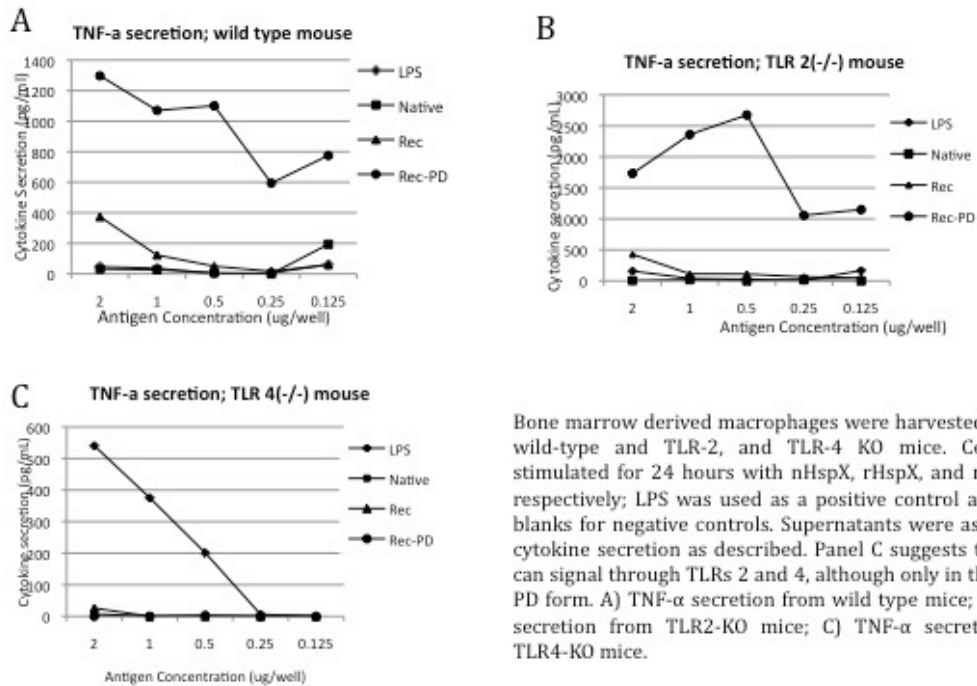
Bone marrow derived macrophages were harvested from B6 wild-type and TLR-2, and TLR-4 KO mice. Cells were stimulated for 24 hours with nHspX, rHspX, and rHspX-PD, respectively; LPS was used as a positive control and media blanks for negative controls. Supernatants were assayed for cytokine secretion as described. Panel C suggests that HspX can signal through TLRs 2 and 4, although only in the rHspX-PD form. A) TNF-α secretion from wild type mice; B) TNF-α secretion from TLR2-KO mice; C) TNF-α secretion from TLR4-KO mice.

2.4 Discussion

To our knowledge, this is the first demonstration of a variation in vaccine protection efficacies between natively purified and recombinant purified antigens from *Mtb* and *E coli*, respectively. We have shown in this study that native HspX is able to protect as well as BCG when given as a subunit vaccine, whereas the recombinant HspX was not able to (Figure 2-3). Additionally, rHspX that was incubated with ΔHspX WCL prior to purification was also able to protect mice from pulmonary TB, supporting our hypothesis that HspX requires its binding partners to remain biologically active as a vaccine.

The pull-down assay described that was performed with the recombinant HspX was an attempt to pick-up any binding partners that native HspX may be carrying in *Mtb*, and absent in *E*

coli. We have previously shown in our laboratory with MALDI-TOF mass spectrometry that when incubated with H37Rv WCL, rHspX bound to itself, i.e., native HspX from *Mtb* (unpublished data). Thus incubating rHspX with ΔHspX WCL eliminated this self-binding exhibited by HspX and allows for interaction of any potential binding partners to recombinant HspX, which is easily re-purified.

The functional difference between these two protein constructs is important to work out in terms of vaccine development. The native 16kDa is time consuming and expensive to purify. It also requires very large amounts of *Mtb* culture to be grown under a BSL-3 level facility. Realistically, a native protein would be near impossible to produce as a vaccine because of the extremely low yields and challenges to purify. A recombinant protein is much cheaper and faster to produce. The protein can be expressed a purified in a non-pathogenic, fast growing organism, such as *E coli,* and the protein yields are much higher since expression is induced at an optimal time during growth. Our pull-down preparation could realistically be applied for vaccine production, thus it was included as one of our vaccine groups.

HspX belongs to the family of small heat shock proteins that function as molecular chaperones and share an α-crystallin core of 90-100 amino acids [94]. The chaperoning ability is shared among all α-crystallins; in the human, an acr homologue is found in the eye of the lens to maintain transparency by preventing protein aggregation [77]. We hypothesize that when purified in a recombinant form, its function as a molecular chaperone or its final three-dimensional structure is altered such that HspX is no longer able to function *in vivo* as a vaccine (figure 1-3). The protection seen by the native and pull-down vaccines (figure 2-3) supports this hypothesis.

Initially comparing native and recombinant HspX with SDS-PAGE and western blotting did not reveal any obvious differences (figure 2-2). *Mtb* has the ability to glycosylate proteins and HspX is not post-translationally modified [96]. Initial analysis of HspX with mass spectrometry on a quadropole time-of-flight (QTOF) instrument did not reveal any similarities between the native and pull-down HspX (and absent in the recombinant) when examining unidentified

peptides with *de novo* sequencing. Further analysis of these protein constructs with mass spectrometry is on going.

The mouse model gives very good immunological data in terms of specific responses to antigens and vaccines because of the availability of antibodies, reagents for immunological assays and an annotated mouse genome [42]. This model also allows for testing of a broader range of vaccine or drug candidates that can then be narrowed down and subsequently move on to other animal models if successful.

The immunological data presented here suggests that HspX-PD in particular was able to induce the secretion of the Th1-type cytokine TNF-α through TLR-4 (figure 2-5), and none were able to induce IL-12 secretion (data not shown). A separate study in our laboratory revealed that HspX was able to stimulate IFN-γ from infected murine splenocytes, when measured by ELISA and intracellular staining (Taylor and Wieczorek, et al; (2011) in revisions) suggesting that HspX is able to induce a Th1 type immune response.

Most vaccines in clinical trials are targeted toward early-secreted antigens [12, 17], however TB also expresses many other antigens later in disease [76]. It would make logical sense to target these antigens for vaccines and diagnostics since the primary reservoirs for TB are latently infected persons [17]. HspX is up-regulated during times of hypoxia and nutrient starvation *in vitro*, and is preferentially recognized by latently infected patients [59] thus makes a tempting vaccine target against latency.

The current idea is that late antigens could be used both as a prophylactic vaccine and as a post-exposure vaccine to prevent re-activation by boosting the immune response that is already in place in latently infected patients [17]. Major obstacles are going to have to be over come to develop a successful post-exposure vaccine. Many people who are latently infected with TB are HIV-co-infected, with low CD4+ T cell numbers; thus testing a post-exposure vaccine will be very difficult as far as reassuring safety in immunocompromised individuals [38]. Although

"Koch" reactions [38] are not as common today, that is still a major fear when giving people a vaccine to an organism/antigen their T cells have already seen. One obstacle to overcome is that there is not a well established animal that accurately represents latent infection [39], and a previous study revealed that a DNA post-exposure vaccine was responsible for lung pathology and thus more severe disease [97]. A safer approach may end up being vaccinating with multi-stage vaccines prophylactically with the hope that the late antigen will help induce a long term, memory response.

Taken together, our results further support that HspX functions a molecular chaperone and that HspX requires its chaperoned molecules to remain biologically active as a vaccine. We also report that subunit vaccination based on latency antigens is able to protect mice from pulmonary tuberculosis and is able to mount an appropriate immune response; however one realistic consideration that needs to be bared in mind is that vaccine production and manufacturing utilizing native TB antigens will be very difficult to produce in large quantities. Recombinant DNA and protein technologies has allowed for the isolation of products that would otherwise be challenging, if not impossible to isolate. However, as we show here, our recombinant pull-down HspX was able to protect and thus further elucidation of the HspX constructs protein from *Mtb* will be required to fully understand the protective effects. We thus present a study that reveals differences between native and recombinant HspX proteins in term of vaccine protection in the mouse model of tuberculosis, and thus functions of antigens in their native form (for use in subunit-based vaccination) should be considered.

3   HspX in the Guinea Pig Model of Tuberculosis

3.1   Introduction:

Latent tuberculosis infection (LTBI) remains a challenge to prevent, diagnose and treat properly. Caused by the bacterium *Mycobacterium tuberculosis (Mtb);* with upwards of 2 billion people latently infected, this population needs to be considered when developing new vaccines; as is evidence by the fact that the current vaccine Bacille Calmette-Guerin (BCG) does not protect adults from pulmonary TB, nor does it prevent reactivation from latent tuberculosis [9]. BCG has been in use since its development the 1920s, with many countries still vaccinating at birth, with the exception of the US and most of Western Europe. It is the most widely given vaccine in the world and for these reasons has an excellent track record for safety; this is particularly important in TB vaccines because much of the target population already suffers from poor healthcare infrastructure and HIV co-infection [9]. Therefore TB vaccine development is twofold: developing a recombinant BCG strain that over-expresses known immunogenic antigens or, developing a new generation subunit vaccine to be given as a booster to the already administered BCG [10]. The latter method will take advantage of the fact that BCG is already given at birth and does offer protection against severe childhood forms of TB [10], with the hopes that a new subunit will boost enough protection to be life-long and specific for TB.

One such candidate antigen for subunit vaccination is the small heat shock protein HspX (acr, α-crystallin, Rv2031c) from *Mtb* due to fact that it is up-regulated during stress *in vitro* during stationary growth, which is assumed to mimic the microenvironment in the lung during chronic TB infection [82]; and is preferentially recognized by latently infected persons when PMBCs were re-stimulated *in vitro* with the antigen [59]. There have been several adaptations of vaccines utilizing HspX in small animal models with initial promising results [69, 73, 74, 83]. It

is obvious that BCG does not protect against reactivation from latency, and most vaccines currently in clinical trials do not contain latency associate antigens (see ref [3] for a review).

Like all α-crystallins, HspX is an ATP independent molecular chaperone that functions to by preventing improper folding and unfolding of other proteins [77, 81]. In vertebrates, and acr homologue can be found in the eye lens to maintain transparency. During *Mtb* infection, it can be found in aggregates on the inner side of the cell wall and has been linked to cell wall thickening [98] and its chaperoning activity has been experimentally verified [81]. Previously when given to the mouse, we found the native form of HspX (nHspX) purified from *Mycobacterium tuberculosis* was able to protect against pulmonary TB, whereas when mice were given recombinant HspX expressed and purified from *Escherichia coli* (*E coli*), it did not protect (See: HspX in the mouse model of tuberculosis).

With the knowledge that HspX functions as a molecular chaperone, this lead us to hypothesize that HspX requires its binding partners (i.e., any molecule its chaperoning) to properly function biologically, and that these binding partners are absent in *E coli*. We are assuming that when HspX is purified from *Mtb* in its native form it is bound to the molecule that it is potentially chaperoning. We will examine this hypothesis by vaccinating guinea pigs with three formulations of the HspX protein, including the native from *Mtb*, the recombinant from *E coli*, and another recombinant form from *E coli* that was incubated with an HspX knock-strain (ΔHspX) lysate and re-purified with any potential binding partners. We have termed this formulation recombinant HspX pull-down (rHspX-PD) since it was subjected to a pull-down assay with the lysate. After an appropriate waiting period post vaccination, guinea pigs were then challenged with either a ΔHspX strain or with the wild-type H37Rv and monitored for survival.

A previous study of ours (Taylor and Wieczorek, 2011, in revisions) in the mouse revealed that native HspX was protective against pulmonary TB, but the recombinant form of the protein was not. For this study, we have chosen to further evaluate the protective effects of HspX in the guinea pig model of TB. This is a normal progression for pre-clinical vaccine testing; generally a

vaccine will go through multiple animal models before entering human trials. Whereas mice are more resistant to *Mtb* infection by generating a stronger acquired immune response that leads to long-term control of infection [99], guinea pigs initially develop immunity but this later fails and the animals then enter a chronic state of infection with more severe pathology than the mouse thus mimicking human pathology more accurately [46]. Guinea pigs live longer than mice and BCG is protective in these animals, and since they are more susceptible to TB, they represent a good model for survival studies for vaccine protection and efficacy [11]. We have vaccinated guinea pigs with three preparations of HspX and subsequently challenged them with H37Rv and an HspX mutant, ΔHspX, to evaluate the efficacy of HspX as a vaccine, and also, to test our hypothesis of HspX as a molecular chaperone requiring its binding partners to function properly *in vivo* as a vaccine.

3.2   Materials and Methods

3.2.1   *Generation of ΔHspX*

ΔHspX was generated and validated as described previously in section 3.2.1: Generation of ΔHspX

3.2.2   *Growth of Organisms*

3.2.2.1   *Preparation of seed stocks*

Each strain of *Mtb* (H37Rv and ΔHspX) were grown for seed stocks as described previously in section 2.2.2.1: Preparation of seed stocks.

3.2.2.2   Preparation of Infectivity (working) stocks

Working stocks for each strain of *Mtb* for use in aerosol challenge studies were grown and prepared as described previously in section 2.2.2.2: Preparation of Infectivity (working) stocks

3.2.3   Protein purification

Native HspX (nHspX), recombinant HspX (rHspX), and rHspX pull-down (rHspX-PD) proteins were purified and validated for vaccine quality as described previously in section 2.2.3: Protein purification.

3.2.4   Guinea pigs

Pathogen free, female outbred Hartley guinea pigs (~500g in weight) were purchased from Charles River Laboratories (North Wilmington, MA) and held under bio-safety level 3 barrier conditions. The animals had unlimited access to guinea pig chow and clean water. Upon arrival, guinea pigs were rested for 14 days, chipped for identification purposes, and rested for another 14 days before vaccination.

3.2.5   Vaccination procedure

Groups of 10 (n=10) guinea pigs were vaccinated with nHspX, rHspX or rHspX-PD, respectively, in three 20µg doses three weeks apart intramuscularly (IM) in the hind legs; BCG was given at the same as the third subunit vaccination at 1×10^4 CFU intradermally (ID) on the underside of the belly. Just prior to vaccination, the HspX subunit vaccines were emulsified with an adjuvant formulation of 250µg dioctadecylammonium bromide (DDA) and 25µg monophosphoryl lipid A (MPL). A saline only and adjuvant only group were included as controls an administered with the same method as the subunit vaccines.

3.2.6   Aerosol challenge

Following a 10 week resting period after the last vaccination, guinea pigs were challenged by using a Madison chamber aerosol generation device (Madison, WI) to deliver a low dose of ~20 bacilli per animal of *Mycobacterium tuberculosis* H37Rv or ΔHspX. Five non-vaccinated guinea pigs per infected group were included for a day 30 CFU counts by plating lung and spleen homogenates on nutrient 7H11 agar with OADC enrichment and incubating at 37°C for three weeks. The remaining animals were monitored weekly and subjected to CFU counts upon death.

3.2.7   Quantitative Real-Time PCR

3.2.7.1   Growth of organisms for QPCR ΔHspX knockout verification

Mycobacterium tuberculosis working stocks were plated onto 7H11+OADC media and following incubation for 14 days at 37°C; one colony from each strain was selected and inoculated into 100mL of 7H9+OADC+0.1% Tween-80 and allowed to grow for 14 days at 37°C with shaking; from which 3 new 100mL cultures for each strain were inoculated at an OD600 of 0.05 into 7H9+OADC+0.1% Tween-80. Cultures were allowed to grow at 37°C with shaking and harvested by centrifugation when the OD read between 0.4-0.6 (~48 hrs). Cell pellets were washed three times with PBS and resuspended in 10mL of TRIzol® reagent (Invitrogen; Carlsbad, CA) and always stored at -80°C or kept on ice.

For verification of HspX expression in the lungs of infected guinea pigs, upon necropsy, 1mL of whole lung homogenate from 5 saline vaccinated guinea pigs from each group (H37Rv or ΔHspX) was saved and placed into 9mL Trizol reagent until RNA extraction, as described below.

3.2.7.2 RNA extraction

Cells were broken by probe sonication and chloroform was added to the broken cells at 0.2 x volume and centrifuged at 27,000 x *g* for 30 minutes. The upper organic layer was recovered and precipitated in 0.5 X volume isopropyl alcohol. Following over-night incubation at -20°C, precipitates were recovered by centrifugation and pellets resuspended in 80% ethanol; RNA was extracted as per manufacturers instructions (RNeasy© spin kit, Qiagen; Valencia, CA). To confirm RNA integrity, 5 μg was run on a 1% agarose gel and visualized with ethidium bromide for the presence of 16s and 23s RNA subunits. Concentration was determined by measuring A260:A280 ratio on a Nanodrop® spectrophotometer (Thermo; Waltham, MA); pure RNA has an A260:A280 ratio between 1.9-2.1. 100 μg RNA was reverse transcribed to cDNA using Invitrogen Superscript III First Strand Synthesis SuperMix (Carlsbad, CA), as per manufacturers instructions.

For verification of HspX expression in the lungs of infected, saline vaccinated guinea pigs, 1 mL homogenate was placed into 9mL Trizol reagent and stored at -80°C. The solution was probe sonicated as described above; and whole (mammalian and bacterial) RNA was purified and reverse transcribed to cDNA as described above. A BLAST search was performed on the primers against the guinea pig genome to verify that the primers would not bind to guinea pig DNA; and quantitative PCR was performed, as described below.

3.2.7.3 Quantitative PCR

Primers were designed for 5 genes within the HspX region: Rv2030c: forward-5'TTATCTACCGGCAACGGAAC 3' reverse- 5'CTGATCGATGTGGATCATGG 3'; Rv2031c (HspX): forward- 5'TAAGGCCACCTACGACAAGG 3'; reverse-5'CCGGATCTGAATGTGCTTTT 3'; Rv2032 (acg): forward-5'CTGACCCACATCACCGAACT 3' reverse- 5'TAGCCCGAACGTGAAACACT 3'; Rv0251c (acr2): forward- 5'CCATCGCGGCTTCCTATG 3' reverse- 5'CTACTTCGTGATGGCGATGC 3'; Rv2703 (sigA, housekeeping gene): forward- 5'CGACGACGAAGACCACGAAG 3' reverse-

5'TGTCCTTTTCGGTGGGTTCA 3'. RNA polymerase sigma factor A was used as a housekeeping gene and genomic DNA from *M. tuberculosis* was used to set a standard curve. Reactions were set up in 96 well plates in triplicate; 3 biological replicates for each strain and 3 technical replicates for each gene/primer pair: 10μL SYBR green (Quanta Biosciences, cat no: 84067), 2μL forward primer @ 10mM, 2μL reverse primer @ 10mM, 2μL DNA sample and QS to 20μL with water. The program was run on a BioRad® iQ5 (Hercules, CA) instrument: 2 min @ 55°C; 2 min @ 95°C; 40 cycles of 95°C for 15 sec; 60°C for 30 sec; 72°C for 45 sec; and finally 5 min @ 72°C then 10 min @ 55°C; gene expression profiles were analyzed with the accompanying program software.

3.3   Results

3.3.1   *ΔHspX Mutant conformation*
Please refer to section 3.3.1 for results.

3.3.2   *Conformation of protein purity*
All HspX protein preparation to be used for vaccination studies were verified for purity as described, see section 3.3.2: Conformation of protein purity.

3.3.3   *Day 30 CFU enumeration*
Ten guinea pigs from each challenge group were not vaccinated, challenged, and assessed for day 30 CFU counts on whole lung homogenates. Figure 3-1 shows that ΔHspX had significantly less bacterial burden in terms of CFU at 30 days post infection when compared to H37Rv (p<0.01). Bacterial burden in the spleen was not found to be significantly different (data not shown). These initial findings suggest that ΔHspX is less virulent in guinea pig at 30 days post challenge.

Lung CFU: day 30
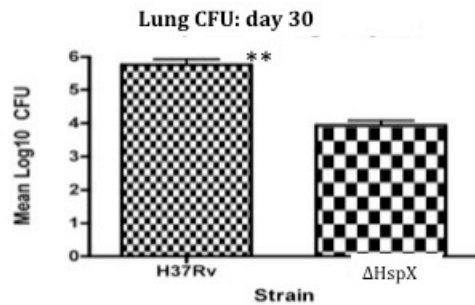
Mean log CFU recovered from whole-lung homogenate of non-vaccinated, H37Rv and ΔHspX challenged guinea pigs. Animals were challenged with a low-dose aerosol of TB and sacrificed 30 days post infection for CFU enumeration. Whole organ homogenates were plated and enumerated. The mutant strain shows decreased bacterial burden which is of statistical significance (**p<0.01), suggesting an avirulent phenotype

Figure 3-1

3.3.4   HspX protection in the guinea pig

In the mouse, when vaccinated with nHspX, rHspX, and rHspX-PD, and challenged with H37Rv and ΔHspX, the native and pull-down HspX preparations were protective, (figure 2-3) and the recombinant HspX alone was found not to be protective. We therefore set out to repeat that experiment in the guinea pig model of tuberculosis, which are more susceptible to TB than the mouse. Guinea pigs were vaccinated with each HspX preparation, were then challenged with

a low-dose aerosol of H37Rv or ΔHspX, and then monitored for survival. Vaccine protection is compared to the protective efficacy of BCG, the current vaccine. Groups of 10 guinea pigs each (for each HspX vaccine and controls), were vaccinated and challenged after a 10 week wait. Figure 3-2 is a Kaplan-Meier survival plot of the wild-type H37Rv infected guinea pigs. At 53 weeks post challenge, none of our HspX vaccines were able to protect when compared to BCG (p>0.05, log-rank test).

The survival rate of the ΔHspX infected group are shown in figure 3-3 and tells a slightly different story. As with the wild-type group, none of the HspX vaccines were able to significantly protect when compared to BCG (p>0.05). However a higher proportion of the saline vaccinated, ΔHspX infected animals were still alive at 53 weeks post challenge, which is inconsistent with virulent *Mtb* infection in guinea pigs. In contrast to the mouse, we speculate that ΔHspX is less virulent in the guinea pig model of TB; chapter 5 details a virulence assay to address this issue.

Figure 3-2

Survival of H37Rv infected guinea pigs
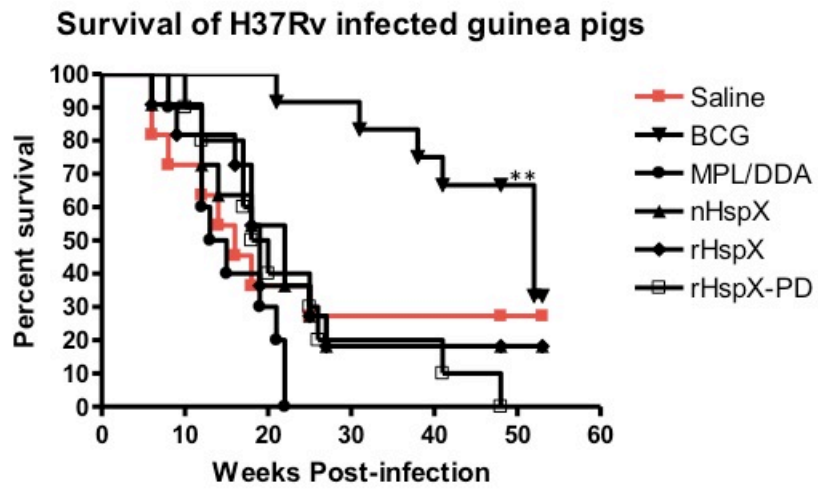
Survival of H37Rv infected guinea pigs at 53 weeks post infection (PI): up until 48 weeks post infection, BCG was the only vaccine able to significantly protect against pulmonary disease in these animals (downward pointing closed triangles; **p<0.01). At 53 weeks PI, none of the vaccines were able to significantly protect, including BCG (p>0.05).

Figure 3-3

Survival of ΔHspX infected guinea pigs
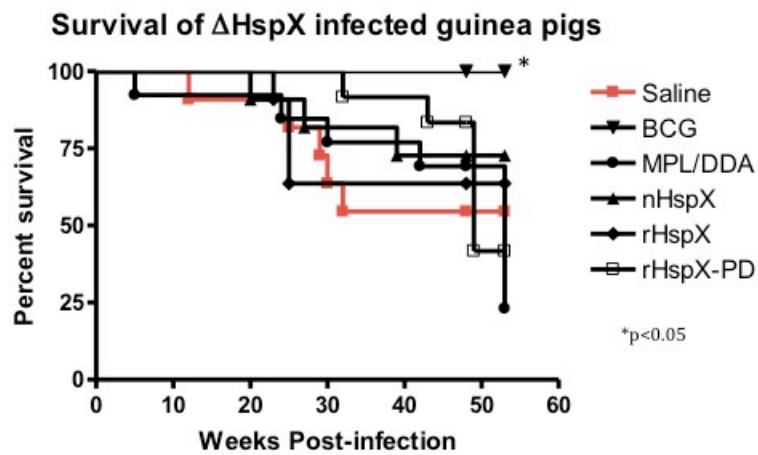
Survival of ΔHspX infected guinea pigs 53 weeks post infection (PI). The only vaccine found to significantly protect was BCG (downward pointing closed triangle; p<0.05). None of the HspX subunit vaccines were able to significantly protect these animals from pulmonary disease (p>0.05 in each case). In contrast to the H37Rv infect guinea pigs, the survival rate of the ΔHspX infected animals suggests that this mutant exhibits a loss-of-virulence phenotype. Guinea pigs will generally succumb to disease with H37Rv at ~25 weeks PI, and our data here shows that ~50% of the animals in this group were still alive at 53 PI.

3.3.5 Gene expression in ΔHspX

Because the results in figure 3-3 were somewhat unexpected, the expression levels of HspX and other genes within immediate proximity were assessed with quantitative PCR (QPCR). The *hspX* gene lies within close proximity to gene Rv2032, a putative nitrate reductase termed acg (for acr co-regulated gene) [100], and Rv2030c, a hypothetical protein with unknown function (figure 2-1). Along with Rv2031c (HspX), these genes are all under control of the of dosR regulon (Rv3133c) for expression and seem to be induced under hypoxia in standing vs. shaking cultures [80], whose promoters contain a common DNA motif [8]. It has been suggested that reactive nitrogen intermediates (RNI) and nitric oxide (NO) are effective anti-mycobacterial agents *in vivo* and can influence the expression of other latency related genes, including *hspX* [101-103]. It was therefore important to determine if *acg* is expressing in the knock-out strain for our studies. Acr2 (Rv0251c, an acr homologue in *Mtb*) was also included in the QPCR study because it is also highly up-regulated during heat-shock and stress, although its location on the genome is not in the DosR region as the other listed genes.

Figure 3-4 reveals that all three genes, Rv2030c, Rv2031c (*hspX*), and Rv2032 (*acg*) were knocked out in our ΔHspX strain, and acr2 (Rv0251c) was still expressing which is not unexpected, given its location on the genome relative to the other genes. We therefore cannot assume that the reduced virulence observed in this strain in the guinea pig was due to the loss of HspX (Rv2031c) alone. It is very likely that the attenuated virulence is due to the loss of *acg* (Rv 2032c) and HspX. The wild type strain however showed that all genes were intact (figure 3-4). A recent study revealed that an *Mtb* strain with a clean knock-out of only *acg* (and not Rv2030c or Rv2031c, HspX), displayed an attenuated phenotype [102]. That same study showed that the loss of Rv2030c alone did not results in a loss of virulence, as SCID mice were able to survive infection. Section 5 details a survival study comparing these two strains in the guinea pig.

Relative gene expression
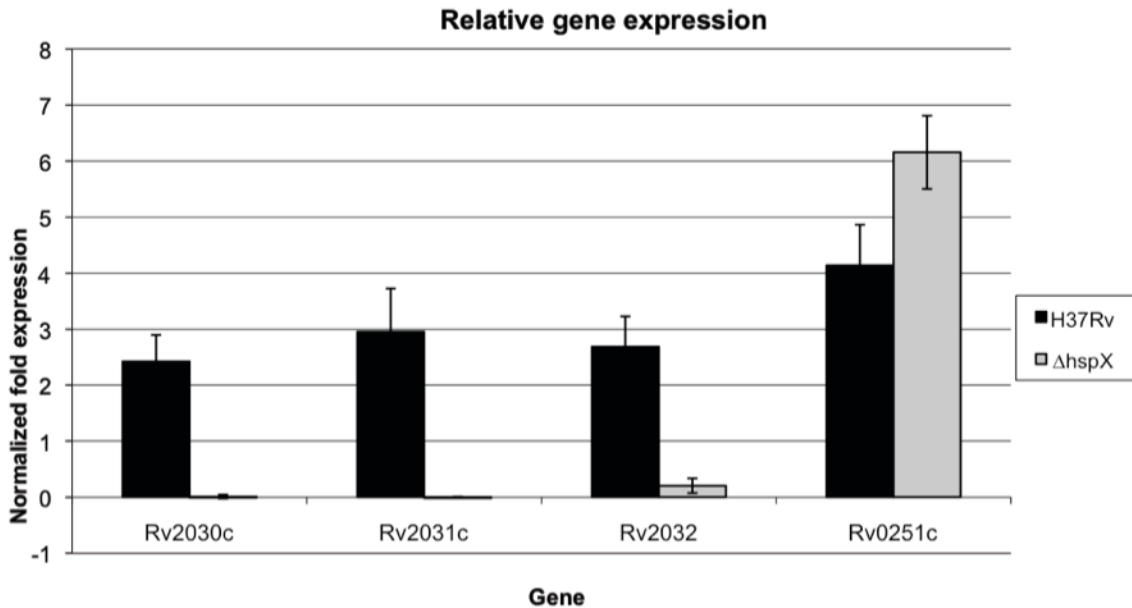
Normalized gene expression level of Rv2030c, Rv2031c (*hspX*), Rv2032c (*acg*, nitrate reductase), and Rv0251c (*acr2*). Gene expression levels were normalized to the TB housekeeping gene, RNA sigma factor A, *sigA*. In addition to the lack of HspX, ΔHspX is not expressing Rv2030c, and the expression levels acg (Rv2032c) were significantly reduced. Acr2 expression levels remained intact. We therefore can not assume that the loss-of-virulence phenotype we observed in the guinea pigs is due to the loss of the *hspX* gene alone.

Figure 3-4

3.4   Discussion

Our data here suggests that, in contrast of studies performed in the mouse model of TB (chapter 2), HspX is not protective in the guinea pig model of tuberculosis. Probably a major set-back was that our HspX was not a clean knock out, with acg being knocked-out along with HspX. Our survival data and day 30 CFU enumeration suggests attenuated virulence. There have been many discrepancies with HspX mutant strains in small animal models [104, 105], and it was recently demonstrated that a clean acg knock-out is attenuated [102]. Recall that HspX lies in close proximity to other latency-associate genes that are under transcriptional control of the same regulon [8]. If acg was expressing in our mutant, then perhaps the bacilli would be more virulent

and thus put more pressure on the host. In addition, we speculate that HspX may not be expressing in the lungs of guinea pigs. Our data suggests that the physiology of *Mtb* may differ in these two animal models when it comes to latent-gene expression.

We speculate that *Mtb* may not be under enough environmental stress to induce expression of HspX in the lungs of guinea pigs. Chronic infection is difficult to match in animal models, and bacteria grown *in vitro* versus *in vivo* can have big differences in protein expression due to completely different environments. Thus accurately measuring expression of latency-associated antigens *in vivo* comes with many challenges, since the genes in the DosR region in particular, can have transcriptional influences on each other. Current studies are on-going to validate *in vivo* HspX expression in the lungs of guinea pigs.

Vaccine discovery and design requires screening in multiple animal models. Whereas the mouse will model immunity very well, guinea pigs will model protection efficacy and survival well because they are more prone to the disease than mice [46]. It is not entirely uncommon to see vaccine candidates do well in the mouse and then subsequently fail in other models. Unfortunately, if a candidate fails multiple times in the guinea pig, then it is probably not a strong enough candidate to warrant further vaccine design.

Subunit vaccine design utilizes few antigens that are delivered with an adjuvant to mount a strong cell-mediated response. The beauty of recombinant gene technologies is that multiple antigens can be put together into one vaccine component. Perhaps HspX would be protective if it was paired with an early antigen in a subunit. These "two-stage" vaccines would then be able to mount responses against early and late antigens [37]. In addition to this strategy HspX should still be considered as a vaccine for latency based antigens as a post-exposure vaccine, a model that was not tested here. In this case, one may consider combining the 2 acr proteins.

Like HspX, acr2 is an alpha-crystallin homologue found in *Mtb* that shows approximately 25% homology to *acr* [106]. This gene is highly up-regulated during heat shock at 45°C and is also the most strongly up-regulated gene following uptake by macrophages and along with acr1,

has been implicated in mycobacterial persistence [107]. An *acr2* mutant in the mouse model didn't reveal any differences in CFU lung pathology versus the wild type, but there was a significant decrease in CD4+ T cells and CD8+ T cells numbers in the lungs at the site of damage; and *in vitro* acr2 KO strains were more susceptible to H_2O_2 treatment [106]. Studies aimed at understanding the utility of the conformation of HspX and other *Mtb* proteins thus remain an area of focus in our laboratory.

4 ΔHspX displays attenuated virulence in the guinea pig

4.1 Introduction

Tuberculosis is spread via aerosol droplets and infects and replicates within alveolar macrophages of the lung. Upon exposure, ~90% of people will control the infection and not develop active TB, with the other 10% developing latent disease, with a 10% chance of reactivation within their lifetimes [3]. During latency, the bacilli are walled off within the hallmark granuloma in the lung tissue and patients do not exhibit any symptoms, nor are they contagious. The bacilli residing in the granuloma are believed to be in a metabolically inactive state, but live bacteria can be isolated from granulomas of the lungs of infected persons [5]. Latent tuberculosis is very difficult to treat, requiring a long drug regimen with multiple and expensive antibiotics, which often present side effects, leading to issues with compliance, thus for long-term control, a vaccine seems to be a more realistic answer. Given that the majority of people infected with TB are latently infected, this population should be considered during vaccine design.

Previous experiments with *Mtb* ΔHspX in the mouse model showed ΔHspX to be hypervirulent with an increased CFU burden and increased pathology in the lungs of infected animals [104]. Our previous study in the guinea pig (section 4) suggested that ΔHspX is attenuated. There have been mixed results regarding the virulence of this strain in small animal models [79, 104, 105], thus we were interested in evaluating the virulence of our ΔHspX strain in the guinea pig model of tuberculosis. Guinea pigs are a widely used model for tuberculosis because they mimic human disease more accurately than the mouse in terms of lung pathology [108]. They are also widely used for TB vaccine testing due to their susceptibility to TB when

compared to the mouse [47]. The guinea pig model will allow assessment of survival and pathology to estimate differences in virulence between the wild type H37Rv and ΔHspX strains. These experiments will in turn provide data for the usefulness of this strain in the guinea pig model for studies using HspX as a vaccine.

It is believed that the environment in the granuloma is harsh with hypoxia, nutrient starvation and the production of reactive nitrogen intermediates (RNIs) by the host [82]. The small heat shock protein from Mtb, HspX (a.k.a 16kDa, α-crystallin, acr) has previously been shown to be highly expressed during hypoxia and stress [34] and under these conditions it can comprise of up to 25% of the total protein expressed by Mtb [59]. HspX functions as a molecular chaperone and has a homologous structure to α-crystallins [81, 94]. All alpha-crystallins function as molecular chaperones and in vertebrates act to suppress the denaturation and aggregation of other proteins to maintain the transparency of the human eye [77]. HspX from Mtb has been shown to stabilize other proteins in vivo and has activity comparable with that of bovine α-crystallin isolated from the eye lens [81]. Although its role during TB infection has yet to be fully elucidated, there have been reports that HspX is preferentially recognized by latently infected persons, and it is not recognized by BCG vaccinated persons, suggesting that BCG cannot mount T cell responses against this latency antigen [28].

In this study, ΔHspX was compared to wild type H37Rv *Mtb* to evaluate its virulence in the guinea pig model of TB. The characteristic of this knock-out in the guinea pig model will provide a platform for the evaluation of HspX as a vaccine candidate, versus protection afforded via its role as a molecular chaperone. Guinea pigs were vaccinated with either of three preparation of HspX and were subsequently challenged to access vaccine protection based on survival rates.

4.2   Materials and Methods

4.2.1   Generation of ΔHspX

ΔHspX was generated and validated as described previously in section 3.2.1: Generation of ΔHspX

4.2.2   Growth of organisms for challenge studies

4.2.2.1   Preparation of seed stock

Each strain of *Mtb* (H37Rv and ΔHspX) were grown for seed stocks as described previously in section 3.2.2.1: Preparation of seed stocks.

4.2.2.2   Preparation of infectivity stocks

Working stocks for each strain of *Mtb* for use in aerosol challenge studies were grown and prepared as described previously in section 3.2.2.2: Preparation of Infectivity (working) stocks

4.2.2.3   Guinea pig infections

Pathogen-free, female outbred Hartley guinea pigs (~500g in weight) were purchased from Charles River Laboratories (North Wilmington, MA) and held under bio-safety level 3 barrier conditions. The animals had unlimited access to guinea pig chow and clean water. Upon arrival, guinea pigs were rested for 14 days, chipped for identification purposes, and rested for another 14 days. Thirteen guinea pigs (n=13) were challenged with H37Rv or ΔHspX respectively, via the aerosol route with a Madison Chamber to deliver ~20 bacilli per animal. Animals were monitored weekly for any signs of distress and were sacrificed if the disease progression was deemed too severe. Guinea pigs were sacrificed day 30 post infection (PI) to obtain CFU counts in the lung and spleen. The remaining guinea pigs (survival group) were also subjected to CFU counting upon death. CFU counts were performed by plating tissue

homogenates serially on 7H11 agar with OADC after incubation at 37°C for three weeks. All protocols have been approved the Colorado State University Animal Care and Use Committee.

4.2.3   *Histopathology assessment*

Tissues (lung, liver, spleen) were fixed in 10% neutral-buffered formaldehyde or 4% paraformaldehyde and routine-processed through paraffin embedding. Hematoxylin and eosin (H&E) stained sections were assessed by a pathologist (HBO) blinded to the treatments and groupings. Lung lesions were scored based on criteria previously published [109], including an assessment of extend of overall lung involvement, extent of primary and secondary lesions, necrosis, mineralization and fibrosis. Maximum possible score for lung pathology would be 24. Liver and spleen lesions were scored based on two criteria: (i) lesion type and (ii) extent of any change(s) seen. For liver the lesions types were: 0 = no apparent lesion in section; 1 = mild sinusoidal infiltration of macrophages and granulocytes accompanying degeneration and necrosis of few individual hepatocytes; 2 = focal/multifocal coagulation necrosis with or without mild infiltration of macrophages and neutrophils; 3 = granulomatous inflammation with or without bile duct proliferation and minimal fibrosis; 4 = granulomatous inflammation with moderate to severe fibrosis and/or mineralization and/or necrosis. For spleen the lesions types were: 0 = no apparent lesion in section; 1 = small clusters of epitheloid macrophages with or without the presence of a few multinucleated giant cells; 2 = granulomas without necrosis or mineralization; 3 = granulomas with necrosis but without mineralization; 4 = granulomas with necrosis/mineralization and with or without fibrosis. For both liver and spleen the extent scores were as follows: 1 = < 10% involvement of parenchyma; 2 = 11-30% involvement of parenchyma; 3 = 31-60% parenchyma involved; 4 = >60% of parenchyma involved. For both organs the maximum possible score would be 8.

4.2.4   Quantitative Real-time PCR

Quantitative RT-PCR was performed as described previously in section 4.2.7: Quantitative Real-Time PCR.

4.2.5   Statistical analysis

All data represent two experimental groups of WT infected (n=13) or ΔHspX infected (n=13). Of the 13 total guinea pigs per group, 5 were sacrificed for day 30 CFU and the rest were assayed for survival. Survival was analyzed using the Kaplan Meier Log-Rank test; CFU data was analyzed using the student's t-test. Any values of p<0.05 were considered significant. All data represent two experimental groups of WT infected (n=13) or ΔHspX (n=13) infected. Of the 13 total guinea pigs per group, 5 were sacrificed for day 30 CFU and the rest were assayed for survival. All data was analyzed using Prism Graph-pad Soft ware (La Jolla, CA); survival data was analyzed with the log-rank test and CFU were analyzed using the student's *t*-test. Any values were p<0.05 were considered significant.

4.3   Results

4.3.1   ΔHspx Mutant conformation

Please refer to section 3.3.1 for results.

4.3.2   Day 30 disease pathology

Twenty-six female guinea pigs (13 per strain, 5 for a day 30 time point and 8 for survival) were challenged with a low dose aerosol to deliver ~20 bacilli per animal and were monitored for survival. A physical examination was given twice weekly and weight checked once weekly. Animals were monitored for signs of distress, such as, sudden weight loss, respiratory rate, and ear color (indicating fever), and any animals that were too ill or appeared uncomfortable

were ethically euthanized. A full necropsy was performed upon death for each animal; lungs and spleens were harvested, homogenized, and plated for CFU enumeration and tissue samples were fixed for histopathology assessment by a veterinary pathologist.

By day 30, the wild type infected animals had higher bacilliary loads in the lungs and spleen (Figure 4-1), although the CFU counts in the spleens were not found to be significant between the two groups, suggesting that the dissemination ΔHspX is similar to that of H37Rv. These day 30 data are consistent with what we found previously thus our ΔHspX strain is not as virulent as H37Rv, with almost a full log reduction in lung CFU.

Figure 4-1
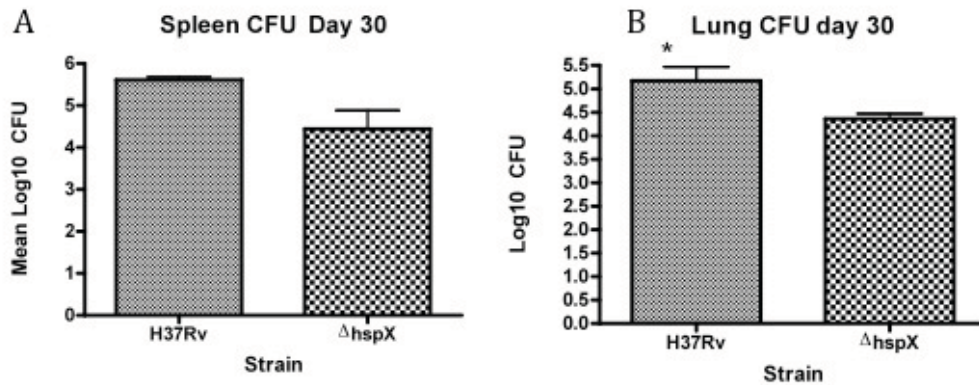
A) Mean log CFU recovered from spleens of infected animals; not significant. B) Mean log CFU from lungs of infected animals; *p<0.05 and is statistically significant. Organs were harvested upon death and whole organ homogenates were plated and enumerated for CFU following 14 days incubation.

4.3.3   Histopathology

Lungs and spleen of day 30 infected animals from H37Rv and ΔHspX animals were examined by a veterinary pathologist who was blinded to the groups. The H37Rv infected groups shows more evidence of tissue destruction with secondary and occasional primary granulomas with central mineralization (PL-in panel A; B-insert; C-arrow; D), and a dense infiltration of lymphocytes and macrophages. The ΔHspX infected animals showed much less pathology with only occasional primary legions and smaller regions of secondary legions involving heterophil (neutrophil) infiltration and less developed granulomas (figures 4-2 thru 4-4). These contrasts can also be seen in the spleens of infected animals with more parenchyma involvement in the H37Rv infected group, Figure 4-4). Overall, the ΔHspX infected animals displayed less organ pathology than H37Rv with less over-all involvement of infected tissues. Our histology report here is consistent with the CFU levels reported (Figure 4-1), further affirming that ΔHspX is less virulent in the guinea pig.
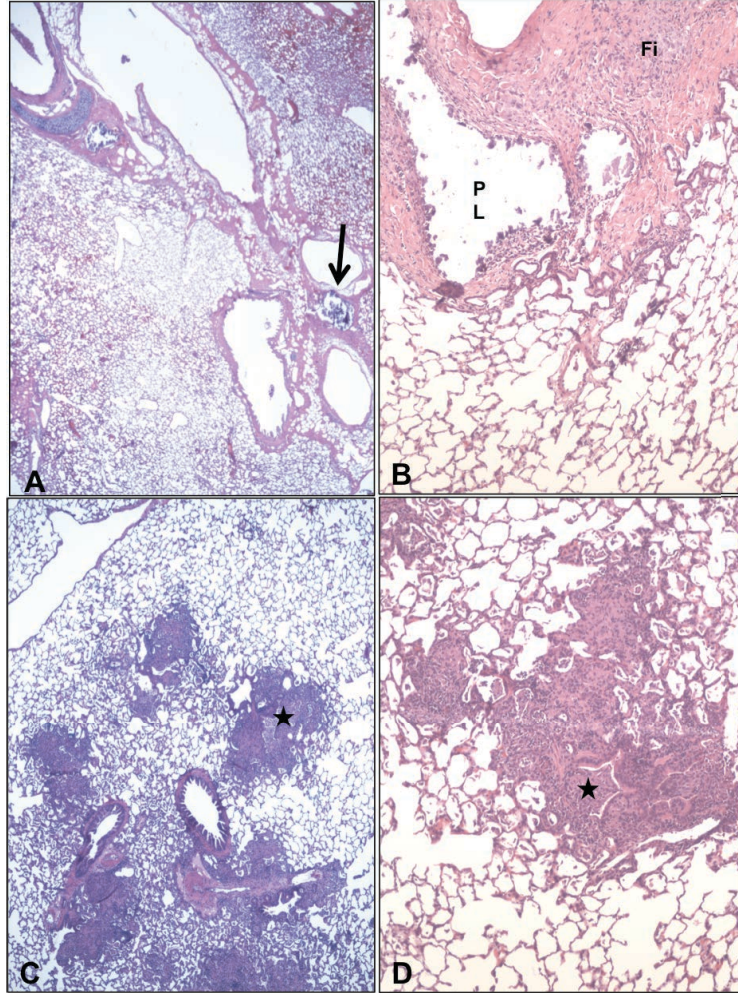
ΔHspX infected– lung. A, B Occasional remnants of primary lesions (PL, arrow) are apparent in the interstitium supporting bronchioles and larger pulmonary vessels. In panel B the mineralized material, characteristic of late-stage primary lesions, have fallen out during tissue processing. Such lesions are often accompanied by marked fibrosis (Fi). C,D Small/restricted areas of secondary lesions are seen in some lungs of this group and characterized by a mild to moderate, mostly compact lympho-histiocytic infiltrate in the alveoli and bronchiolitis (stars) also involving hetrophil-infiltration.

Figure 4-2
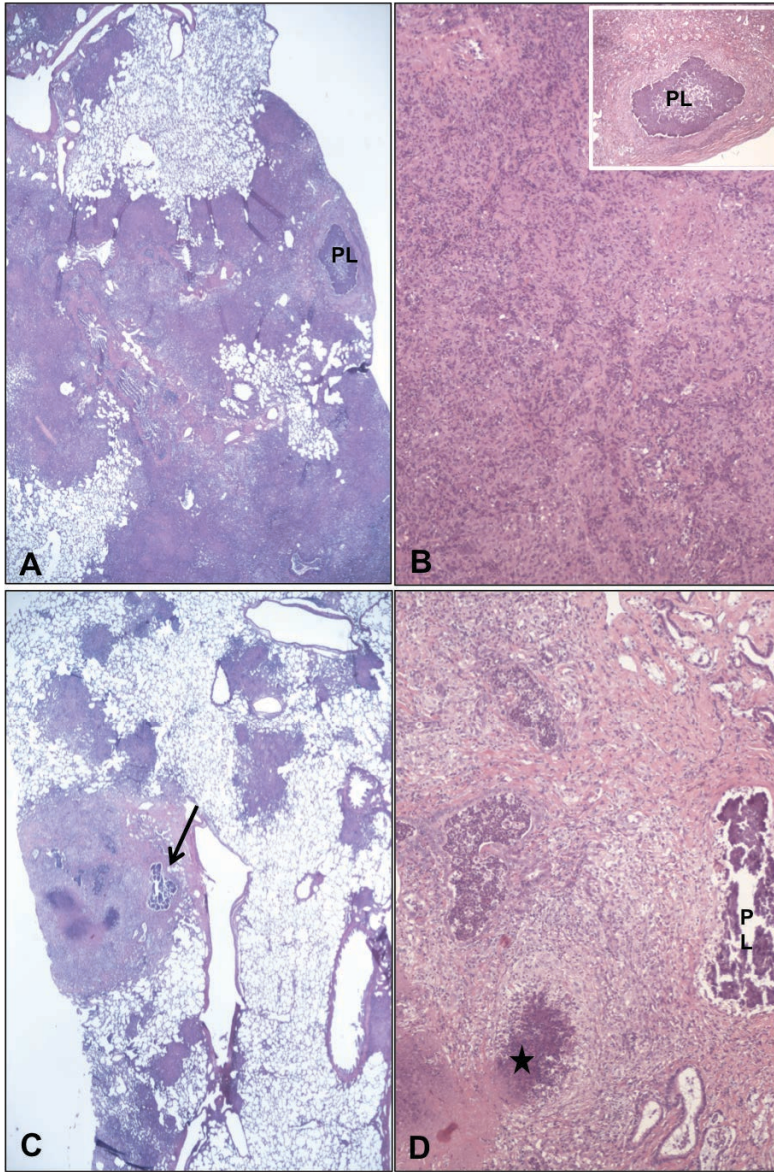
H37Rv infected – lung. The pathological changes are dominated by secondary lesions with dense, multifocal (C) to coalescing (A) infiltrates of lymphocytes and macrophages. Occasional primary granulomas with central mineralization (PL in A, B-insert, D; arrow in C) are seen within the extensive secondary changes. Areas of necrosis (star in D) and variable fibrosis characterize the secondary lesions (minimal fibrosis in A,B; moderate fibrosis in D).

Figure 4-3

Figure 4-4

ΔHspX (A, B) & H37Rv (C, D) – spleen. In group 1 no or only occasional small granulomatous foci (star in A, B) were seen, involving less than 10% of the parenchyma. In contrast, in group 2 more than 30% and often more than 60% of the spleen parenchyma was involved, as illustrated in panels C and D. The multifocal to coalescing cellular infiltrates are composed mainly of epitheloid macrophages with variable admixtures of lymphocytes and occasional multinucleated giant cells.

4.3.4   *Survival*

Although our day 30 results revealed that ΔHspX is less virulent, it is useful to measure

survival rates in guinea pig studies. Guinea pigs are susceptible to tuberculosis and will succumb

to disease within ~20 weeks post infection [46]. As can be seen in Figure 4-5, as expected, all of

the H37Rv infected animals died by the average time point, with over 95% of the ΔHspX still

alive by this point, and 95% still alive at the end of the study at 35 weeks. At this point, our

statistical p-value was very significant (p<0.001) and the experiment was terminated at 35 weeks. Taken together, our results suggest that ΔHspX is less virulent in the guinea pig than H37Rv. The mutant displayed consistently less CFU burden in the lungs and the animals were much healthier than the H37Rv infected animals.

Figure 4-5
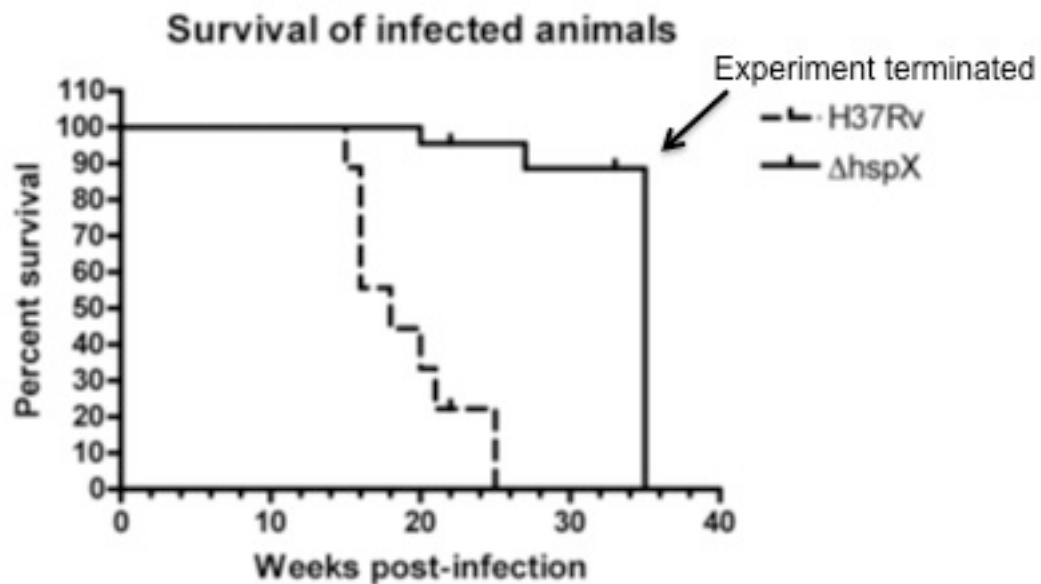
Survival of H37Rv and ΔHspX challenged guinea pigs. Thirteen guinea pigs (n=13) were challenged with either H37Rv or ΔHspX; 5 per group were sacrificed for day 30 CFU and the remaining 8 per group were carried out to survival. As expected, the H37Rv infected animals died by 26 weeks PI. By 36 PI, almost 90% of the ΔHspX animals were still alive and our p value was very significant (p<0.001), thus we terminated the experiment at 36 weeks PI. These results support the rest of our data that ΔHspX used for this study has attenuated virulence in the guinea pig.

4.3.5   *Whole genome sequencing of ΔHspX*

In addition to the QPCR performed, a whole genome sequence analysis on ΔHspX to confirm that only the targeted HspX operon was deleted. Our analysis verified that HspX was absent (not shown). In addition, 84 total single nucleotide polymorphisms (SNPs) were identified, of which 37 were unique to the mutant and could attribute to the loss of virulence phenotype. Figure 4-6 below is a chart of the SNPs found and which genes they are referenced to in the H37Rv genome.

ref reads	reference position	snp reads	description	start	end	strand	aa	NP/YP	GeneID	symbol	Rv	COG	CLSK/PRK	name
13C	174978C/T	13	short-chain type dehydrogenase/ reductase	17483 3	17569 3	+	286	NP_214662.1	886845		Rv0148	COG1028IQR	CLSK79D302	Rv0148
9C	234496C/T	20	oxidoreductase	23223 1	23451 9	+	762	NP_214711.1	886758		Rv0197	COG0243C	CLSK867466	Rv0197
0A	459399C	191	hypothetical protein Rv0383c	45846 1	45931 5	-	284	NP_214897.1	886458		Rv0383c	-	CLSK79D481	Rv0383c
			endopeptidase ATP binding protein	45945 6	46200 2	-	848	NP_214898.1	886440	clpB	Rv0384c	COG0542O	CLSK79D482	clpB
1C	635633T	517	hypothetical protein Rv0543c	63557 3	63587 5	-	100	NP_215057.1	887494		Rv0543c	-	CLSK790583	Rv0543c
2A	636533G	56	inorganic phosphate transporter	63621 0	63746 3	-	417	NP_215059.1	887517	pitA	Rv0545c	COG0306P	CLSK871824	pitA
1C	708430C/T	2	hypothetical protein Rv0613c	70694 5	70951 5	-	855	NP_215127.1	887913		Rv0613c	-	CLSK79D633	Rv0613c
2C	713117C/G	5	galactokinase	71271 6	71380 7	+	363	NP_215134.1	887936	galK	Rv0620	COG0153G	PRK00555	galK
0C	958922A	434	DNA helicase ErcC3	95852 3	96015 1	-	542	NP_215376.1	885425	ercc3	Rv0861c	COG1061KL	CLSK871863	ercc3
11C	107117 0C/G	11	magnesium chelatase	10698 83	10712 62	+	459	NP_215473.1	885405		Rv0958	COG1239H	CLSK871876	Rv0958
8G	108965 4A/G	10	hypothetical protein Rv0976c	10884 93	10901 75	-	560	NP_215491.1	885860		Rv0976c	-	CLSK867600	Rv0976c
0G	118505 5T	185	hypothetical protein Rv1062	118488 3	118574 0	+	285	NP_215578.1	887129		Rv1062	COG1752R	CLSK790927	Rv1062
6G	119945 9C/G	6	hypothetical protein Rv1075c	119942 6	12003 70	-	314	NP_215591.1	887110		Rv1075c	COG2755E	CLSK790934	Rv1075c
4G	133598 7A/G	7	acyl-CoA synthetase	13357 94	13372 15	+	473	NP_215709.1	886074	fadD36	Rv1193	COG0318IQ	PRK0787	fadD36
1C	146392 3T	213	F0F1 ATP synthase subunit alpha	14632 28	14648 77	+	549	NP_215824.1	886936	atpA	Rv1308	COG0056C	PRK09281	atpA
1C	147165 9T	918	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	14703 21	14715 77	+	418	NP_215831.1	886921	murA	Rv1315	COG0766M	PRK093 69	murA
			methylated-DNA--protein-cysteine methyltransferase	14771 34	14776 31	-	165	NP_215832.1	886913	ogt	Rv1316c	COG0350L	CLSK791095	ogt
0C	171162 7T	194	sugar transferase	171102 8	17120 68	+	346	NP_216036.1	886447		Rv1520	COG0463M	CLSK791221	Rv1520
2C	199009 3C/T	2	PE-PGRS family protein	19898 33	19925 77	-	914	YP_177831.1	885325	wag22	Rv1759c		CLSK824795	wag22
0A	200603 2G	721	oxidoreductase	20051 61	20064 47	-	428	NP_216287.1	885441		Rv1771	COG0277C	CLSK791383	Rv1771
3A	215341 0G	447	hypothetical protein Rv1907c	21532 35	21538 82	-	215	NP_216423.1	885402		Rv1907c	-	CLSK791476	Rv1907c
4T	216748 9C	660	PPE family protein	21629 32	21673 11	-	1459	YP_177655.1	885362	PPE34	Rv1917c	COG5651N	CLSK799851	PPE34
			PPE family protein	21676 49	21706 12	-	987	YP_177850.1	885506	PPE35	Rv1918c	COG5651N	CLSK23D1106	PPE35
0A	225199 9G	463	hypothetical protein Rv2005c	22509 96	22518 83	-	295	NP_216521.1	888831		Rv2005c	COG0589T	CLSK791556	Rv2005c
			trehalose-6-phosphate phosphatase OtsB1	22520 02	22559 85	+	1327	YP_177855.1	888943	otsB1	Rv2006	COG1554G	CLSK799862	otsB1
0A	250591 9G	411	hypothetical protein Rv2231c	25046 05	25056 99	-	364	NP_216747.1	888337	cobC	Rv2231c	COG0079E	PRK07908	cobC
			hypothetical protein Rv2232	25062 78	25071 53	+	291	NP_216748.2	887597	-	Rv2232	COG0546R	CLSK799885	Rv2232
1T	271885 2G	572	nicotinic acid mononucleotide adenylyltransferase	27181 73	27188 08	-	211	NP_216937.1	885457	nadD	Rv2421c	COG1057H	PRK00071	nadD
			hypothetical protein Rv2422	27190 83	27193 55	+	90	NP_216938.1	885531	-	Rv2422	-	CLSK791841	Rv2422
12C	280943 9A/C	24	branched-chain alpha-keto acid dehydrogenase subunit E2	28087 58	28099 39	-	393	NP_217011.1	888237	pdhC	Rv2495c	COG0508C	PRK11856	pdhC
0G	325220 6T	245	phenolpthiocerol synthesis type-I polyketide synthase PPSB	32510 72	32556 88	+	1538	NP_217448.1	888023	ppsB	Rv2932	COG3321Q	CLSK792170	ppsB
16C	329684 1A/C	24	polyketide synthase PKS15	32963 50	32978 40	-	496	NP_217463.1	887291	pks15	Rv2947c	COG3321Q	CLSK792183	pks15
3G	329968 2A	160	polyketide synthase PKS15	32963 50	32978 40	-	496	NP_217463.1	887291	pks15	Rv2947c	COG3321Q	CLSK792183	pks15
12G	333973 3C/G	23	hypothetical protein Rv2983	333911 8	33397 62	+	214	NP_217499.1	888161	-	Rv2983	COG1920S	CLSK792205	Rv2983
0G	337976 3A	101	PPE family protein	33793 76	33804 52	-	358	YP_177920.1	888924	PPE47	Rv3021c	COG5651N		PPE47
0G	389874 1A	348	hypothetical protein Rv3479	38958 20	38988 85	+	1021	NP_217996.2	888478	-	Rv3479		CLSK799994	Rv3479
3A	425759 8A/G	4	polyketide synthase PKS13	42559 45	426114 6	-	1733	NP_218117.1	886133	pks13	Rv3800c	COG3321Q	CLSK792729	pks13

Figure 4-6

4.4 Discussion

Our data here shows that ΔHspX has a loss of virulence phenotype, and that this loss of virulence may be due to the loss of HspX, acg, or Rv2030c expression. In addition 37 SNPs were identified which could also contribute to the loss of virulence phenotype that was observed. Each SNP will be analyzed for its potential to contribute to gene function in order to determine the relevance of these SNPs.

Our study in the mouse (section 3) revealed that native HspX protected mice from tuberculosis, while recombinant HspX was not able to. Although we hypothesize that nHspX requires its binding partners to remain biologically active, at the present time we do not know what it is chaperoning and other experiments to determine this are currently in progress.

To further test the hypothesis of HspX requiring its binding partners, an HspX mutant strain was developed for *in vivo* models. Because our strain has not been analyzed in the guinea pig, we aimed to establish a survival curve comparing our knockout to the H37Rv wild type. Figure 4-5 shows that ΔHspX infected guinea pigs survived significantly longer than wild type infected guinea pigs. Our histology and CFU counts from infected organ tissue support our survival data that ΔHspX is attenuated in the guinea pig (figures 4-1 thru 4-4).

There have been contrary results in previous studies using ΔHspX *in vivo* [79, 104, 105]. Our histopathology and CFU clearly show a difference between the two infected groups; however we cannot rule out that something else besides the lack of HspX may have caused the attenuation, such as the lack of *acg* (Rb2032c) [102], as is shown in our QPCR results (figure 3-4). The HspX mutant that showed attenuated growth in macrophages by Yuan et al in 1998 [58], was made before the function of other genes in the proximity of Rv2031c (HspX) were known, and Rv2032 (acg) was later shown to be knocked out [102]. Nitric oxide and other reactive nitrogen intermediates are generated by TB activated macrophages as a major defense mechanism against infection with tuberculosis [110]. Acg has been described as putative nitrate reductase, which is a known virulence determinant in bacteria [111] and it has recently been shown that a clean acg

knockout strain with HspX and Rv2030c intact, significantly reduced the virulence of the bacteria, while the Rv2030c knock out did not, thereby suggesting that acg is needed for virulence and growth [102]. Caution should be taken when evaluating strains for virulence and pathology, particularly in knockout mutants.

HspX is an α-crystallin, all of which contain a conserved homologous sequence of a 90-100 amino acid "core" that is very stable and are found in prokaryotes as well as higher organisms and vertebrates [78]. Alpha-crystallins are believed to maintain homeostasis in the cell during times of stress due its ability to prevent aggregates of other proteins, prevent unfolding and aid with re-folding of other proteins in the cell [77]. A homologue can be found in the pathogenic *M leprae* and in the *M bovis* vaccine strain [112]. HspX from *Mtb* contains this conserved α-crystallin core and forms a tetrahedral arrangement of monomers to form a final dodecameric structure [107]. If the chaperoned molecule is at all dependant on the α-crystallin core for proper binding to function, then it is possible that recombinant HspX purified from *E coli* may have a different three-dimensional structure than its native counter part, thus hindering the chaperoning ability and the ability to function properly *in vivo* as a vaccine.

The idea for using HspX as a vaccine target is tempting because it is up-regulated during hypoxia, can be found in the sera of latently infected patients [58] and is preferentially recognized by latently infected people [59]. At the present time, the only vaccine available for use in humans is the live, attenuated strain of Mycobacterium bovis Bacille-Calmette Guerin (BCG), which offers very limited protection against adult pulmonary TB [9]. Current vaccine strategies against tuberculosis utilizes two approaches: 1) producing a recombinant BCG (rBCG) that over-expresses immunogenic antigens to replace the current BCG, or 2) boosting already administered BCG with a new-generation subunit vaccine [9]. This "prime-boost" strategy involves the development and characterization of purified antigens from tuberculosis to be given as a booster to BCG in the form of a subunit vaccine, either prophylactically or also as a post-exposure

vaccine to target latent TB. Subunit vaccines are comprised of just antigens, usually a protein or a polyprotein delivered with an appropriate adjuvant formulation, to mount an antigen specific T cell response against TB [10].

It has been suggested that the current BCG vaccine cannot recognize and respond to HspX because when PBMCs from TB uninfected, BCG vaccinated patients were re-stimulated with HspX, they did not give a response when measured by ELISPOT [59]. Since BCG does not protect against reactivation from latency, mycobacterial antigens that can differentiate from latency and induce specific T cell responses to these antigens can be studied in new generation sub-unit vaccines.

Along with HspX as a latency antigen vaccine candidate, it has been suggested as a new diagnostic tool to more accurately diagnose latent TB from BCG vaccination. The most common way to diagnose tuberculosis is with a purified protein derivative (PPD) of secreted TB antigens injected under the skin [3]. If the patient has been exposed to TB, a delayed-type hypersensitivity reaction will occur under the skin and a measurable induration will form [7]. BCG vaccinated individuals will give a positive reaction to PPD, regardless of infection state, however PBMCs recovered from BCG vaccinated, TB negative patients did not respond to HspX when re-stimulated [28]. Although the precise role of HspX during TB infection has yet to be elucidated, it clearly is an important antigen that deserves further analysis as a vaccine and a diagnostic tool.

5   Concluding remarks

Biologically speaking, *Mycobacterium tuberculosis* is a very successful organism and pathogen. Few diseases can be traced back as far as TB in human history and indeed TB has developed a perfect niche in its host throughout time. Vaccine design is daunting and challenging, but with the combination of new technologies and antigen discovery we can begin to move forward. The only tuberculosis vaccine available to humans is BCG and its efficacy is clearly not enough, as is evident by the very high numbers of latent TB carriers throughout the world. New vaccine design against TB has therefore shifted to new technologies of antigen discovery, and applying these to subunit vaccine constructs, to initiate a specific T cell response.

We have presented here a subunit vaccine design based on the latency-associated antigen, HspX, expressed in *M tuberculosis.* This antigen has been linked to latency during tuberculosis and is recognized by LTB infected patients, thus it was chosen for further investigation as a vaccine to prevent latency. Initial studies in the mouse showed us that the native protein purified from *Mtb* was protective, but the recombinant protein expressed and purified from *E coli* was not protective. However, mice that were vaccinated with the rHspX-PD vaccine were protected (figure 2-3), supporting our hypothesis that HspX requires its binding partners to remain biologically active.

Our mouse protection data (chapter 2) reveals that the pull-down was protective in mice challenged against both strains, in addition to the native HspX, supporting our hypothesis. In contrast, our protection data from the guinea pig (chapter 3) suggested that HspX was not able to protect guinea pigs. This suggests that the establishment of tuberculosis disease in these animal models differs physiologically. Perhaps HspX is not being expressed in the lungs of guinea pigs, and we have these studies on-going. Regardless, the protection seen in the mouse is promising

and a repeat experiment in the guinea pig with an HspX mutant that retains virulence would be a more accurate way of truly measuring protection of HspX (and its binding partners) in the guinea pig.

Our data in chapter 5 reveals that our mutant strain, ΔHspX was severely attenuated, and that this loss of attenuation was likely not due to the absence of HspX alone. However, our pull-down vaccine was able to protect mice significantly against disease in the ΔHspX infected animals (figure 2-3), therefore characterizing HspX binding partners is a priority in our laboratory.

We have presented here a new vaccine design for TB, but unfortunately the production of a native antigen from tuberculosis as a vaccine is not practical. Recombinant protein technologies are a relatively simple way of purifying these types of products that would otherwise be difficult to achieve in high quantities. Since rHspX did not protect, this is an issue that needs to be resolved in terms of vaccine production. We have formulated another recombinant HspX where the protein could be potentially carrying native products from *Mtb*. This pull-down formulation was able to protect in the mouse model, potentially resolving this short-fall. With that being said, care should be taken when utilizing recombinant proteins that have unique functions in their native hosts. Additionally, care should be taken when making knock-out strains to ensure clean gene deletions. This is of particular importance to our study because the other genes in close proximity to HspX may have influences on each other [110].

Between the studies presented here and previously published reports about HspX, the 16kDa heat shock protein from *Mycobacterium tuberculosis* is able to protect mice from pulmonary TB, is a latency-associated antigen, and has potential as a diagnostic tool. Although HspX did not corroborate our mouse vaccine studies, we showed that there are differences in the protection levels of native versus recombinant antigens. The differences that we saw in protection between these two animal models suggest that *Mtb* may be established differently in the lungs of

these animals. Further, complete elucidation of what HspX is chaperoning will be required to fully characterize this antigen and its protective characteristics.

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