Identifying new biomarkers for the development of a more effective prime-boost vaccine strategy against Latent Tuberculosis: A case for ESAT-6 and HspX proteins as indicators of early-and-late stage infection

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**Introduction**

*Mycobacterium tuberculosis* (MTB), a gram-positive bacterium that causes tuberculosis, is one of the most successful and complex human pathogens in medical history. This is the result of the sophisticated interactions between this bacteria and the human immune system, as MTB has evolved several host-evasion mechanisms that allow it to persist in human tissues for extended periods. Robert Koch first discovered MTB in 1882 [1], and since then many scientists have studied the phenotypic and mechanistic properties of this pathogen in order to better understand disease progression and possible avenues for therapeutic interventions. However, despite intensive research, tuberculosis remains a serious public health emergency as critical gaps in our knowledge prevent the effective control and prevention of TB.

It is estimated that over 2 billion people, or roughly one-third the world’s population, is currently infected with latent tuberculosis, a state of decreased *M. tuberculosis* replication resulting in dormancy [2]. This latently infected population represents a huge potential reservoir for future disease outbreaks. With this global distribution of infection, there is no wonder that there is a staggering 8 million new tuberculosis cases every year (1 new case every 4 seconds) with approximately 2 million deaths per year (1 death every 10 seconds) [3]. As a reference point, around 5%-10% of infected individuals will develop active TB; half within the first few years after infection and half in later life as a result of reinfection or reactivation of an original infection [4]. However, most people (around 90%) who become infected with *M. tuberculosis* do not develop disease or demonstrate clinical symptoms over a lifetime. The co-pandemic with HIV/AIDS patients has caused the synergistic effects of the two with disheartening
outcomes for disease: HIV increases in the proportion of TB disease reactivation from 5%-10% in a lifetime to 5-10% per life-year [5].

In order to achieve the UN Millennium Development Goal of halving TB prevalence and incidence by 2015, with the eventual objective of eliminating TB worldwide by 2050, there must be optimization of existing diagnostic tools and antibiotics. But most importantly, investment in new therapeutic strategies with an emphasis on developing a novel vaccine against adult pulmonary TB is critical for the realization of any true progress against tuberculosis.

**Specific Aims**

Developing better vaccines is a critical component of any prevention and control strategy for TB. One of the most crucial objectives of this proposal is to seek more funding for the development and identification of new biomarkers that are preferentially expressed during specific stages of tuberculosis infection. More specifically, my research team wants to examine ESAT-6 and HspX as biomarkers for the development of a more efficacious prime-boost vaccine strategy, in which recombinant BCG over-expresses these antigens in an attempt to elicit a comprehensive immune response that contains M. tuberculosis and interrupts the transmission cycle. HspX is a small heat shock protein that is required for mycobacterial persistence within the macrophage and is dominantly expressed in the bacterial stationary phase and under reduced oxygen levels [13]. ESAT-6 is a protein that is preferentially expressed during the early stage of infection and is essential for M. tuberculosis to survive and spread in vivo [16]. The ultimate objective of our research is to eradicate and prevent disease for the lifetime of individuals infected with M. tuberculosis by characterizing the immunogenic potential of these biomarkers in
animal models such as mice and guinea pigs, with the intention to develop this vaccine for use in human clinical trials.

**Background**

Currently, the vaccine of choice to treat tuberculosis is BCG, which is an attenuated strain of *M. bovis*, a mycobacterium that infects cattle. Developed by French scientists Albert Calmette and Camille Guerin, the BCG vaccine became the most widely distributed vaccine to impede global tuberculosis progression with almost 3 billion doses administered since 1921 [6]. Approximately 115 million doses are given each year, providing almost 89% coverage of infants worldwide [6]. However, BCG is not used in the US because of the inability of the vaccine to prevent the establishment of latent TB or reactivation and progression to adult pulmonary TB. BCG has been extensively evaluated and demonstrated variable protective efficacies ranging from 0 to 85% in different field trials [7]. This issue of varying efficacy and the estimate that BCG prevents only 5% of all the potentially vaccine-preventable deaths due to tuberculosis [8] has caused increased interest in vaccine research. Typical vaccine approaches to generating immunity against *M. tuberculosis* have relied upon three main methods: live, attenuated or recombinant vaccines, DNA vaccines, or sub-unit vaccines.

Even with these promising new approaches, there is still a dire need to further characterize the intricate relationship between *M. tuberculosis* and the human immune system. The transmission cycle of *M. tuberculosis* starts with the aerosolization of infectious droplets containing bacilli from an individual with pulmonary TB, in which the bacteria are inhaled and taken up by resident alveolar macrophages. Although tuberculosis can manifest itself at any tissue site, the lung represents both the main port of
entry and an important site of disease manifestation [9]. After macrophage engulfment, this phagocyte begins to release cytokines such as IFN-γ, which induces a confined pro-inflammatory response that leads to the recruitment of other immune effector cells from blood vessels. Hence, a granuloma is formed; a well-organized structure that contains infected macrophages in the center, surrounded by CD4+ and CD8+ T cells. This represents the containment phase of the infection, during which there are no overt signs of disease and host transmission is inhibited [10].

*M. tuberculosis* is able to persist in this hostile environment of the granuloma, characterized by nutrient starvation and depleted oxygen, by using several immune evasion strategies such as arresting the phagosome at an early stage of maturation and preventing fusion with lysosomes [3]. In addition, *M. tuberculosis* can secrete decoy antigens that dampen the immune response of functional helper T cells [11]. This granuloma can persist for decades as *M. tuberculosis* lies in a state of non-replicating persistence [12], during which this pathogen differentially expresses certain genes. Hence, an effective balance is established between *M. tuberculosis* and the human immune system in which some immunosuppressive event, such as acquiring HIV/AIDS, perturbs this delicate balance in favor of the pathogen, leading to the breakdown of the granuloma causing viable infectious bacilli to be released into the airways, thus leading to the development of pulmonary tuberculosis [10]. The unique feature of tuberculosis of differential transcription ironically has provided many scientists with a new means of vaccine development: strategies aimed at these early and late stage antigens encoded by *M. tuberculosis* as potential vaccine candidates.
Since the sequencing of the *M. tuberculosis* genome, there is increasing interest in the identification of novel *M. tuberculosis* antigens that are immunogenic. Lin et al. studied human T-cell responses to DosR regulon encoded antigens of *M. tuberculosis* and observed the preferential recognition of latency antigens by patients with latent tuberculosis compared to patients with active TB disease. The authors’ found that BCG-vaccinated adults with exposure to *M. tuberculosis* showed increased levels of IFN-y to selected latency antigens, particularly HspX, whereas patients with *M. tuberculosis* (past or active) recognized early secreted antigens such as ESAT-6.

To corroborate this finding, Demissie et al. compared immune responses to HspX with responses to ESAT-6 in tuberculosis patients and healthy individuals with and without evidence of prior infection. They suggested that immune responses to ESAT-6 and HspX may be characteristic of different phases of *M. tuberculosis* infection, with the possibility for a therapeutic vaccine against TB as well as for differential immunodiagnostics that can track progression of disease by determining the ratio of responses to these biomarkers not used in the vaccine strategy, but are characteristic of tuberculosis infection. However, the most important study by Brooks et al. provided evidence that BCG fails to protect against tuberculosis due to waning efficacy as a result of increased age. They found that mice vaccinated with BCG exhibited a lack of protective immunity, whereas mice that received a booster immunization with an early stage biomarker 9 months and 15 months after inoculation with a recombinant BCG vaccine had a reduction in bacterial load and demonstrated improved resistance when challenged with *M. tuberculosis*. 
Taken together these studies provide poignant scientific evidence that ESAT-6 and HspX are highly immunogenic biomarkers that have proven efficacy in various animal models of tuberculosis. These findings establish the basis for my proposal of a prime-boost vaccine strategy using these biomarkers in a recombinant BCG vaccine that overexpresses these antigens in an attempt to disrupt the natural pathogenesis of *M. tuberculosis*.

**Experimental/Programmatic Design**

My research team decided to use ESAT-6 and HspX as part of the prime-boost strategy because of their aforementioned immunogenicity, they are prominent biomarkers that are excreted in large amounts in culture filtrates [4], and the fact that during the attenuation of BCG, the gene segments that encode these biomarkers were deleted, thus leading us to believe that they are important for *M. tuberculosis* pathogenesis and persistence [11]. We hypothesize that priming with a live, recombinant form of BCG with a follow up booster immunization with ESAT-6 and HspX will induce a robust immune response that will lead to the generation of long-lasting immunity against *M. tuberculosis*. To test this hypothesis, our research team seeks funding to implement the use of animal models, such as mice and guinea pigs, which accurately mimic the pathogenesis of *M. tuberculosis* in humans. This recombinant BCG vaccine contains a DNA plasmid encoding the regions for ESAT-6 and HspX, which will be over-expressed in vivo. In order to develop a vaccine for use in human clinical trials, the prime-boost strategy must demonstrate four indicators of efficacy:

1. The ability to elicit cell-mediated immunity.
Experiment: Take mice and guinea pigs and intradermally inject them with BCG or rBCG6X to induce a local inflammatory response that is consistent with the delayed-type hypersensitivity response, which is caused by antigen-specific effector T cells. If such a response occurs to a comparable reaction with BCG, then we can conclude that rBCG6X is an effective activator of cellular immunity.

*ii. The ability of rBCG6X to establish protective immunity.*

Experiment: Immunize mice and guinea pigs to prime with BCG or rBCG6X with ESAT-6:HspX booster several months later, and challenging them via the aerosol route with *M. tuberculosis*. Our goal is to focus on the long-term progression of disease by taking time points several months after immunization and assaying for the production of TH1-cytokines such as IFN-γ using ELISA testing. Then, we plan to perform immunohistochemistry in order to examine lung tissue for disease pathology, principally the formation of tubercles, or lung lesions, and also to quantify the amount of bacterial load in the spleen and lungs, as indicated by colonizing forming units (CFUs). Follow up experiment 1 by assaying for the ability of rBCG6X to protect against death attributed to *M. tuberculosis* infection after immunization by using death curves to track changes in mortality. Then, we plan to repeat these experiments to validate our results.

We hypothesize that the rBCG6X vaccine with ESAT-6:HspX booster strategy will result in optimum protective immunity by decreasing bacterial load and increasing the activation of B and T-cells as a result of enhanced cytokine production, when compared with wild-type and BCG vaccinated animals. In addition, we propose that rBCG6X immunized animals survive significantly longer than both other research conditions.
iii. Demonstrate safety of rBCG6X when administered.

Experiment: Immunize mice and guinea pigs with either vaccine and observe the impact on overall health due to vaccination alone. In addition, we plan to immunize different mice and guinea pigs and then infect them with microorganisms to assess whether basic immunological function is affected by rBCG6X administration in terms of clearing these microorganisms that usually do not cause disease in wild-type animals. We expect to see no adverse health effects due to the administration of the vaccines, thus demonstrating their safety in animal models with potential for use in human clinical trials.

iv. Demonstrate stability of the plasmid vector to continue expression of ESAT-6 and HspX.

Experiment: In vitro, culture *M. tuberculosis* that is transformed with DNA plasmid and assay for continued secretion of ESAT-6 and HspX in the media to determine level of expression over extended periods by performing an ELISA test. Also, do the same in vivo by vaccinating animal models with rBCG6X and then removing the lungs and spleen after euthanizing the mice and guinea pigs at different monthly time points post-vaccination. After culturing the cells from these organs, we plan to again assay for our biomarkers by performing the same ELISA test. We expect that our plasmid stably expresses our desired antigens at sufficient levels to provide protection against *M. tuberculosis* infection.

After performing these series of experiments, we are confident that these four indicators will be clearly established by our rBCG6X prime vaccine with ESAT-6:HspX
booster strategy in the animal models. Hence, we can move forward to testing in higher-primate species, such as Macques, with the eventual objective of progressing to phase I and phase II clinical trials in humans. The rationale behind our unique prime-boost strategy is to prime with rBCG6X during infancy, then boost with the ESAT-6:HspX fusion throughout life every ten years in order to optimize the immune response to this pathogen by keeping immunity above disease thresholds. The creation of this fusion plasmid is to allow multiple epitopes and diversified activation of B and T cell subsets, thus leading to long-lasting immunological memory involving both cell-mediated and humoral immune responses in a multifaceted immune attack on the pathogen to not only prevent disease, but potentially infection.

**Conclusion**

Recent advances in the clinical testing of new vaccines show much promise and should be regarded as a sign of significant progress. For example, in Asia and sub-Saharan Africa, regions with the greatest disease burden, tuberculosis vaccines can reduce deaths by as much as 62%, saving millions of lives [14]. Furthermore, tuberculosis vaccine development represents a significant opportunity for industry investment with potential global markets of US $450 million to nearly $1 billion [14]. However, tuberculosis vaccine development is an arduous task that requires financial commitment to improving the current state of infectious disease around the world. The process should be dynamic in that clinical testing and basic science research should reinforce the improvement in identifying more effective biomarkers and directing research towards these areas. An effective vaccine must provide multiple epitopes in
order to account for the genetically heterogeneous population afflicted with disease, thus ensuring broad coverage [15].

Most importantly, it is paramount that equity issues be addressed so that our prime-boost strategy can be made affordable to vulnerable populations, since 90% of the estimated deaths from tuberculosis and 95% of the estimated eight million new cases of tuberculosis each year occur in developing countries [6]. Despite the stringent regulatory policies for clinical trials and the enormous investment of financial resources and intellectual capital, vaccine development remains a necessary and rational alternative to the prevention and control of tuberculosis.
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Works Referenced and Consulted for Background and Experimental Design Section

