

Role of Isoprenoid Biosynthesis in Listeria-based Tuberculosis Vaccine Efficacy

BY

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THESIS

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To Dominic Conti, Fiori Conti,
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Without whom none of this would
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Contribution of Authors

Chapter 1 is a literature review that places my dissertation question in the context of the larger field and highlights the significance of my research question. Chapter 2 represents the materials and methods used to generate the data for these experiments. Sections of the materials and methods have been published in manuscripts that I am first author or co-first author, including **J. Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing *Listeria* mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. *J Leukoc Biol.* 2014 Dec;96(6):957-67 and **J. Frencher**, B. Ryan-Passeyeur, D. Huang, R. Wang, P. McMullen, N. Letvin, W. Collins, N. Freitag, M. Malkovsky, C. Chen, L. Shen, Z. Chen "SHIV Antigen Immunization Alters Patterns of Immune Responses to SHIV/malaria Co-infection and Protects against Life-threatening SHIV-related Malaria." *J Infect Dis.* 2013 Jul 15;208(2):260-70 B. Ryan-Passeyeur, and **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*." *J. Immunol* 2012 Aug 1;189(3):1285-93. Dr. Bridget Ryan-Passeyeur contributed to the writing of B. Ryan-Passeyeur, and **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*, while I produced figures 3-6. Dr. Zheng Wei Chen revised all of the above manuscripts in preparation for their submission. Dr. Crystal Chen, Dr. Dan Huang, and Dr. Ri Cheng Wang assisted in the preparation of all gross pathologic samples for processing and analysis, and thus contributed to the generation of figures 16 and 17. Arwa Qaqish assisted in the daily husbandry and clinical monitoring of the rhesus macaques. Chapter 3 represents a published manuscript (B. Ryan-Passeyeur, **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*." *J. Immunol* 2012 Aug 1;189(3):1285-93.) for which I was the co-first author. I generated figures 2-6 and assisted in the majority of the manuscript. Chapter 4 represents a combination of figures from a previously published work for which I am first author, (**J. Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing *Listeria* mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. *J Leukoc Biol.* 2014 Dec;96(6):957-67.) and work that is currently unpublished and part of a manuscript that is currently submitted for publication (**J. Frencher**, A. Qaqish, L. Shen, Z. Zhang, D. Huang, C. Chen, R. Wang, Z. Chen. "Combined peptide Ag-phosphoantigen immunization of rhesus macaques by respiratory route induced detectable protection against tuberculosis" (submitted). Chapter 5 represents a set of unpublished figures that have been put together for a manuscript that is in preparation for submission for which I am the first author (**J. Frencher**, A. Qaqish, L. Shen, Z. Zhang, D. Huang, C. Chen, R. Wang, A. Rizzo, Z. Chen. "Repeated phosphoantigen exposure reduces efficacy of *Listeria*-based T-cell targeting vaccine" (manuscript in preparation). The future directions and significance of this work to the field are discussed in chapter 6.

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LIST OF ABBREVIATIONS

<i>M.tb.</i>	<i>Mycobacterium tuberculosis</i>
CFU	Colony Forming Units
HIV	Human Immunodeficiency Virus
TNF	Tumor Necrosis Factor
HAART	Highly Active Antiretroviral Therapy
TB	Tuberculosis
MDR	Multi-Drug Resistant
BCG	<i>Mycobacterium bovis</i> -Bacillus Calmette Guerin
WHO	World Health Organization
CD	Cluster of Differentiation
MHC	Major Histocompatibility Complex
Th1	T-helper 1
IFN γ	Interferon- γ
IL-2	Interleukin-2
PPD	Purified-Protein Derivative
ESAT-6	Early Secreted Antigen of Tuberculosis 6
Ag85A	Antigen 85A
Ag85B	Antigen 85B
Ag85C	Antigen 85C
MEP	2-C-methyl-D-erythritol 4-phosphate
DOXP	1-deoxy-D-xylulose 5-phosphate
TCR	T cell receptor

LIST OF ABBREVIATIONS (Continued)

HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP	Isopentenyl pyrophosphate
Lm	Listeria monocytogenes
rLm	recombinant Listeria monocytogenes
LLO	Listeriolysin O
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
BHI	Brain Heart Infusion
RBC	Red Blood Cell
OD	Optical Density
RPMI	Roswell Park Memorial Institute media
R10	Roswell Park Memorial Institute media containing 10% Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
PE	phycoerythrin
Cy7	Cyanine 7
APC	Allophycocyanin
PB	Pacific Blue
AF700	Alexafluor700
IACUC	Institution of Animal Care and Use Committee

LIST OF ABBREVIATIONS (Continued)

IBC	Institutional Biosafety Committee
SIV	Simian Immunodeficiency Virus
mg	milligram
kg	kilogram
HCl	Hydrochloric acid
CBC	Complete Blood Count
BAL	Bronchoalveolar Lavage
μg	microgram
ml	milliliter
CO ₂	Carbon Dioxide
MOI	Multiplicity of Infection
pg	picogram
ng	nanogram
U	Units
rh-IL-2	Recombinant Human IL-2
IV	intravenous
IM	intramuscular
LN	Lymph node
Tdap	Tetanus-Diphtheria and acellular Pertussis Vaccine

SUMMARY

Tuberculosis is among the leading infectious causes of death worldwide. Antibiotics used to treat the causative agent, *Mycobacterium tuberculosis*, are becoming less effective as the bacterium develops resistance mutations to the 4-drug first-line treatment regimen. The only vaccine used for this pathogen worldwide shows variable levels of protection and this immunity progressively declines with age, showing no demonstrable efficacy in adults. Our studies have focused on the development of alternative vaccine strategies to induce protective immunity against *Mycobacterim tuberculosis* strains. The studies within the Chen lab focus on the use of attenuated strains of the gastrointestinal pathogen *Listeria monocytogenes* to deliver immunogenic proteins to the cytoplasm of antigen presenting cells, allowing for the development of strong Helper and Killer T cell responses. The current studies focus on the examination of the T cell responses generated by respiratory administration of this *Listeria*-based Tuberculosis Vaccine, and the potential protective effect it generates against primary progressive Tuberculosis infection. In the studies describe here, we examine the protective effect of respiratory administration of a *Listeria*-based Tuberculosis vaccine, and demonstrate that 1 intratracheal dose provides significant improvement of bacterial burden and pulmonary pathology following respiratory *Mycobacterium tuberculosis* challenge, while 3 doses of the same vaccine provide minimal protection against Tuberculosis-mediated pathology. Examination of the T cell responses generated by the vaccine demonstrated that CD4 and CD8 T cells specific for select *Mycobacterim tuberculosis* proteins accumulate in both the blood and airways following vaccination. In addition, a subset of T cells specific for isoprenoid synthesis pathway intermediates accumulate in massive numbers in the blood and airways after a single administration of the vaccine, but the number and activity of these cells declines following

repeated administration of the vaccine. In addition, these isoprenoid-sensitive T cells demonstrate markers of exhaustion and apoptosis following repeated stimulation with the vaccine, suggesting that repeated high dose vaccination could be inducing activation-induced T cell exhaustion and cell death. Using molecular biology techniques, we decrease the ability of our vaccine to generate isoprenoid intermediates that are potent stimulators of these cells. The *Listeria*-based vaccine that only produces low potency isoprenoids shows a reduced ability to stimulate isoprenoid-sensitive T cells, decreased expression of exhaustion markers followed repeated respiratory administration, and a restored ability to protect against pulmonary Tuberculosis. Collectively, these data support the hypothesis that *Listeria*-based vaccines given in repeated high doses induce exhaustion and cell death of isoprenoid-sensitive T cells. As these T cells have been demonstrated to be important components of the early response to Tuberculosis and other isoprenoid-producing bacterial infections, this may provide a mechanism through which repeated bacterial infection or repeated BCG infection result in poorer outcomes of Tuberculosis infection.

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis

1.1.1. Tuberculosis Epidemiology and Pathogenesis

Tuberculosis is the leading infectious cause of death worldwide, killing an estimated 1.4 million people in 2011¹. Exposure to the causative agent, *Mycobacterium tuberculosis* most commonly occurs through inhalation of aerosolized bacilli². Although the minimum dose of *Mycobacterium tuberculosis* bacilli required to cause infection is not known, research in mice and macaques suggest that inhalation of as few as 10 live bacilli may be sufficient to induce pulmonary tuberculosis³. Indeed, previous studies examining rhesus macaques inoculated intrabronchially with 10 colony forming units (CFU) of a clinical isolate of *Mycobacterium tuberculosis* resulted in significant progressive pulmonary disease in these animals⁴. Clinically patients with pulmonary tuberculosis will develop recurrent fevers, night sweats, weight loss and a productive cough that will contain acid-fast staining bacilli. Dissemination of the bacillus following pulmonary infection can occur, resulting in colonization of any organ².

The clinical outcome following exposure to *Mycobacterium tuberculosis* can vary considerably depending on the strain of *Mycobacterium tuberculosis* and host factors^{3,5-7}. It is currently believed that a low percentage of people initially exposed present with progressive primary tuberculosis. It is generally accepted that the vast majority of individuals exposed to

Mycobacterium tuberculosis bacilli develop a clinically latent infection⁸. These patients are infected, develop strong adaptive immune responses to protein antigens that are capable of suppressing replication of the bacillus and show no clinical signs of infection, but are incapable of completely clearing the organism⁹. Because of the strong antigen-specific responses generated, clinically asymptomatic tuberculosis carriers are commonly screened for using the tuberculin-skin test, or the more recently approved QuantiFERON blood test¹⁰. HIV infection, administration of corticosteroids, chemotherapy, or Tumor Necrosis Factor (TNF) inhibitors causes immune suppression and reactivation of *Mycobacterium tuberculosis* resulting in a progressive pulmonary disease, dissemination, and death without treatment¹¹⁻¹³. The increasing prevalence of HIV in *Mycobacterium tuberculosis* endemic regions, increased use of corticosteroids and TNF inhibitors in the treatment of allergy and autoimmunity, and increased resistance of *Mycobacterium tuberculosis* to drugs in southeast Asia and Sub-Saharan Africa suggests that new drugs and vaccines will be necessary for the treatment and possible eradication of *Mycobacterium tuberculosis*.

1.1.1. Current Anti-tubercular Agents

Due to the slow growth patterns of *Mycobacterium tuberculosis*, treatment for Tuberculosis infection requires an intensive administration of four drug cocktail for at least 8 weeks, followed by continuation of 2 drugs for up to 6 months^{2,14}. If sputum cultures remain positive at the 8 week period, drug treatment may have to continue for up to 9 months¹⁴. The regimen for most patients with drug-resistant Tuberculosis lasts 20 months. Many of the patients in Sub-Saharan Africa and Southeast Asia infected with *Mycobacterium tuberculosis* are concurrently

infected with HIV and require the highly active anti-retroviral therapeutic (HAART) drug cocktail to maintain immunocompetence¹⁵.

Due to the long length of treatment, side-effects and toxicities of the multi-drug cocktails, and drug/drug interactions (especially in cases of HIV-TB co-infection), adherence to drug treatment remains a problem in managing the disease¹⁶. Up to half of all patients with TB do not complete treatment¹⁷. This incomplete treatment of Tuberculosis infection can result in the production of multi-drug resistant organisms. Indeed, isolates of multi-drug resistant Tuberculosis (MDR-TB; defined as demonstrating resistance to at least 2 of the 4 drugs primarily used to treat M.tb.) has been isolated from patients previously diagnosed with drug-sensitive Tuberculosis who discontinued therapy, and MDR-TB isolates are more commonly associated with patients containing physician reports of poor adherence to treatment in their chart¹⁸. The number of MDR-TB cases continues to rise, reaching 60,000 notified cases worldwide in 2011². Estimates suggest that more than 5 times that amount, or over 300,000 cases worldwide per year, are multi-drug resistant¹⁹. Thus, there is an increasing need for new drug treatments and or immunotherapeutic regimens that will decrease the length of treatment time for patients, and have fewer drug interactions with currently prescribed medications.

The best hope for decreasing the burden of tuberculosis would be the creation of an effective vaccine. The current vaccine for *Mycobacterium tuberculosis* is an attenuated form of a related slow-growing mycobacterial species, *Mycobacterium bovis*-Calmette Guerin (BCG). Although it is not used in the United States, the BCG vaccine is administered in the majority of countries and has been used to vaccinate against Tuberculosis since 1921²⁰. The BCG vaccine has shown

promising efficacy in preventing severe disseminated tuberculosis in children, but has not proven to be effective in decreasing the incidence or complication of tuberculosis in adults. In the United States, BCG has been an effective treatment for a number of cancers and multiple sclerosis^{21,22}. Like *Mycobacterium tuberculosis*, *Mycobacterium bovis* is a persistent pathogen, and incomplete clearance of BCG and reactivation is possible under certain circumstances. Indeed, bladder cancer patients who are immunocompromised are at increased risk for BCG dissemination and granulomatous pathology following intravesicular BCG administration for bladder cancer treatment²³. Similarly, the WHO no longer advocates the administration of the BCG vaccine to infants with HIV even in high Tuberculosis endemic areas due to the high risk of disseminated BCG infection in these children²⁴. As a result, one of the most widely available tools to fight the spread of tuberculosis is becoming less widely used due to the HIV epidemic in these tuberculosis endemic regions. A vaccine with a decreased risk of reactivation would assist in decreasing overall tuberculosis burden in these areas.

1.1 Immune correlates of protection against *Mycobacterium tuberculosis*

1.2.1. CD4 T cells

Many studies have established the key role CD4 T cells play in host protection against *Mycobacterium tuberculosis*²⁵. Tuberculosis infection of mice deficient for CD4 or MHC class II molecules that present antigens to CD4 T cells result in increased bacterial burdens and increased susceptibility to intravenous tuberculosis infection, establishing that these cells contribute to keeping bacterial burden following *Mycobacterium tuberculosis* exposure low²⁶. Decreases in CD4 T cell number due to HIV infection result in reactivation of *Mycobacterium*

*tuberculosis*¹². Studies in macaques using CD4 depleting antibodies have demonstrated that CD4 T cells play an important role in preventing severe pulmonary pathology and dissemination throughout the body²⁷. CD4 T cells mediate protective effects through the production of the Th1 cytokines IFN γ , TNF α , and IL-2²⁸. Pharmaceutical agents that inhibit TNF α have shown to cause reactivation of *M.tb.* in macaques and humans^{29,30}. Adoptive transfer of T cells in mouse models of Tuberculosis have shown that IFN γ production specifically from CD4 T cells are necessary for minimizing bacterial burdens following aerosolized exposure to *M.tb*³¹. While initial studies examining immune responses following *Mycobacterium tuberculosis* infection demonstrated a role of each of these Th1 cytokines individually, the development of flow cytometer capable of using multiple lasers and detecting cells stained with many antibodies has allowed for the examination of T cell production of multiple cytokines following stimulation^{32,33}. More recent studies examining these multifunctional or polyfunctional CD4 T cells have shown strong correlations between the percentage or number of CD4 T cells producing 2 or 3 Th1 cytokines and low bacterial burdens³⁴. Indeed, *in vitro* studies have shown a synergistic effect of IFN γ used in combination with TNF α to inhibit the intracellular growth of *Mycobacterium tuberculosis*.

1.2.1. CD8 T cells

In addition to CD4 T cell-mediated protection against *M.tb.*, antigen-specific CD8 T cells have also shown to be responsible for mediating some protection against Tuberculosis^{35,36}. CD8 T cells accumulate in the granulomas of infected individuals³⁷. Genetic deletion of CD8 or MHC class I molecules in mice or mice have established that T cell recognition of antigens through

MHC class I presentation is necessary for control of *M.tb.* infection³⁸. CD8 depleting antibodies have been used in macaques vaccinated with BCG to demonstrate that BCG vaccine-mediated protection against *Mycobacterium tuberculosis* involves CD8 T cell responses to invading bacilli³⁹. CD8 T cells are commonly characterized by their strong cytolytic activity, mediated through the production of perforin, granzyme A, and granulysin⁴⁰⁻⁴². Cytolytic granulysin has been shown to directly cause lysis of extracellular tubercle bacilli, but is incapable of entering intact cells. Perforin punches holes in the cell membrane of target cells, allowing for the entry of cytolytic granulysin to enter cells and kill intracellular *M.tb.* In addition to their cytolytic activity, CD8 T cells from PPD+ individuals secrete large amounts of IFN γ and TNF α in response to mycobacterium re-exposure, suggesting that they could contribute to activation of macrophages and inhibition of intracellular growth of *M.tb.* Recent studies have demonstrated that IFN γ -producing CD4 T cells augment *M.tb.*-specific CD8 T cell degranulation and cytokine function following antigen re-exposure³¹. Recombinant forms of the BCG vaccine that express the listeria hemolysin Listeriolysin O or the *Clostridium perfringens* toxin perfringolysin O demonstrated increased immunogenicity and protection against *M.tb.* challenge^{43,44}. These new vaccines allow BCG to escape the endosome and produce antigens in the cytoplasm, allowing for increased cytoplasmic antigen processing and presentation to CD8 T cells⁴⁵. Vaccination strategies combining activation of CD4 and CD8 T cells may provide optimal synergy to minimize spread of *M.tb.*

1.2.3. Early Secreted Antigen of Tuberculosis 6 (ESAT-6)

Because a large percentage of the world population has been vaccinated with the BCG vaccine, a number of investigators have sought approaches to administer a booster vaccine to augment the vaccine-induced *M.tb.*-specific responses generated by the BCG vaccine in order to generate a vaccine regimen that would induce protection against Tuberculosis in children and adults^{46,47}. Many of the early studies examined cultured filtrates of *Mycobacterium tuberculosis* lysates in order to find antigens that are highly immunogenic⁴⁸. Among the proteins discovered, early secreted antigen of tuberculosis 6 (ESAT-6) has been shown to induce high levels of IFN γ secretion from T cells derived from *M.tb.*-infected mice⁴⁸. ESAT-6 is produced by clinical isolates of *M.tb.* as well as virulent strains of *Mycobacterium bovis*, but is not secreted by a number of strains of the BCG vaccine due to a genomic deletion that eliminates the gene encoding ESAT-6⁴⁹. The attenuated laboratory strain of *Mycobacterium tuberculosis*, H37Ra, is also deficient in ESAT-6 secretion because of a defect in its regulator protein, *phoP*^{50,51}. Transfection of wildtype *phoP* in to the H37Ra strain of *Mycobacterium tuberculosis* results in a recovery of virulence and secretion of ESAT-6, suggesting that ESAT-6 may contribute to virulence⁵⁰. Indeed, *in vitro* studies in amoeba have demonstrated that ESAT-6 mediates lysis and apoptosis of lung epithelial cells and macrophages and permits cell-to-cell spread of *M.tb.* without lysing infected cells through eliciting formation of cellular ejectosomes⁵².

Because ESAT-6 elicits strong T cell responses in mice and is not present in the BCG vaccine, it has been viewed as a strong candidate for boosting the BCG-vaccine induced response.

Recombinant forms of the BCG vaccine have been created that induce ESAT-6 production and

secretion by the vaccine vector. Additionally, subunit vaccines based on ESAT-6 have conferred protection against *M.tb.* challenge in mice. These data suggest that stimulation of ESAT-6-specific T cells could play a strong role in inducing anti-tuberculosis immunity.

1.2.4. Antigen 85B (Ag85B)

Proteins actively secreted by *Mycobacterium tuberculosis* have been examined as potential candidates of immunogens that could stimulate CD4 and CD8 T cells to respond to cells infected by this intracellular organism. Among the secreted proteins produced by *M.tb.* the proteins of the Antigen 85 complex are the most common proteins in culture supernatants of *M.tb.* The antigen 85 complex proteins are encoded by 3 genes and produce 3 products, Ag85A, Ag85B, and Ag85C. These proteins have been shown to be highly immunogenic in natural *M.tb.* infection as well as many experimental models. Ag85B immunization in mice has been shown to induce partial protection against intravenous *M.tb.* challenge and significant protection in a guinea pig model of aerosolized *M.tb.* exposure. Humans and animals produce strong T cell responses following exposure to Ag85B. Studies using recombinant forms of BCG that express and secrete the *M.tb.* gene for Ag85B has shown superior protection compared to the parental BCG strain, indicating that Ag85B is capable of augmenting immune responses induced by the BCG vaccine. Because the Ag85B encoded by BCG is highly homologous to the Ag85B expressed by *M.tb.*, differing in only 2 amino acids, it is hypothesized that the increased protection seen in recombinant BCG expressing *M.tb.* Ag85B is due to the heightened expression of Ag85B in the recombinant BCG strain. Collectively, these studies suggest that delivery and antigen presentation of Ag85B to the host would strengthen immune protection against *M.tb.*

1.2.5. $\gamma\delta$ T cells

In addition to CD4 and CD8 T cells, $\gamma\delta$ T cells have increasingly been considered a major player in playing a role in the early phase of protection against *Mycobacterium tuberculosis*. $\gamma\delta$ T cells are considered an innate lymphocyte, capable of rapid activation and cytokine production following exposure to the antigens they recognize. $\gamma\delta$ T cells increase in large number following *Mycobacterium tuberculosis* infection and have been identified in the granulomas of infected individuals through immunohistochemistry. Figure 1 lists many of the described anti-mycobacterial activities that $\gamma\delta$ T cells possess. Following activation, the majority of $\gamma\delta$ T cells secrete Th1-related cytokines such as IFN γ and TNF α . Some subsets of $\gamma\delta$ T cells have been shown to produce perforin, granzymes, and granulysin similar to CD8 T cells, and kill or inhibit the intracellular growth of *Mycobacterium tuberculosis*. Although these cells are similar to innate immune cells in their ability to recognize and respond to patterns, some $\gamma\delta$ T cells are capable of developing a memory response, responding to antigen faster and at a higher magnitude following the second response than the first. Vaccines that induce and maintain strong CD4, CD8 and $\gamma\delta$ T cell responses may be strong candidates against *Mycobacterium tuberculosis* challenge.

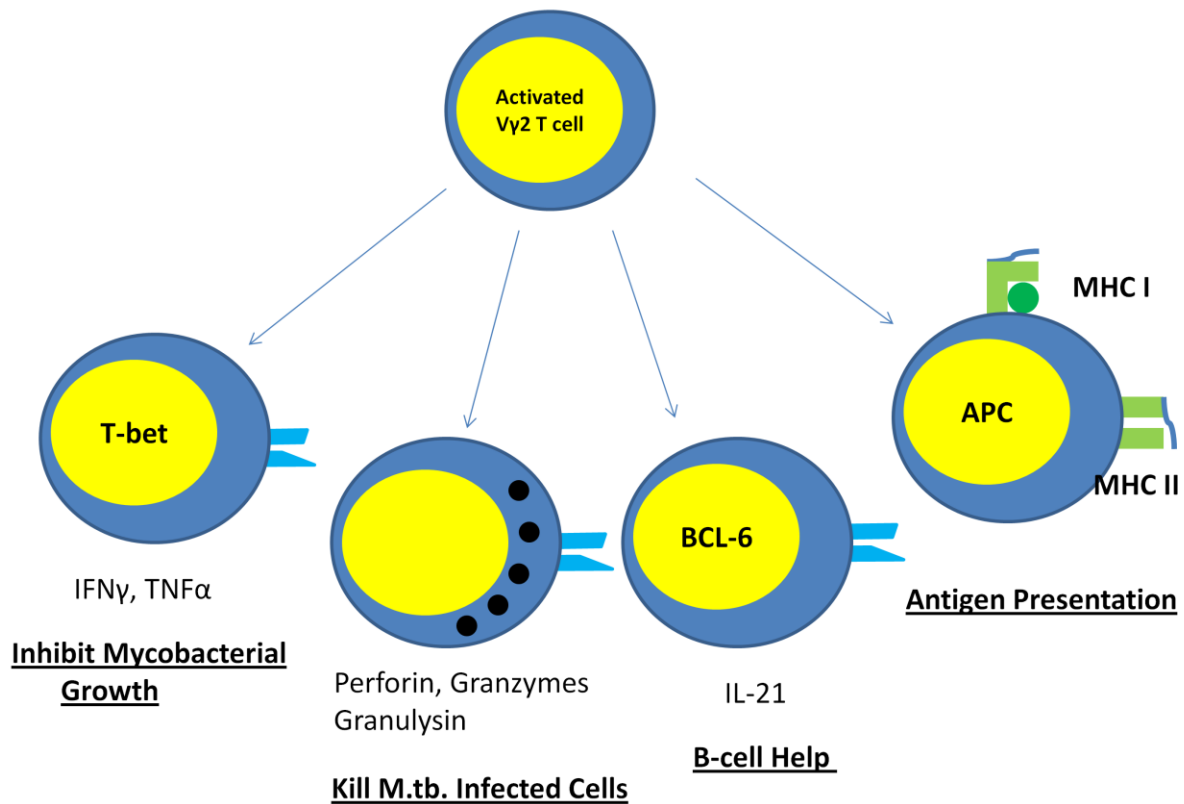


Figure 1. **Anti-microbial Functions of Vγ2Vδ2 T cells**

Summary of the functional activities described for Vγ2Vδ2 T cells that may contribute to elimination of *Mycobacterium tuberculosis* following *in vivo* infection. Vγ2Vδ2 T cell secretion of Th1 and Tfh cytokines cytolytic granule production has been described both *in vitro* and *in vivo*, while antigen presentation activity of Vγ2Vδ2 T cells has only been described through *in vitro* experimentation.

1.3. Isoprenoid Biosynthesis and Host Response

1.3.1. Isoprenoid Biosynthesis

Isoprenoid biosynthesis is essential for life in all known archaea, eukarya, and bacteria⁵³. There are 2 main pathways that can be used to generate isoprenoids. Many Bacteria and algae, including *Mycobacterium tuberculosis*, use the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway for isoprenoid production, while most eukaryotes use the mevalonate pathway of isoprenoid biosynthesis. Isoprenoids make up the precursors of molecules necessary for protein prenylation, cell membrane maintenance, steroid hormone synthesis, protein anchoring, and N-glycosylation.

1.3.2. V γ 2V δ 2 T cell Response to Isoprenoids

Studies using human and non-human primate white blood cells have shown that a subset of lymphocytes, V γ 2V δ 2 T cells are capable of responding to isoprenoids^{54,55}. These cells are exclusively found in human and non-human primates and comprise 65-90% of total circulating human $\gamma\delta$ T cells⁵⁶. Using an oligoclonal T cell receptor (TCR) repertoire derived from the γ 2 and δ 2 TCR loci, these cells detect MEP pathway intermediate (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) or mevalonate pathway intermediate isopentenyl pyrophosphate (IPP) presented by some unknown antigen presenting molecule (see Figure 2). While high concentrations of IPP are required to activate V γ 2V δ 2 T cells, HMBPP is a ~1000-fold more potent than IPP for *in vitro* activation of V γ 2V δ 2 T cells through detection of MEP pathway HMBPP, V γ 2V δ 2 T cells are capable of responding to a variety of bacterial pathogens⁵⁶. Figure 3

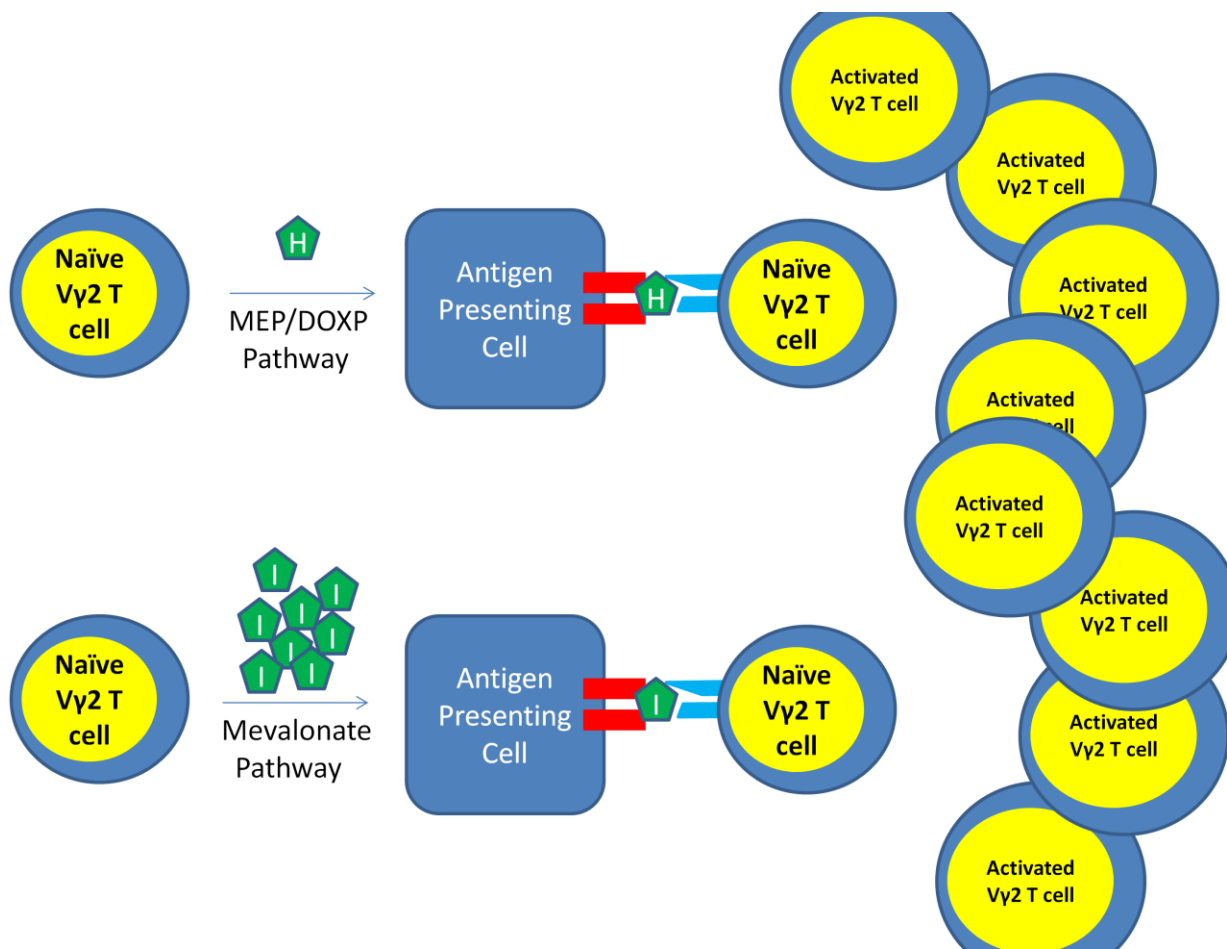


Figure 2. **Diagram of activation of Vγ2Vδ2 T cells by phosphoantigens**

Natural Phosphoantigens HMBPP (H), derived from the MEP/DOXP pathway, or IPP (I), an intermediate common to the MEP/DOXP pathway and the mevalonate pathway, are both sufficient for activation of naïve Vγ2Vδ2 T cells *in vitro*. Administration of low doses of purified H or high doses of purified I result in activation and proliferation of Vγ2Vδ2 T cells. Vγ2Vδ2 T cell activation occurs through TCR-mediated recognition of the phosphoantigen in the context of an unknown antigen presenting molecule. TCR of the Vγ2Vδ2 T cell is shown in blue, and unknown antigen presenting molecule on the antigen presenting cell is shown in red.

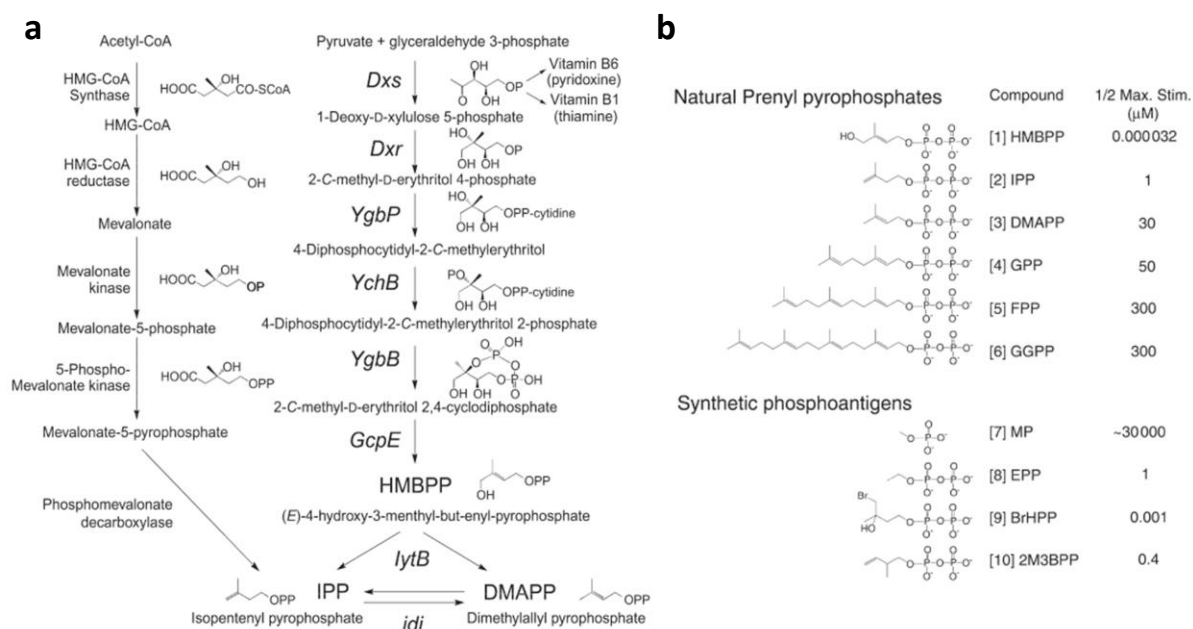


Figure 3. **Potency of Isoprenoid Biosynthesis pathways in Vy2Vδ2 T cell induction**

A) Diagram of mevalonate and MEP pathways of isoprenoid biosynthesis. Mevalonate pathway of isoprenoid biosynthesis predominantly utilized by eukaryotes to generate IPP, while the MEP pathway of isoprenoid biosynthesis is used by a number of Bacteria species and protozoans to generate isoprenoids. B) Diagram of potency of natural and synthetic isoprenoids in their ability to stimulate activation of Vy2Vδ2 T cells *in vivo*

lists the relative potency of natural and synthetic antigens that have been used *in vitro* to stimulate V γ 2V δ 2 T cells, demonstrating that the MEP pathway intermediate HMBPP is one of the most potent stimulators of phosphoantigen-responsive T cells. In addition to bacteria-mediated phosphoantigen stimulation, host cell infection with viruses and oncogenic transformation appears to activate V γ 2V δ 2 T cells through the upregulation of mevalonate pathway intermediates⁵⁷. Indeed, inhibition of the mevalonate pathway upstream of IPP production through HMG-CoA Reductase inhibitors results in decreased response of V γ 2V δ 2 T cells to tumors or influenza virus infected cells⁵⁸.

In vitro activated V γ 2V δ 2 T cells can produce Th1 cytokines IFN γ and TNF α , lyse infected cells or tumors through release of cytolytic effectors and inhibit the intracellular growth of bacteria⁵⁹. Some of these *in vitro* findings have been replicated *in vivo* by administration of IL-2 plus HMBPP or equivalents^{60,61}. Infections of humans and nonhuman primates with HMBPP-producing bacteria and parasites have been shown to induce *in vivo* activation and expansion of V γ 2V δ 2 T cells^{56,57}. Activated V γ 2V δ 2 T cells can traffic to and accumulate in the pulmonary compartment and intestinal mucosal interface^{60,62}. Following immune clearance of pathogens, some V γ 2V δ 2 T cells can express central and effector memory phenotypes similar to those seen in CD8+ $\alpha\beta$ T cells, suggesting an adaptive immune response of these phosphoantigen-specific $\gamma\delta$ T cells^{62,63}. Consistent with these memory phenotypes, robust, accelerated recall-like expansion and rapid production of anti-microbial cytokines can be seen after *in vivo* re-exposure to HMBPP-producing pathogens such as Mtb, BCG or *Listeria monocytogenes*^{62,64}.

Virtually, rapid recall-like expansion of V γ 2V δ 2 T cells after Mtb challenge of BCG-vaccinated macaques coincides with the vaccine-induced protection against severe form of tuberculosis.

1.4. *Listeria monocytogenes*

1.4.1. *Listeria monocytogenes*-based immunogen delivery

Listeria monocytogenes is a bacterium that has been investigated as a possible vaccine-delivery vector for over 20 years⁶⁵⁻⁶⁸. A food-borne pathogen most commonly associated with gastroenteritis in the immunocompetent, sepsis and abortion in pregnant women, and meningitis in infants and the immunocompromised, *Listeria monocytogenes* has developed efficient mechanisms to invade and infect phagocytes as well as many other cell types^{69,70}. Following endocytosis by the host cell, *Listeria monocytogenes* can use phospholipases and its secreted hemolysin, Listeriolysin O (LLO) to escape the phagosome and survive in the cytoplasm⁷¹. While in the cytoplasm, *Listeria* can travel throughout the cell and even invade neighboring cells using *actA*-mediated actin polymerization^{72,73}.

Extensive study has been performed on the mechanisms of virulence of *Listeria* using genetic manipulation of the organism⁷⁰. Deletion of the *actA* gene from laboratory strains of *Listeria monocytogenes* demonstrated that the pathogen is greatly attenuated in the absence of its ability to polymerize actin, suggesting this attenuated bacterial organism could be used as a means to deliver foreign proteins or DNA to the cytoplasm of cells⁶⁵. Indeed, various investigators have used forms of *Listeria* deficient for actin polymerization, alanine metabolism,

or *Listeria* capable of rapid autolysis as a delivery system for foreign proteins, DNA or RNA in various mouse models⁷⁴⁻⁷⁶.

Because there are many tools for the modification of *Listeria monocytogenes*, there has been increased interest in its use for vaccine development^{45,66,77}. Attenuated forms of *Listeria monocytogenes* that produces immunogens involved in cancer are being tested in clinical trials for a variety of cancers, including prostate, breast, cervical cancer, mesothelioma and glioblastoma⁷⁸⁻⁸². Many of these vaccines have been administered through intravenous routes to cancer patients and were safely tolerated in patients⁷⁹. As *Listeria* is a non-persistent pathogen that can efficiently be cleared through antibiotic treatment, individuals who demonstrate poor vaccine clearance can be treated with antibiotics in order to minimize the possibility of vaccine-induced complications. In addition, *Listeria*-based vaccines are in development for parasite infections such as malaria and a variety of infectious diseases⁷⁸.

Listeria-based vaccine vectors allow for immunogen delivery to the cytoplasm of host cells, providing for robust antigen presentation to CD8 T cells^{83,84}. Genetic modifications to *Listeria* transcription factor *prfA* that allow constitutive activation of the transcription factor allow for dramatically increased production of proteins that are under the regulation of this transcription factor⁸⁵. Immunogens placed under the Listeriolysin O promoter demonstrate significantly increased production of the immunogen and stronger immune responses generated^{76,85}. A *Listeria*-based vaccine capable of autolysis expressing *Mycobacterium tuberculosis* antigens demonstrated similar vaccine efficacy as the BCG vaccine in a mouse model of Tuberculosis infection⁷⁵. Because *Listeria*-based vaccines will stimulate both CD4 and CD8 T cell responses

and are readily controllable with antibiotic treatment, this vaccine delivery system may serve as a safer alternative to BCG vaccination in immunocompromised patients.

1.4.2. Isoprenoid Synthesis in *Listeria monocytogenes*

While most microbes contain either the MEP/DOXP or mevalonate pathway of isoprenoid synthesis, *Listeria monocytogenes* is unique in that it contains functional enzymes for both the MEP and mevalonate pathways⁸⁶. Because of the dual pathways of isoprenoid synthesis in *Listeria*, bacilli genetically modified to lack MEP pathway genes remain viable, allowing for the examination of host responses to *Listeria* lacking the ability to produce the potent V γ 2V δ 2 T cell intermediate, HMBPP⁸⁷. Because V γ 2V δ 2 T cells have been shown to provide an early source of Th1 cytokines, produce cytolytic enzymes and kill infected cells, and present antigens to CD4 and CD8 T cells, it is important to determine if the isoprenoids synthesized by *Listeria* contribute to the vaccine-induced adaptive immune responses and overall vaccine-induced protection seen in studies using *Listeria*-based vaccines⁶². In addition, it is currently unknown whether *Listeria monocytogenes* is capable of V γ 2V δ 2 T cell stimulation in the absence of HMBPP production. Because *Listeria monocytogenes* can also produce IPP through the mevalonate pathway, it is possible that bacteria-produced IPP would be sufficient to activate V γ 2V δ 2 T cells *in vivo* and produce a memory T cell response (see Figure 4A). Also, because *Listeria monocytogenes* is an intracellular pathogen, it may induce host cell stress that induces an increased production of IPP similar to that seen in influenza infection (Figure 4B)⁸⁸. Either of these mechanisms could induce V γ 2V δ 2 T cell activation in the absence of HMBPP production.

1.5. Role of V γ 2V δ 2 T cells in *Listeria*-based Vaccine Induced Immune Responses

Taken together, the data suggests that *Listeria monocytogenes* may serve as an efficient vaccination vector for protecting individuals against *Mycobacterium tuberculosis*. This protection may be mediated through the development of memory CD4, CD8, and $\gamma\delta$ T cells. We intend to examine the role that isoprenoid recognizing V γ 2V δ 2 T cell activation and memory development may play in the development of *Listeria*-mediated vaccine responses and Tuberculosis protection through genetic modification of our *Listeria*-based vaccine vector.

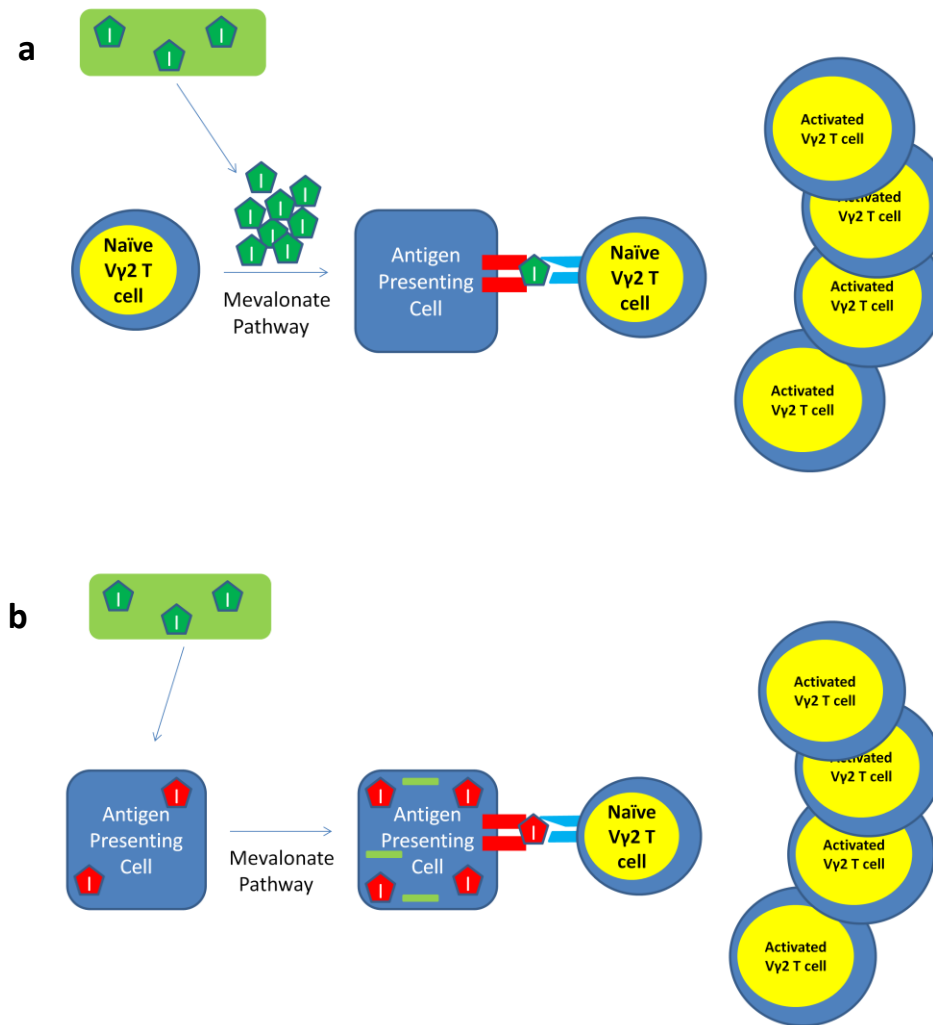


Figure 4. **Proposed mechanism of *Listeria*-mediated activation of Vy2V62 T cells in the absence of HMBPP production**

A) *Listeria monocytogenes* Δ gcpE strains are incapable of HMBPP production but competent for IPP biosynthesis. High IPP production from the *Listeria* bacillus (shown in green) may provide sufficient levels of IPP to the antigen presenting cell to result in IPP presentation to Vy2V62 T cells for activation and expansion *in vitro*. B) Cellular infection of host macrophages by *Listeria monocytogenes* Δ gcpE strains may result in increased production host cell IPP generated through upregulation of the mevalonate pathway (shown in red). Upregulation of host cell IPP production occurs in oncogenic transformation of host cells and host cell infection by some viruses

CHAPTER 2

MATERIALS AND METHODS

*Portions of the text and/or figures were reprinted from **J. Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing *Listeria* mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. *J Leukoc Biol.* 2014 Dec;96(6):957-67. and B. Ryan-Passeyur, **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*." *J. Immunol* 2012 Aug 1;189(3):1285-93.

2.1. Bacterial Strains

The strains of bacteria and their sources are indicated in Table 1. All *Listeria monocytogenes* strains and samples derived from mice or macaques infected with *Listeria monocytogenes* were plated and cultured on BHI media except where indicated. *Mycobacterium tuberculosis* Erdman strain and all samples derived from *M.tb.* infected macaques were placed on Middlebrook 7H11 media plates.

Strain	Source
<i>L.monocytogenes</i> 10403S Δ actA	Gift from Dr. Nancy Freitag (University of Illinois at Chicago College of Medicine)
<i>L.monocytogenes</i> 10403S Δ actA prfA*(G155S)	Gift from Dr. Nancy Freitag (University of Illinois at Chicago College of Medicine)
<i>L.monocytogenes</i> 10403S Δ actA Δ gcpE prfA*(G155S)	Created in this study

<i>Recombinant L.monocytogenes</i> 10403S $\Delta actA$ <i>prfA</i> *(G155S) pPL6-ESAT6	Created in this study
<i>Recombinant L.monocytogenes</i> 10403S $\Delta actA \Delta gcpE$ <i>prfA</i> *(G155S) pPL6-ESAT6	Created in this study
<i>Recombinant L.monocytogenes</i> 10403S $\Delta actA$ <i>prfA</i> *(G155S) pPL6-Ag85B	Created in this study
<i>Recombinant L.monocytogenes</i> 10403S $\Delta actA \Delta gcpE$ <i>prfA</i> *(G155S) pPL6-Ag85B	Created in this study
<i>Mycobacterium tuberculosis</i> Erdman	Gift from Dr. William Jakobs (Albert Einstein College of Medicine)

TABLE I

Bacteria Strains Used for Studies

2.2. Targeted deletion of gcpE from *Listeria monocytogenes* strain 10403S $\Delta actA$ *prfA**

Primers used for generation of *Listeria monocytogenes* $\Delta actA \Delta gcpE$ *prfA** (G155S) are listed in Table II. Amplification of 5' and 3' flanking regions of GcpE were amplified using the following primer pairs: GcpESOE1 and GcpE SOE2 to amplify the 5' upstream region, and GcpE SOE3 and GcpE SOE4 to amplify the downstream coding region. Resulting products were mixed in a 1:1 ratio, and PCR was performed using primers GcpE SOE1 and GcpE SOE4 to generate 1 overlapping product lacking the GcpE coding region. This PCR product and the pKSV7 plasmid was digested with restriction enzymes PstI and XbaI and ligated together using NEB T4 Ligase. The resulting ligation product was transformed into competent E.coli DH5 α and transformed E.coli cells were selected for using LB agar containing 100 μ g/mL ampicillin. Minipreps (Qiagen) of pKSV7 containing GcpEKO PCR product were prepared from positive colonies, and insert was confirmed using restriction digest using PstI and XbaI. Plasmid pKSV7-GcpEKO was electroporated into electrocompetent *Listeria monocytogenes* $\Delta actA$ *prfA** (G155S) (provided by Dr. Nancy Freitag)⁸⁵. *Listeria* containing the plasmid was selected for by plating on BHI plates

containing 5µg/mL chloramphenicol. Select colonies were grown in BHI media in the presence of chloramphenicol at 42°C for 24 hours, then repeatedly passaged at 30°C in the absence of chloramphenicol. The loss of *gcpE* coding region was confirmed by PCR analysis using primers GcpEKO_F and GcpEKO_R.

Primer Name	Sequence
GcpE SOE1	5'-GATTTATTCTTT CTAG ATTTGGCC-3'
GcpE SOE2	5'-GCGAAATATTCTTTCATTCAAAGA-3'
GcpE SOE3	5'-TCTTTGAATGAAAGAATATTTGCCCCGATTAAAGTAGCCGTGCTT-3'
GcpE SOE4	5'-CTACTC CTGCAG CAATACCAA-3'
GcpEKO _F	CTCGAAGGTACCAATGCTTATCTCCTGCCCTACTTG
GcpEKO _R	TTTTCTGAATTCAACGATAATCCCCCTTG

TABLE II

Primers Used in the generation of *Listeria monocytogenes* strain 10403S $\Delta actA \Delta gcpE$ *prfA**

2.3. Complementation of *gcpE* in to *Listeria monocytogenes* strain 10403S $\Delta actA \Delta gcpE$ *prfA**

The Kanamycin resistance containing shuttle plasmid pIMK2 (received from Dr. Nancy Freitag, UIC, Chicago, IL) was used to generate the plasmid pIMK2-*gcpE*. The forward and reverse primers GcpEComp_F and GcpEComp_R were used to amplify the coding region of *gcpE*. PCR product and pIMK2 were digested with *NotI* and *SmaI* (NEB Biolabs). Digested plasmid and insert were combined and ligated using T4 ligase. Resulting ligation product was transformed into E.coli DH5α and transformed cells were selected for on LB agar plates containing 50µg/mL kanamycin. Select colonies were grown up in 4mL LB media

containing kanamycin and MiniPrep kits were used to isolate plasmid DNA. Isolated DNA was digested with NheI and Sall to confirm presence of insert, and 5 colonies were sequenced to confirm no mutations occurred during cloning process.

Primer Name	Sequence
GcpECompF	TAGTCTAC CCATGGT TGGTCTCTTTGAATGAAAG
GcpECompR	TTTTCT GTCGACA ACGATAATCCCCCTTG

Table III

Primers used in generation of *Listeria monocytogenes* strain 10403S

*ΔactA ΔgcpE prfA*plMK2-gcpE*

2.4. Peripheral Blood Mononuclear Cell (PBMC) Isolation

Freshly collected EDTA anti-coagulated blood was centrifuged and the buffy coat was removed and diluted in PBS. Diluted buffy coats were layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) and centrifuged to separate PBMCs from red blood cells (RBCs) and granulocytes. PBMCs were aspirated from top layer of Ficoll-Paque and contaminating RBCs were lysed using RBC lysis buffer (eBioscience). Purified PBMCs were washed twice and counted using a hemacytometer.

2.5. Preparation of Low-molecular weight lysates of *Listeria monocytogenes* strains

Listeria monocytogenes strains were grown in BHI media to an OD of 0.4, washed twice with PBS and resuspended in 1mL PBS and sonicated to lyse bacteria. Sonicated cells were filtered through a 10kDa Centrifugal Filter Units (EMD Millipore). The bottom fraction was collected and stored in -80°C until use.

2.6. In-vitro Stimulation of Vy2Vδ2 T cells in PBMC by *Listeria monocytogenes* supernatants

100µL of PBMCs at a concentration of 5×10^6 PBMC/mL R10 media containing 500U/mL IL-2 were placed in round-bottom 96-well plates and stimulated with indicated dilutions of filtered low molecular weight lysates of indicated *Listeria monocytogenes* strains. Cells were stimulated for 7 days prior to analysis. On day 7, cells were stained with CD3-PE-Cy7 (SP34-2; BD Biosciences) and Vy2-FITC (7A5; Thermo Scientific) for 15 minutes at RT. Cells washed with PBS and fixed in 2% formalin. Cells were analyzed using a CyAn Flow Cytometer. Lymphocytes were gated based on forward- and side-scatters, and pulse-width and at least 50,000 gated events were analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

2.7. Intravenous Challenge of BALB/c mice with *Listeria monocytogenes* strains

2×10^4 CFU of indicated *Listeria monocytogenes* strains were injected into the tail vein of 8 week old BALB/c mice. 3 days following infection, mice were euthanized, liver and spleen were weighed, harvested, homogenized, and serial dilutions of homogenate were placed on BHI plates to determine bacterial burden.

2.8. Rhesus Macaques for *in vivo* Infection with *Listeria monocytogenes*

Twelve Chinese-origin rhesus macaques, free of simian retrovirus, simian T-lymphotrophic virus type 1, and SIV infection, were used in this study. All animals were maintained and used in accordance with guidelines of the IACUC and IBC of the University of Illinois at Chicago. Animals were anesthetized with 10mg/kg ketamine HCl (Fort Dodge Animal Health) for all blood sampling, infections and treatments. Blood for white blood cell isolation was collected in EDTA tubes and complete blood counts (CBCs) were performed on all harvested blood samples. Day 0 blood was drawn immediately before infection.

2.9. Intratracheal challenge and rechallenge of Rhesus macaques with recombinant *Listeria monocytogenes* strains

10^8 CFU of *Listeria monocytogenes* $\Delta actA$ *prfA** expressing ESAT6 or Ag85B (5×10^7 CFU of recombinant *Listeria* expressing each antigen, 1×10^8 CFU recombinant *Listeria* total) or *Listeria monocytogenes* $\Delta actA$ $\Delta gcpE$ *prfA** expressing ESAT6 or Ag85B (5×10^7 CFU of recombinant *Listeria* expressing each antigen, 1×10^8 CFU recombinant *Listeria* total) was administered intratracheally to 12 Chinese-origin rhesus macaques (6 per group). For intratracheal inoculation, macaques were sedated with Ketamine (10mg/kg) and Xylazine (1-2 mg/kg; Ben Venue Laboratories, Bedford, OH), by intramuscular injection. An endotracheal tube was inserted through the larynx into the trachea and placed at the carina and a 1mL solution containing the inoculum was administered through the endotracheal tube. Seven weeks following initial challenge, macaques were re-challenged with 10^8 CFU of the same strain of

Listeria through the intratracheal route. Macaques were isolated following vaccination and monitored for clinical signs of disease throughout the study.

2.10. Bronchoalveolar Lavage (BAL) and Bronchoalveolar Lavage Fluid (BALF) Collection

BAL was performed as previously described. Briefly, macaques were sedated with Ketamine (10mg/kg) and Xylazine (1-2 mg/kg), by intramuscular injection. An intratracheal tube was inserted through the larynx into the trachea and placed at the carina. Saline solution was instilled and harvested from the lungs through the intratracheal tube. A maximum of 10mg/kg of solution was placed in the lungs of macaques, and the recovery rate was greater than 50% in all cases.

2.11. Phenotyping of PBMC and BAL lymphocytes from Macaques

For cell-surface staining, PBMCs were stained with up to 6 Abs (conjugated to FITC, PE, allophycocyanin, pacific blue, PE-Cy7, and Alexafluor-700) for 15min. After staining, cells were fixed with 2% formaldehyde-PBS prior to analysis on an LSR Fortessa flow cytometer (BD Biosciences). Lymphocytes were gated based on forward- and side-scatters, and pulse-width and at least 50,000 gated events were analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

2.12. Intracellular Cytokine Staining of Mononuclear Cells

0.5×10^6 PBMC plus mAbs CD28 (1 μ g/ml) and CD49d (1 μ g/ml) were incubated in 100 μ l final volume for 1h at 37°C, 5% CO₂ followed by an additional 5h incubation in the presence of brefeldin A (GolgiPlug, BD). After staining for cell-surface markers (CD3, CD4, $\gamma\delta$) for 15 min, cells were permeabilized for 45 min (Foxp3 Fix/Perm Buffer, Biolegend) and stained for 45 min with cytoplasmic antibodies (IFN γ , TNF α , IL-2), (IFN γ , IL-17, IL-22) or (Perforin, Granzyme B, Granulysin) before fixation in formalin.

2.13. Intracellular Inhibition of Growth Assay

For generation of target cells, monocytes were isolated from PBMC through adherence to flat-bottom 96-well plates. Monocytes were infected with an MOI of 100 of *Listeria monocytogenes* strain 10403S for 1 hour. Cells were washed to remove extracellular bacteria, and media was replaced with R10 media containing 10 μ g/mL gentamicin.

To isolate effector cells, PBMCs isolated from challenged macaques were stained for Vy2-FITC (7A5) for 15 minutes and washed twice with PBS prior to sorting on a MoFlo (Beckman Coulter). Sorted cells were aliquoted into wells containing target cells at the indicated ratios. The total volume of Target cell: Effector cell mixture was 200 μ L in all cases. At indicated time-points following co-culture, cells were centrifuged, media was removed, and cells were lysed using sterile water solution to release bacteria. Solutions were serially diluted and placed on BHI plates containing 200 μ g/mL streptomycin to determine CFU counts of *Listeria*.

2.14. Third challenge of Rhesus macaques with recombinant *Listeria monocytogenes* strains

10^8 CFU of $\Delta actA prfA^*$ *Listeria monocytogenes* expressing ESAT6 or Ag85B (5×10^7 CFU of recombinant *Listeria* expressing each antigen, 1×10^8 CFU recombinant *Listeria* total) or $\Delta actA \Delta gcpE prfA^*$ *Listeria monocytogenes* expressing ESAT6 or Ag85B (5×10^7 CFU of recombinant *Listeria* expressing each antigen, 1×10^8 CFU recombinant *Listeria* total) was administered intratracheally to 12 Chinese-origin rhesus macaques (6 per group) as previously described.

2.15. V γ 2V δ 2 T Cell Proliferation Assay

PBMCs were isolated blood collected from macaques prior to infection, 7 weeks following initial *Listeria* vaccination, 7 weeks following second administration of the *Listeria*-based vaccine, or 7 weeks following the 3rd administration of the *Listeria*-based vaccine. PBMCs were stained with CellTracker Violet Dye (Invitrogen) and seeded at the bottom of round-bottom 96-well plates at a concentration of 5×10^6 cells/mL and stimulated with 40ng/mL of HMBPP, 4ng/mL HMBPP, 400pg/mL of HMBPP, or 40pg/mL of HMBPP in the presence of 100U/mL rh-IL-2 (Sigma). These cells were grown in culture for 7 days, then stained with antibodies against CD3 (SP34-2; BD Biosciences) and V γ 2 (7A5 Thermo Scientific) for 15 minutes at room temperature. These cells were fixed with 2% formalin, then analyzed by flow cytometry using the LSR Fortessa. 50,000 lymphocytes were counted for all samples.

2.16. V γ 2V δ 2 T Phosphoantigen Sensitivity Assay

PBMCs were isolated from blood collected from macaques prior to infection, 7 weeks following initial *Listeria* vaccination, 7 weeks following second administration of the *Listeria*-based vaccine, or 7 weeks following the 3rd administration of the *Listeria*-based vaccine. PBMCs were seeded at the bottom of round-bottom 96-well plates at a concentration of 5×10^6 cells/mL in R10 media containing 1 μ g/mL CD28 and CD49d and stimulated with 40 ng/mL of HMBPP, 4 ng/mL HMBPP, 400 pg/mL HMBPP, 40 pg/mL HMBPP, or left unstimulated for 1 hour. 1 hour following HMBPP stimulation, protein transport was inhibited with Brefeldin A (GolgiPlug, BD Biosciences) and cells were cultured for 5 more hours. After the 5 hour incubation period, cells were stained with surface markers (CD3, V γ 2) for 15 minutes, washed twice, then fixed and permeabilized with Foxp3 Fix/Perm Buffer (Biolegend) for 30 minutes. Fix/Perm buffer was washed off, cells were washed twice with Foxp3 Perm buffer, then incubated with intracellular antibodies (IFN γ , TNF α) for 45 minutes at room temperature. Cells were washed twice, fixed in 2% formalin, and analyzed using the LSR Fortessa. 5,000 V γ 2+ events were counted for each sample.

2.17. Rhesus Macaques for *in vivo* Infection with *Mycobacterium tuberculosis*

Two weight-matched Chinese-origin rhesus macaques, free of simian retrovirus, simian T-lymphotrophic virus type 1, and SIV infection, were challenged with *Mycobacterium tuberculosis* in addition to the two Rhesus macaques vaccinated and boosted with *Listeria monocytogenes* Δ actA prfA* (G155S) expressing ESAT6 and Ag85B and three macaques vaccinated and boosted with *Listeria monocytogenes* Δ actA Δ gcpE prfA* (G155S) as previously

described. All animals were maintained and used in accordance with guidelines of the IACUC. Animals were anesthetized with 10mg/kg ketamine HCl (Fort Dodge Animal Health) for all blood sampling, infections and treatments. Blood for white blood cell isolation was collected in EDTA tubes. Day 0 blood was drawn immediately before infection.

2.18. Challenge of Rhesus Macaques with *Mycobacterium tuberculosis*

Rhesus macaques were sedated with Ketamine (10mg/kg) and Xylazine (1-2 mg/kg), by intramuscular injection. A pediatric bronchoscope was inserted into the right caudal lung lobe, and 80 CFU of *Mycobacterium tuberculosis* Erdman strain was administered in 3mL of saline solution. Pediatric bronchoscope was subsequently flushed with 3mL of saline followed by 3mL bolus of air to ensure full inoculum was administered to the macaque. Macaques were monitored for clinical signs of pulmonary Tuberculosis 3 times weekly following infection and weighed once weekly to determine the extent of weight loss following infection.

2.19. Gross pathologic analysis of Tuberculosis-induced lesions

8 weeks following infection, macaques were euthanized with pentobarbital (37.5mg/kg IV) and necropsies were performed immediately following euthanasia. Standard gross pathologic evaluation procedures were followed, and steps were recorded and gross pathologic specimens were photographed. Lung lobes, bronchial, axillary, and mesenteric lymph nodes were collected and labeled. Multiple specimens from all tissues with gross lesions were harvested. Gross

observations of the presence, size, location, number and distribution of lesions were recorded.

A scoring system used previously in the Chen lab was excised to calculate gross pathology scores for TB lesions in lungs. For each lobe of the lung, granuloma presence was scored 0-4 for (0) no visible granulomas, (1) 1-3 visible granulomas, (2) 4-10 visible granulomas, (3) >10 discrete granulomas, and (4) military pattern of granulomas, respectively. Granuloma size was scored 0-3 for (0) none present, (1) <1-2 mm, (2) 3-4 mm, and (3) >4mm, respectively.

Pulmonary consolidation or atelectasis as viewed from organ exterior and cut surfaces were scored 0-2 for (0) absent, (1) present focally in 1 lobe, (2) extensive within 1 lobe or involving multiple lobes. One score was also given for the presence of tuberculosis-related focal parietal pleural adhesions, pleural thickening and opacification, or pulmonary parenchymal cavitation.

For Hilar lymph nodes, enlargements were scored 0-3 for (0) visible but not enlarged, (1) visibly enlarged unilaterally (<2cm), (2) visibly enlarged bilaterally (<2cm), (3) visibly enlarged unilaterally or bilaterally (>2cm). Tuberculosis lesions in hilar lymph nodes were scored 0-4 for (0) no granulomas visible on capsular or cut surface, (1) focal or multifocal, circumscribed, non-coalescing granulomas, <2mm, (2) coalescing solid or caseating granulomas occupying <50% of nodal architecture, (3) coalescing solid or caseating granulomas occupying >50% of nodal architecture with residual nodal components still recognizable, (4) complete granulomatous nodal effacement and caseation. Tuberculosis lesions in each of the extrathoracic organs were scored similarly as each lung lobe. Pathology scoring of infected tissues was conducted in a blinded fashion.

2.20. Determining Bacterial Colony Forming Unit (CFU) counts in organ tissue homogenates

1 cubic centimeter tissue sections harvested from right caudal, right medial, and left caudal lung lobes and liver, spleen, and kidney of necropsied macaques were taken after gross pathological analysis was completed. If there were gross TB lesions in the respective lobe, a half of the lung tissue containing approximately 50% lesions and 50% healthy tissue was taken. If no visible lesions were present in the respective lobe, a random piece of tissue was taken for tissue homogenization. Tissue homogenates were prepared using a homogenizer (PRO 200, PRO Scientific INC, CT) and diluting the tissue in sterile PBS + 0.05% Tween-80. 5-fold serial dilutions of tissue homogenate samples were plated on Middlebrook 7H11 plates (Remel).

2.21. Statistical Analysis

Statistical analysis was done using paired two-tailed Student's *t* test, unpaired two-tailed Student's *t* test, or Mann-Whitney U test using Graphpad software (Prism, La Jolla, CA). Data compared were based on percentage, unless otherwise stated.

CHAPTER 3

Altered *in vivo* V γ 2V δ 2 T cell Expansion and Memory Formation by *Listeria monocytogenes* deficient for HMBPP production

*Portions of the text and/or figures were reprinted from J. **Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing *Listeria* mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. *J Leukoc Biol.* 2014 Dec;96(6):957-67. and B. Ryan-Passey, J. **Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*." *J. Immunol* 2012 Aug 1;189(3):1285-93.

3.1. Rationale

V γ 2V δ 2 T cells exist only in humans and non-human primates, and comprise 65-90% of total circulating human $\gamma\delta$ T cells. V γ 2V δ 2 T cells can be activated by naturally occurring metabolites often termed phosphoantigens, which include isopentenyl pyrophosphate (IPP) from the mevalonate pathway and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) from the microbial 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) isoprenoid synthesis pathway^{55,89}. HMBPP is produced in selected microbes including *Mycobacterium tuberculosis* (Mtb), *M. bovis* BCG, *Listeria monocytogenes* and malaria parasites (2, 3). IPP is synthesized in large amounts in stressed host cells due to infection or oncogenic transformation^{90,91}. While high concentrations of IPP are required to activate V γ 2V δ 2 T cells,

HMBPP is a ~1000-fold more potent than IPP for *in vitro* activation of V γ 2V δ 2 T cells⁹²⁻⁹⁴. *In vitro* activated V γ 2V δ 2 T cells can produce Th1 cytokines IFN γ and TNF α , lyse infected cells or tumors through release of cytolytic effectors and inhibit the intracellular growth of bacteria^{54-56,64}. Some of these *in vitro* findings have been replicated *in vivo* by administration of IL-2 plus HMBPP or equivalents^{60,95,96}.

Infections of humans and primates with HMBPP-producing bacteria and parasites have been shown to induce *in vivo* activation and expansion of V γ 2V δ 2 T cells^{56,57}. Activated V γ 2V δ 2 T cells can traffic to and accumulate in the pulmonary compartment and intestinal mucosal interface^{60,62}. Following immune clearance of pathogens, some V γ 2V δ 2 T cells can express memory phenotypes similar to those seen in CD8+ $\alpha\beta$ T cells, suggesting an adaptive immune response of these phosphoantigen-specific $\gamma\delta$ T cells^{62,63}. Consistent with these memory phenotypes, robust, accelerated recall-like expansion and rapid production of anti-microbial cytokines can be seen after *in vivo* re-exposure to HMBPP-producing pathogens such as Mtb, BCG or *Listeria monocytogenes*^{62,64}. Rapid recall-like expansion of V γ 2V δ 2 T cells after Mtb challenge of BCG-vaccinated macaques coincides with the vaccine-induced protection against severe form of tuberculosis⁶². It is therefore critical to elucidate relative importance of isoprenoids in inducing immune responses of V γ 2V δ 2 T cells in infection.

It is now well known that many microbes and parasites produce HMBPP via the MEP pathway, and infections with these pathogens activate and expand V γ 2V δ 2 T cells, but it has yet to be determined whether bacterial organisms deficient for HMBPP production

are capable of stimulating V γ 2V δ 2 T cells, and if these V γ 2V δ 2 T cells activated *in vivo* in the absence of HMBPP exposure would generate similar effector and memory T cell responses. In the absence of HMBPP exposure, *Listeria monocytogenes* may be incapable of stimulating V γ 2V δ 2 T cell responses due to an insufficient production of stimulatory phosphoantigen IPP (Figure 5A). Alternatively, the IPP production by *Listeria monocytogenes* or the host upregulation of the MEP pathway by intracellular infection by *Listeria* may be sufficient to stimulate V γ 2V δ 2 T cell activation and expansion. Addressing this fundamental immunologic question will enhance our understanding of biology of V γ 2V δ 2 T cells in infections, and help to design $\gamma\delta$ T-cell-targeted genetic manipulation of *Listeria monocytogenes* and macaque models^{64,76,85} to test the hypothesis that V γ 2V δ 2 T cells could be activated and expanded by IPP-producing *Listeria* deficient for HMBPP production, and that these cells would differ phenotypically from those generated by HMBPP/IPP co-production in *Listeria*. *Listeria monocytogenes* is the only pathogen known to possess both the mevalonate and MEP pathways of isoprenoid biosynthesis, making it possible to knock out the MEP pathway without a loss of *Listeria* viability⁸⁷. We used homologous recombination to delete the *gcpE* gene encoding HMBPP synthase from our attenuated strain of *Listeria monocytogenes*, and compared phenotypic and functional differences in V γ 2V δ 2 T cells stimulated by these *Listeria* strains.

3.2. HMBPP synthase-deficient *Listeria monocytogenes* $\Delta actA \Delta gcpE$ *prfA** exhibited reduced ability to expand V γ 2V δ 2 T cells in vitro.

A number of bacterial and parasitic pathogens produce HMBPP, and infections of humans and primates with these HMBPP-producing pathogens induce significant activation and expansion of V γ 2V δ 2 T cells^{59,62,64}. However, it remains unknown whether bacteria-derived HMBPP-induced activation of V γ 2V δ 2 T cells results in similar development of V γ 2V δ 2 T cell effector and memory cells as IPP-driven activation of V γ 2V δ 2 T cells. The research community needs to address this fundamental immunologic question, as an optimal V γ 2V δ 2 T cell production protocol may require stimulation of V γ 2V δ 2 T cells through a coordinated use of HMBPP and IPP in combination or in tandem.

To determine the contribution of HMBPP to V γ 2V δ 2 T cell expansion during microbial infections, we sought to develop infectious mutant bacteria deleted of *gcpE* gene encoding HMBPP synthase and to use it as an infection tool for comparative studies in macaques. While this genetic approach was attractive for comparing bacterial HMBPP-induced activation of V γ 2V δ 2 T cells during infections to IPP-induced activation of V γ 2V δ 2 T cells, most bacterial pathogens express either the MEP pathway or the mevalonate pathway of isoprenoid synthesis, and not both. Although *Listeria monocytogenes* is the only pathogen carrying both mevalonate and MEP pathways for critical bacterial metabolism, *gcpE*-knockout *Listeria* is significantly attenuated, and non-comparable to wild-type *Listeria* strain for infection⁸⁶. To overcome these problems, we made use of our experience with genetic modification of attenuated *Listeria monocytogenes* (*Listeria* $\Delta actA$ *prfA**) and *Listeria* infection of macaques^{64,76,85}. We presumed

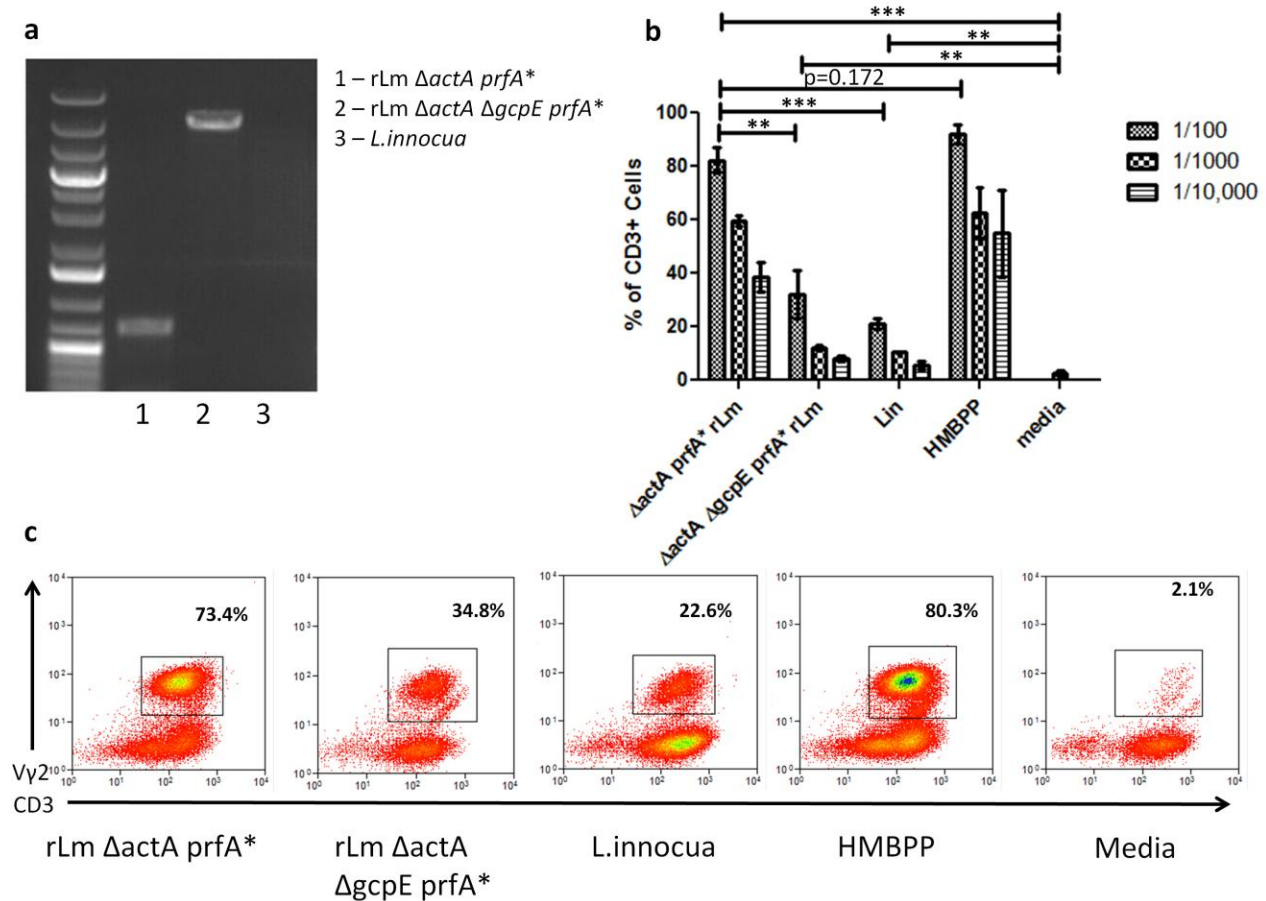


Figure 6. Deletion of *gcpE* gene encoding HMBPP synthase from *Listeria* $\Delta actA prfA^*$ vaccine strain reduced *Listeria*-mediated *in-vitro* expansion of V γ 2V δ 2 T cells.

A) PCR products amplified from the HMBPP-synthase gene *gcpE* sequence from indicated genomic *Listeria monocytogenes* DNA strains. PCR products were generated using PCR primers indicated in Materials and Methods, and run on a 0.8% agarose gel with GeneRuler 100bp plus ladder. B) Mean percentages of expanded $\gamma\delta$ T cells following *in vitro* stimulation using *Listeria monocytogenes* lysates, and representative flow histograms of CD3+V γ 2+ T cell percentages (bottom). PBMCs were isolated from healthy naïve rhesus macaques and stimulated with sonicated lysates of *Listeria monocytogenes* strains for 7 days in the presence of 500U/ml recombinant human IL-2, then stained with CD3 and V γ 2 prior to flow cytometry analysis. Lysates from *Listeria innocua*, known to lack *gcpE*, were used as a negative control. Serial dilutions of HMBPP (40ng/ml) were used as a positive control. **, $p < 0.01$; ***, $p < 0.001$

that since *Listeria* $\Delta actA$ *prfA** strain is already remarkably attenuated, knock out of *gcpE* from this mutant would not further attenuate its infectivity/immunogenicity, and thus allowed us to compare the activation of V γ 2V δ 2 T cells in the context of bacterially-produced HMBPP to IPP-induced V γ 2V δ 2 T cells activation⁶⁴.

We used homologous recombination to remove *gcpE* gene from our *Listeria* $\Delta actA$ *prfA** strain. This approach allowed us to successfully develop *gcpE* knock-out *Listeria* $\Delta actA$ *prfA** (*Listeria* $\Delta actA$ $\Delta gcpE$ *prfA**) (Figure 6A). Whereas HMBPP-containing, low-molecular-weight lysate from *Listeria* typically stimulates an expansion of V γ 2V δ 2 T cells in macaque PBMC culture, culture with lysate from HMBPP-synthase-deficient *Listeria* $\Delta actA$ $\Delta gcpE$ *prfA** mutant led to a significantly reduced expansion of V γ 2V δ 2 T cells compared to parental *Listeria* $\Delta actA$ *prfA** (Figure 6B). *Listeria innocua*, a nonpathogenic species of *Listeria* that lacks the *gcpE* locus (Figure 6A) was inefficient as well in stimulating V γ 2V δ 2 T-cell expansion (Figure 6B), while synthetic HMBPP was a potent inducer of V γ 2V δ 2 T cell expansion (Figure 6B). Low molecular weight lysates from both *Listeria* $\Delta actA$ $\Delta gcpE$ *prfA** and nonpathogenic *Listeria innocua* induced V γ 2V δ 2 T-cell expansion above media control, suggesting that IPP production from these pathogens is capable of activation and expansion of V γ 2V δ 2 T-cells (Figure 6B). Taken together, these data suggest *Listeria* $\Delta actA$ $\Delta gcpE$ *prfA** deficient in HMBPP expanded V γ 2V δ 2 T-cells to a lesser degree than the parent strain, suggesting that although HMBPP is the dominant contributor to V γ 2V δ 2 T cell expansion *in vitro*, *Listeria*-induced IPP and HMBPP can both contribute to V γ 2V δ 2 T cell expansion.

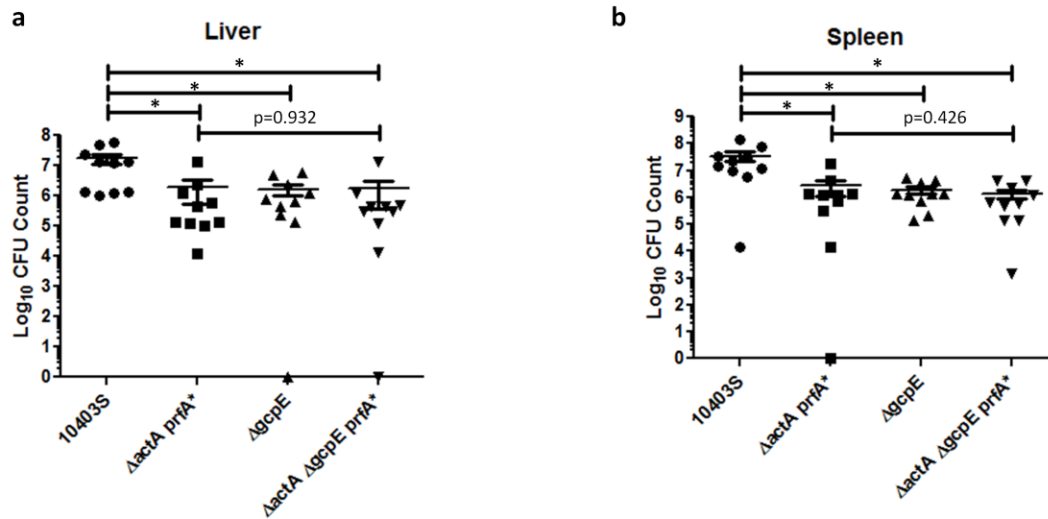


Figure 7. A loss of *gcpE* encoding HMBPP synthase in *Listeria* Δ actA Δ gcpE prfA* did not further attenuate *Listeria* Δ actA prfA* in mice

Bacterial burdens in livers (A) and spleens (B) of mice infected through intravenous inoculation with 2×10^4 CFU of the indicated *Listeria monocytogenes* strains. Livers and spleens of mice were harvested 3 days post infection. *, $p < 0.05$

3.3. *A loss of gcpE encoding HMBPP synthase in Listeria $\Delta actA \Delta gcpE$ prfA* did not further attenuate Listeria $\Delta actA$ prfA* in mice*

We then sought to determine whether removing *gcpE* from our attenuated strain of *Listeria* would further attenuate bacterial replication and infection *in vivo*, since a previous study showed that elimination of HMBPP synthase in a wildtype strain of *Listeria* resulted in a decreased virulence in mice⁸⁶. While *gcpE* deletion in a wildtype strain of *Listeria*, 10403S, resulted in decreases in bacterial loads in spleen and liver following intravenous challenge of mice (Fig.2a, 2b, columns 1 and 3), *gcpE* deletion from the attenuated *Listeria $\Delta actA$ prfA** did not appear to attenuate infectivity further as this *gcpE*-deficient *Listeria $\Delta actA \Delta gcpE$ prfA** strain did not cause a significant decrease in bacterial burdens at 3 days following intravenous infection of mice compared to the parental *Listeria $\Delta actA$ prfA** (Figure 7A, 7B, columns 2 and 4). These results demonstrated that *Listeria $\Delta actA \Delta gcpE$ prfA** resembled *Listeria $\Delta actA$ prfA** in replication and infection in mice, suggesting a utility of this mutant for mechanistic studies of a role of listerial HMBPP in infection of macaques.

3.4. *HMBPP- producing Listeria $\Delta actA$ prfA* and HMBPP-deficient Listeria $\Delta actA \Delta gcpE$ prfA* significantly expand pulmonary of V γ 2V δ 2 T cells after Listeria exposure or re-exposure.*

To determine if IPP-producing, HMBPP-deficient *Listeria $\Delta actA \Delta gcpE$ prfA** lost an ability to activate or expand V γ 2V δ 2 T cells *in vivo*, rhesus macaques were inoculated intratracheally with 10⁸ CFU of *Listeria $\Delta actA$ prfA** and *Listeria $\Delta actA \Delta gcpE$ prfA** mutant, respectively. We elected

to perform an intratracheal challenge with *Listeria* since we were interested in dissecting pulmonary and systemic immune responses of HMBPP-driven V γ 2V δ 2 T cells after microbial invasion to lungs. Previous studies of respiratory infections with HMBPP-producing pathogens including *Mycobacterium tuberculosis* and *Yersinia pestis* have demonstrated that expanded V γ 2V δ 2 T cells can accumulate in lungs and correlate with improved clinical outcomes after pulmonary infections with these pathogens⁵⁹. Because these organisms only have the MEP pathway of isoprenoid biosynthesis, elimination of *gcpE* and other genes in the

MEP pathway in these pathogens renders these organisms unviable. The ability of *Listeria* $\Delta actA \Delta gcpE prfA^*$ to survive in the absence of a MEP pathway gives us the unique opportunity to examine V γ 2V δ 2 T-cell response to HMBPP and IPP in respiratory exposure to an HMBPP-producing organism.

No clinical signs of disease or distress were seen in rhesus macaques challenged with *Listeria* $\Delta actA prfA^*$ or $\Delta actA \Delta gcpE prfA^*$ mutant, and no *Listeria* bacteria were recovered either in cultures of BAL samples or blood collected at 7 days following infection (data not shown), suggesting that attenuated *Listeria* bacteria did not apparently disseminate into the blood stream and was cleared within a week of inoculation. Since activated V γ 2V δ 2 T cells during infection of macaques with HMBPP-producing pathogens such as *Listeria* and Mtb can readily traffic to and accumulate in the pulmonary compartment^{62,64}, we asked if HMBPP was a major microbial factor driving such pulmonary responses of V γ 2V δ 2 T cells. To address this, we determined the dynamics of V γ 2V δ 2 T cells in lymphocytes from bronchoalveolar lavage (BAL) fluid. Macaques exposed to HMBPP-producing *Listeria* showed a significantly increases in these

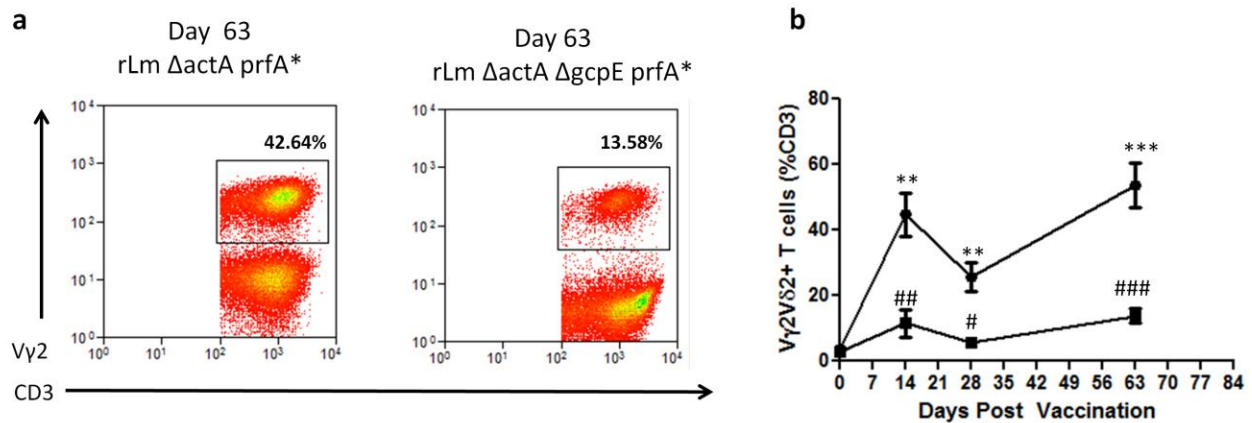


Figure 8. **HMBPP- producing *Listeria* $\Delta actA$ $prfA^*$ and HMBPP-deficient *Listeria* $\Delta actA$ $\Delta gcpE$ $prfA^*$ significantly expand pulmonary V γ 2V δ 2 T cells after *Listeria* exposure or re-exposure.**

A) Representative flow histograms of V γ 2V δ 2 T cells in BAL cells from a macaque exposed to *Listeria* $\Delta actA$ $prfA^*$ on day 63 (2 weeks) after secondary exposure (left) and a macaque exposed to *Listeria* $\Delta actA$ $\Delta gcpE$ $prfA^*$ on day 63 (right). B) Mean percentage numbers of V γ 2V δ 2 T cells in BAL cells at various time points following *Listeria* exposure. Asterisks indicate statistically significant comparisons of time points between the group of macaques exposed to *Listeria* $\Delta actA$ $prfA^*$ and the group exposed to *Listeria* $\Delta actA$ $\Delta gcpE$ $prfA^*$ (asterisks for p values are the same as above); # signs indicate statistically significant comparisons between the indicated time point and Day 0 for the group of macaques exposed to *Listeria* $\Delta actA$ $\Delta gcpE$ $prfA^*$.

cells, reinforcing previous observations that HMBPP is a potent inducer of V γ 2V δ 2 T cell expansion. Interestingly, macaque groups challenged with HMBPP-deficient *Listeria* strains exhibited an increased percentage of V γ 2V δ 2 T cells in the airway (Figure 8A,B). Secondary exposure of macaques to HMBPP-producing *Listeria* resulted in a recall-like increase in V γ 2V δ 2 T cells in both groups. Taken together, these data support the view that both IPP and HMBPP from *Listeria* contribute to expansion of V γ 2V δ 2 T cells in pulmonary compartment during *Listeria* infection.

3.5. HMBPP-synthase deficiency in *Listeria* Δ actA Δ gcpE prfA* significantly reduced the ability of *Listeria* to expand V γ 2V δ 2 T cells in blood circulation following intratracheal listerial exposure

Significant expansion of pulmonary V γ 2V δ 2 T cells in could be seen in rhesus macaques challenged with the attenuated *Listeria* Δ actA prfA* capable of producing HMBPP as early as 14 days post-infection (Figure 8B). We next to determine whether an increase of circulating V γ 2V δ 2 T cells can be seen despite our inability to detect live bacilli in the blood of infected macaques. Significant increases in V γ 2V δ 2 T cells were seen in macaques challenged with *Listeria* Δ actA prfA* as early as 7 days post-challenge. In contrast, macaques challenged with *Listeria* Δ actA **Δ gcpE** prfA* mutant that was deficient in producing HMBPP showed no or subtle increases in circulating V γ 2V δ 2 T cells (Figure 9B,C). Indeed, while circulating V γ 2V δ 2 T cells in macaques exposed to HMBPP-producing *Listeria* were increased in means from baseline <3% up to 24% of all circulating T cells, *Listeria* Δ actA **Δ gcpE** prfA* mutant induced no or subtle expansion of V γ 2V δ 2 T cells following primary challenge (Figure 9B,C). These data suggest that HMBPP in *Listeria* contributes to a more profound expansion of V γ 2V δ 2 T cells following *Listeria*

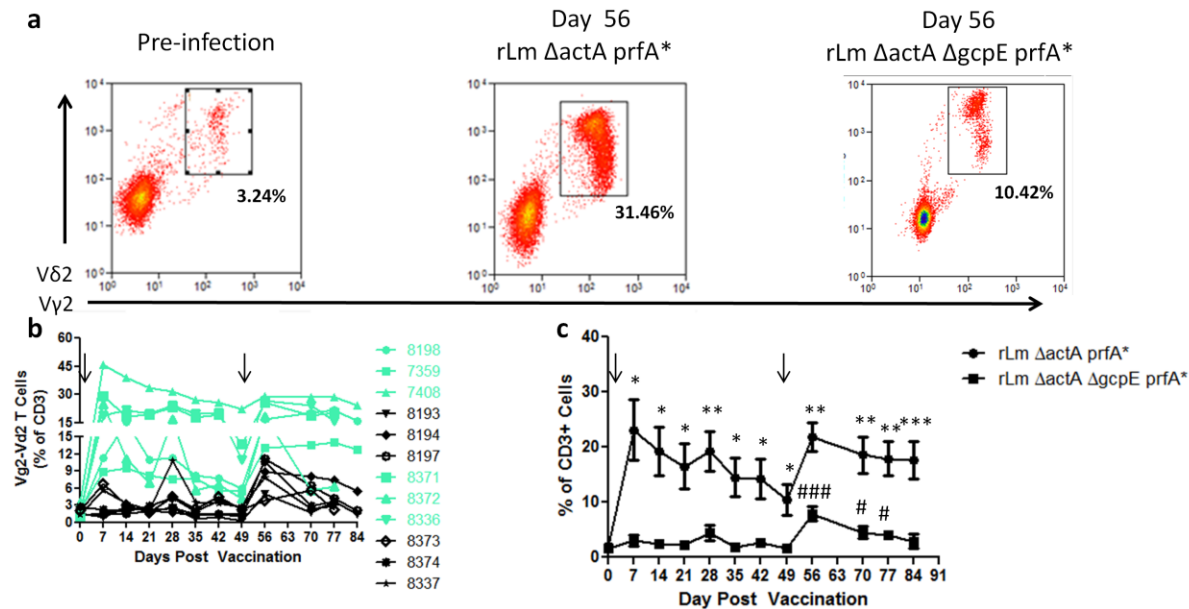


Figure 9. **HMBPP- producing *Listeria* $\Delta actA$ prfA* and HMBPP-deficient *Listeria* $\Delta actA \Delta gcpE$ prfA* significantly expand circulating V γ 2V δ 2 T cells after *Listeria* exposure or re-exposure.**

A) Representative flow histograms of V γ 2V δ 2 T cells in PBMCs from a macaque not exposed to *Listeria* (left, pre), a macaque exposed to *Listeria* $\Delta actA$ prfA* on day 56 [1 week following secondary exposure, (middle)], and a macaque exposed to *Listeria* $\Delta actA \Delta gcpE$ prfA* challenged macaque on day 56 (right), respectively. Flow panels are gated on CD3+ lymphocytes. B) Percentages of V γ 2V δ 2 T cells in blood of individual rhesus macaques (B) or mean numbers (C) at various time points following *Listeria* exposures (arrows). Macaque numbers in light grey indicate animals challenged with *Listeria* $\Delta actA$ prfA*. Asterisks indicate statistically significant comparisons of time points between the group of macaques exposed to *Listeria* $\Delta actA$ prfA* and the group exposed to *Listeria* $\Delta actA \Delta gcpE$ prfA* (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$); # signs indicate statistically significant comparisons between the indicated time point and Day 0 for the group of macaques exposed to *Listeria* $\Delta actA \Delta gcpE$ prfA*

infection of macaques and allows for a larger accumulation of V γ 2V δ 2 T cells throughout the body. Secondary pulmonary exposure to HMBPP-producing *Listeria* $\Delta actA$ *prfA** resulted in a rebound expansion of circulating V γ 2V δ 2 T cells with a peak similar in magnitude to the first expansion (Figure 9C). Interestingly, macaques re-exposed to HMBPP-deficient *Listeria* $\Delta actA$ 10C) although these increases were significantly lower numbers of circulating V γ 2V δ 2 T cells in macaques exposed to the parental strain. These results suggest that although HMBPP in *Listeria* plays a dominant role in driving expansion V γ 2V δ 2 T cells during *Listeria monocytogenes* re-exposure or re-infection of macaques, macaques exposed to HMBPP-deficient strains of *Listeria monocytogenes* exhibit expansion of V γ 2V δ 2 T cells in pulmonary and blood compartments.

3.6. HMBPP-synthase deficiency in *Listeria* did not negatively affect V γ 2V δ 2 T cell differentiation into anti-microbial effector cells producing IFN γ and TNF α Th1 cytokines after *Listeria* exposure

We next sought to determine whether HMBPP from *Listeria* help to differentiate effector functions of V γ 2V δ 2 T cells after exposure to *Listeria*. Typically, V γ 2V δ 2 T cells could produce significant amounts of IFN γ and TNF α Th1 cytokines in response to *in vitro* HMBPP stimulation (Figure 11A). V γ 2V δ 2 T cells from macaques challenged with either HMBPP-producing or HMBPP-deficient $\Delta actA$ ***ΔgcpE*** *prfA** strain of *Listeria* demonstrated a similar percentage of V γ 2V δ 2 T effector cells producing IFN γ (Figure 10A, top panels Figure 10B, upper left histogram) or TNF α (Figure 10A, middle panels, Figure 10B, upper right histogram), much greater total numbers of IFN γ -producing V γ 2V δ 2 T effector cells or percentages of CD3+ T cell population were seen in macaques exposed to HMBPP-producing *Listeria* than those exposed to HMBPP-

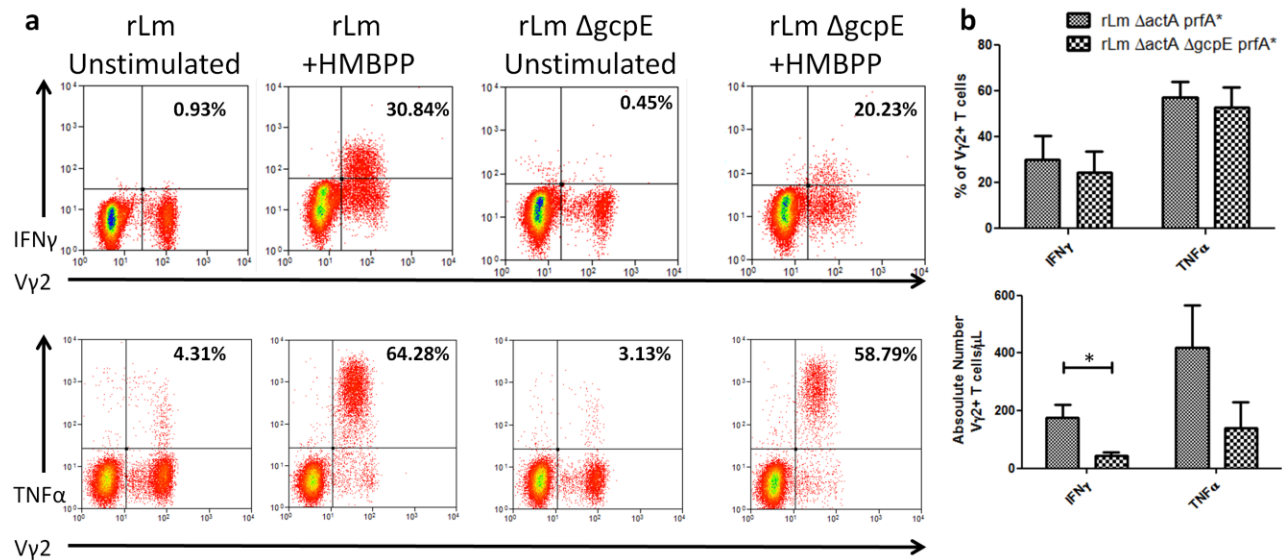


Figure 10. HMBPP-synthase deficiency in *Listeria* inefficiently differentiated V γ 2V δ 2 T cells into anti-microbial effector cells co-producing IFN γ and TNF α Th1 cytokines after listerial exposure

A) Representative flow histograms of IFN γ (top panels) TNF α (bottom panels) producing V γ 2V δ 2T cells at 63 days following primary *Listeria* exposure. Top and middle panels were gated on CD3+ lymphocytes. Bottom panels were gated on V γ 2+CD3+ lymphocytes. Numbers in upper right quadrant indicate the percentages of cells expressing IFN γ + (top), TNF α + (bottom). B) Mean percentage numbers of phosphoantigen-specific IFN γ + or TNF α + cells out of V γ 2+ T cells.

Percentages were generated by subtracting cytokine+ V γ 2+ T cell percentage from unstimulated panel from cytokine+ V γ 2+ T cell percentage in the HMBPP stimulated panel. (*, $p < 0.05$)

deficient *ΔactA ΔgcpE prfA** *Listeria* (Figure 10B, lower histograms) due to increased total numbers of Vγ2Vδ2 T cells. Thus, HMBPP and IPP production can be used interchangeably to induce differentiation of Vγ2Vδ2 T cells into anti-microbial effector cells co-producing IFNγ and TNFα Th1 cytokines after exposure to *Listeria*.

3.7. HMBPP-synthase deficiency in *Listeria* influenced polarization of memory phenotype of Vγ2Vδ2 T cells following *Listeria* exposure

We next sought to determine whether *Listeria*-mediated HMBPP or IPP exposure impacted the development of memory phenotype of Vγ2Vδ2 T cells following exposure to *Listeria monocytogenes*. Surface markers CD27 and CD45RA are commonly used to differentiate the different stages of memory formation for Vγ2Vδ2 T cells (Figure 11A). Following primary exposure with HMBPP-producing *Listeria*, macaques exhibited a significant increase in percentage of circulating Vγ2Vδ2 T cells with memory phenotype, as defined by CD27+ CD45RA- expression (Figure 11B). In contrast, Vγ2Vδ2 T cells from macaques exposed to HMBPP-deficient *Listeria ΔactA ΔgcpE prfA** showed significant lower percentages of circulating memory-type Vγ2Vδ2 T cells and higher percentage of circulating naïve cells (Figure 11C). Although percentages of memory-type Vγ2Vδ2 T cells in the airway following primary exposure to either strain of *Listeria* were similar between groups, macaques re-exposed to HMBPP-producing *Listeria* exhibited a higher percentage of memory-type Vγ2Vδ2 T cells and lower percentage of naïve Vγ2Vδ2 cells in the airway than those animals re-exposed to HMBPP-deficient *Listeria ΔactA ΔgcpE prfA** (Figure 11D,E, p <0.05 for both comparisons after II exposure).

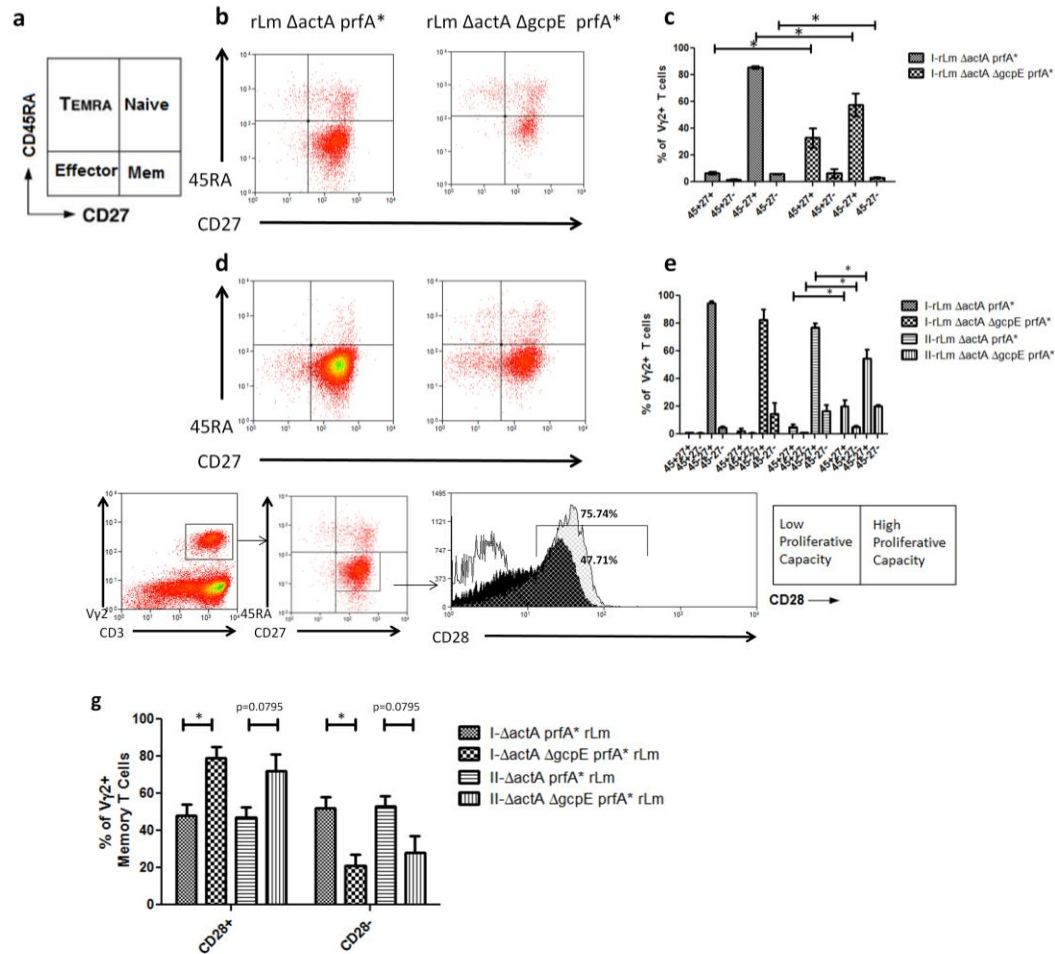


Figure 11. HMBPP-synthase deficiency in *Listeria* influenced polarization of memory phenotype of V γ 2V δ 2 T cells following listerial exposure. A) Diagram of phenotypic subsets of V γ 2V δ 2 T cells based on CD27 and CD45RA expression. B,D) Representative flow histograms of V γ 2V δ 2 T cell subtypes based on CD27 and CD45RA expression in blood (B) and BAL cells (D). Flow histograms are gated on V γ 2⁺ CD3⁺ Lymphocytes. C,E) Graphs of phenotypic subsets of V γ 2V δ 2 T cells in blood (C) and BAL cells (E) following primary (I) or secondary (II) exposure to the indicated strains of *Listeria monocytogenes*. Note that there are greater numbers of memory-type (CD45RA-CD27⁺) V γ 2V δ 2 T cells in macaques exposed to HMBPP-producing *Listeria* than in those exposed to HMBPP-deficient strain (*, p < 0.05). F) Gating strategy for determining CD45RA-CD27⁺CD28⁻ and CD45RA CD27⁺ CD28⁺ subsets of memory V γ 2V δ 2 T cells and representative flow histograms of CD28⁺ V γ 2V δ 2 memory T cells in BAL of a macaque exposed to *Listeria* Δ actA prfA* (black histogram) or a macaque exposed to *Listeria* Δ actA Δ gcpE prfA* (Grey histogram). Isotype control for CD28 expression is shown as white histogram. G) Mean percentages of CD28⁺ and CD28⁻ V γ 2V δ 2 memory T-cell subsets in BAL of macaques following primary (I) or secondary (II) challenge with indicated strains of *Listeria monocytogenes*. Data from primary exposure were generated from blood collected on day 42-post infection, and those from secondary exposure were derived from blood collected on day 84. Significant difference between the groups was seen on day42 (*, p < 0.05), and a similar trend was noted on day 84 despite no statistical significance.

One of the hallmarks of memory T cells is their ability to expand greatly in number following re-exposure to antigen. Previous studies have determined that CD28 co-stimulation to memory T cells correlates significantly with an ability to proliferate following re-exposure to antigen⁹⁷. We compared the expression of CD28 on V γ 2V δ 2 memory T cells in *Listeria* exposed macaques. Interestingly, phenotypic analysis of memory-type V γ 2V δ 2 T cells demonstrated that macaques exposed to HMBPP-producing *Listeria* shifted toward the development of CD28- memory pool in the pulmonary compartment whereas primary and secondary exposure of macaques to HMBPP-deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ displayed a higher percentage of CD28+ memory V γ 2V δ 2 T cells (Figure 11G). Previous studies examining the memory phenotypes of V γ 2V δ 2 T cells have suggested the loss of CD28 expression correlates with a later memory phenotype in these cells⁹⁸, suggesting that HMBPP stimulation skews V γ 2V δ 2 cells to develop later memory phenotypes following primary and secondary exposure, while V γ 2V δ 2 cells activated through *Listeria*-mediated IPP production exhibit development of earlier memory phenotypes.

CHAPTER 4

Determining the effect of repeated *Listeria monocytogenes*-based vaccine exposure on V γ 2V δ 2 T cell phosphoantigen sensitivity

*Portions of the text and/or figures were reprinted from **J. Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing *Listeria* mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. *J Leukoc Biol.* 2014 Dec;96(6):957-67. and B. Ryan-Passey, **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* Δ actA prfA*." *J. Immunol* 2012 Aug 1;189(3):1285-93.

4.1. Rationale

Current vaccination efforts for Tuberculosis involve the administration of a second or third immunogenic stimulus to generate strong memory responses to the antigens in the vaccine^{47,99-101}. These methods are commonly viewed as beneficial to the immune system and help to augment or diversify T cell responses and induce a higher titer of antibody production. Many other vaccination regimens involve repeated administration of the vaccine in order to induce sufficient protection against the invading organism^{102,103}. It has been well-established that maintenance of immunity to the toxins produced by tetanus requires booster doses to prevent waning immunity. In addition, a resurgence in cases of adult pertussis has prompted the CDC to consider the benefits of administering booster doses of the Tdap vaccine to adults to minimize adult pertussis cases as well as adult-to-child transmission of pertussis¹⁰⁴. The hepatitis B vaccine typically requires 3 doses to achieve maximal immunogenicity¹⁰². Immunity to Hepatitis

B also wanes over time, and can require booster doses to augment antibody responses later in life^{102,105}. Similarly, vaccines that target *Neisseria meningitides* and prevent bacterial meningitis require multiple doses to induce sufficient immunity that does not last the life of the host¹⁰⁵. Increasing studies have determined that newer vaccines such as those for cervical cancer may require repeated doses to maintain the protection induced by these constructs¹⁰⁶.

Despite the beneficial effects of repeated vaccine administration in a number of vaccine models known to induce strong antibody responses, BCG revaccination programs has faced predominantly negative results⁹⁹. BCG revaccination has shown little or no demonstrable increase in protection against Tuberculosis following revaccination of adolescents or adults who have received BCG as an infant, nor a beneficial protection of revaccinating adults upon conversion from TST-positive to TST-negative^{107,108-110}. Despite these negative results, immunologic studies have indicated increases in some of the known immune correlates of protection, such as CD4 T cell-mediated IFN γ release following *M.tb.* antigen re-exposure, or increased production of anti-mycobacterial antibodies¹¹¹. Although repeated antigen exposure of B cells results in increased antibody production and affinity maturation, repeated stimulation of antigen-specific T cells is associated with T cell hyporesponsiveness, anergy, and activation-induced cell death^{112,113}. Studies examining chronically infected individuals with Hepatitis C, chronic hepatitis B infection, or HIV infection have demonstrated T cell dysfunction and hyporesponsiveness of antigen-specific T cells¹¹⁴⁻¹¹⁶. A recent cancer vaccination study demonstrated that the persistence of vaccine antigens in the body resulted in T cell migration to the site of vaccination, dysfunction, and clonal deletion of these antigen-specific T cells,

suggesting that chronic or repeated stimulation of these T cells may be detrimental to inducing a strong, long-lasting, protective response¹¹⁷.

Multiple studies have indicated that V γ 2V δ 2 T cell number and responses may be adversely affected by repeated exposure to phosphoantigens^{63,95,118}. Because isoprenoid production is essential for proper growth of bacteria, many organisms produce IPP through the mevalonate pathway or HMBPP through the MEP pathway⁵⁶. Thus, humans are exposed to a variety of pathogenic and non-pathogenic bacteria harboring large doses of these isoprenoids throughout their life. Indeed, studies examining the V γ 2V δ 2 T cell population in the fetus, neonate, adult, and elderly demonstrate a decline in naïve V γ 2V δ 2 T cells and an increase in memory V γ 2V δ 2 T cells as individuals age⁶³. Animal studies that have used repeated administration of synthetic phosphoantigens have shown that the magnitude of V γ 2V δ 2 T cell expansion decreases with repeated high dose stimulation with phosphoantigens, suggesting that although V γ 2V δ 2 T cell stimulation may result in rapid effector function and memory development upon secondary exposure to phosphoantigens, repeated phosphoantigen stimulation could result in decreased V γ 2V δ 2 T cell proliferation and response, rendering these cells less useful for vaccination purposes⁹⁵. Thus, repeated administration of BCG or other HMBPP-producing pathogens, may decrease the host ability to detect and control *M.tb.* infection due to phosphoantigen-induced exhaustion of V γ 2V δ 2 T cells.

Because V γ 2V δ 2 T cells are capable of responding to a number of different phosphoantigen stimuli, we sought to determine whether *in vivo* antigen stimulation of V γ 2V δ 2 T cells through *Listeria monocytogenes*-based vaccine vectors expressing the potent phosphoantigen resulted in T cell hyporesponsiveness. Using *Listeria monocytogenes* strains deficient for HMBPP

synthase and comparing it to the parental strain that contains all the enzymes necessary for HMBPP production intact, we examined the consequences of repeated administration of the *Listeria*-based vaccines on the V γ 2V δ 2 T cell expansion and responses to phosphoantigens re-exposure.

4.2. Decreased expansion and pulmonary accumulation of V γ 2V δ 2 T cells following repeated administration of *Listeria monocytogenes* $\Delta actA prfA^*$

An understanding of the dynamics of activation, expansion, memory formation, and exhaustion of V γ 2V δ 2 T cells is necessary for developing optimal vaccination regimens that would induce strong V γ 2V δ 2 memory T cell responses without inducing V γ 2V δ 2 T cell exhaustion and activation-induced cell death. Previous studies have demonstrated that while a single administration of synthetic phosphoantigen in combination with IL-2 results in massive V γ 2V δ 2 T cell expansion, repeated phosphoantigen administration resulted in decreased expansion of these cells with each administration⁹⁵. We sought to determine whether repeated administration of a vaccine vector capable of producing HMBPP would result in a similar decline in V γ 2V δ 2 T cell expansion following repeated administration. To do this, we examined the kinetics of pulmonary V γ 2V δ 2 T cell responses following intratracheal administration of recombinant *Listeria monocytogenes* $\Delta actA prfA^*$ expressing *M.tb.* antigens ESAT6 and Ag85B. Peak V γ 2V δ 2 T cell percentages in bronchoalveolar lavage fluid rose significantly following the first administration, reaching an average of 42% of all T cells in the lungs (Figure 13B). A second exposure to the *Listeria*-based vaccine induced an expansion of higher magnitude than the first response ($p < 0.05$). Interestingly, a third administration of the *Listeria*-based vaccine induced a

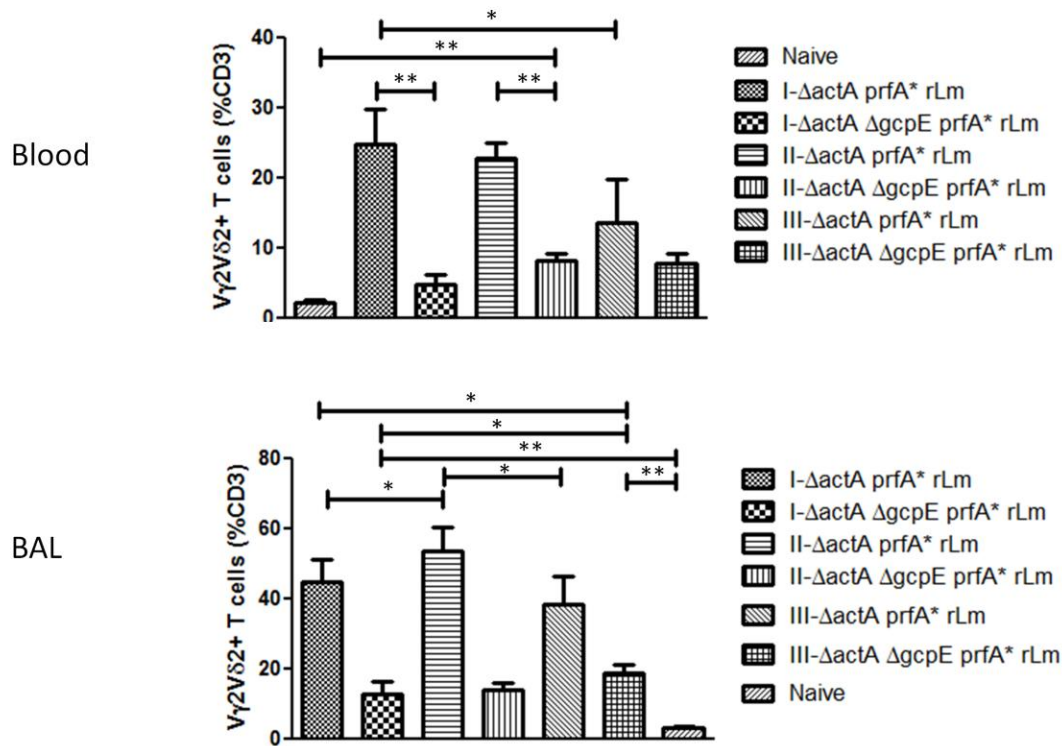


Figure 12. Peak *in vivo* expansion and pulmonary accumulation of Vγ2Vδ2 T cells diminishes with repeated vaccination with HMBPP producing *Listeria ΔactA prfA, but not with HMBPP deficient *Listeria ΔactA ΔgcpE prfA****

Peak percentages of Vγ2Vδ2 T cells in blood (A) and BAL (B) of rhesus macaques after primary (I), secondary (II) and tertiary (III) exposure to intratracheal administration of the indicated *Listeria*-based vaccine strains. Peak Vγ2Vδ2 T cell percentages were determined by surface cell staining of PBMCs with CD3 and Vγ2 antibodies and analysis by flow cytometry. Peak levels of Vγ2+ T cells were typically seen 7 days following inoculation. (*, $p < 0.05$; **, $p < 0.01$)

response of lower magnitude than the second, suggesting that repeated administration of the *Listeria*-based vaccine may have a detrimental effect on V γ 2V δ 2 T cell proliferation following re-stimulation. Circulating V γ 2V δ 2 T cell percentages rose following a single intratracheal administration of V γ 2V δ 2 T cells, but the magnitude of V γ 2V δ 2 T cell expansion in the peripheral blood declines significantly with each additional administration of the vaccine (Figure 12A). These data demonstrate that single administration of the HMBPP producing *Listeria*-based vaccine induces V γ 2V δ 2 T cell expansion with, while repeated administration results in decreasing V γ 2V δ 2 T cell proliferation.

4.3. Increased expansion and pulmonary accumulation of V γ 2V δ 2 T cells following repeated administration of HMBPP-deficient *Listeria monocytogenes* $\Delta actA \Delta gcpE prfA^*$

While both HMBPP producing *Listeria monocytogenes* and high potency phosphoantigens induce decreased proliferative responses from V γ 2V δ 2 T cells with repeated administration, the effect of low-potency phosphoantigens on V γ 2V δ 2 T cell proliferation and exhaustion has not been examined. Because the *Listeria monocytogenes* vaccine vector deficient for HMBPP production can induce activation and expansion of V γ 2V δ 2 T cells, we examined the effect of repeated administration of HMBPP-deficient *Listeria monocytogenes* $\Delta actA \Delta gcpE prfA^*$ expressing ESAT6 and Ag85B on the magnitude of V γ 2V δ 2 T cell responses. Pulmonary V γ 2V δ 2 T cells increase significantly above baseline following primary exposure to HMBPP-deficient *Listeria*-based vaccine (Figure 12B). Secondary exposure to the vaccine showed a comparable increase in V γ 2V δ 2 T cell percentage as the first exposure. Contrary to HMBPP-producing *Listeria*-based vaccine, tertiary administration of HMBPP-deficient *Listeria*-based vaccine

induced an increase in Vγ2Vδ2 T cell percentage compared to the first and second administration, suggesting that Vγ2Vδ2 T cells activated with the HMBPP-deficient *Listeria*-based vaccine maintain high sensitivity to phosphoantigens.

4.4. Decreased sensitivity of Vγ2Vδ2 T cells from *Listeria monocytogenes* ΔactA prfA* vaccinated macaques to ex vivo phosphoantigen stimulation

Because the magnitude of Vγ2Vδ2 T cell expansion decreases over time in macaques vaccinated with the HMBPP-producing *Listeria*-based vaccine, we examined whether cells derived from these macaques show decreased proliferative response following *ex vivo* stimulation with phosphoantigens. Stimulation of PBMCs with phosphoantigens in the presence of IL-2 and HMBPP or other phosphoantigens typically results in massive expansion of Vγ2Vδ2 T cells. Macaques that are naïve for phosphoantigen show potent responses to HMBPP stimulation. Because a previous study performed in our lab had demonstrated a protective effect of administering 1 dose of the *Listeria*-based Tuberculosis vaccine to macaques (Chen et. al. 2014 - manuscript in preparation), we compared the proliferative response of Vγ2Vδ2 T cells following stimulation with various doses of HMBPP in macaques vaccinated with 2 doses of the *Listeria*-based tuberculosis vaccine with macaques exposed to 3 doses. While 40ng/mL HMBPP is sufficient for stimulation and expansion of the vast majority of Vγ2Vδ2 T cells, significant proliferation of Vγ2Vδ2 T cells is seen after culture of PBMCs with lower concentrations of HMBPP (Figure 13A,B). Following repeated vaccination, however, most macaques show decreased percentages of Vγ2Vδ2 T cells proliferating in response to HMBPP stimulation (Figure 13B). Vγ2Vδ2 T cells derived from macaques vaccinated 3 times with HMBPP-producing

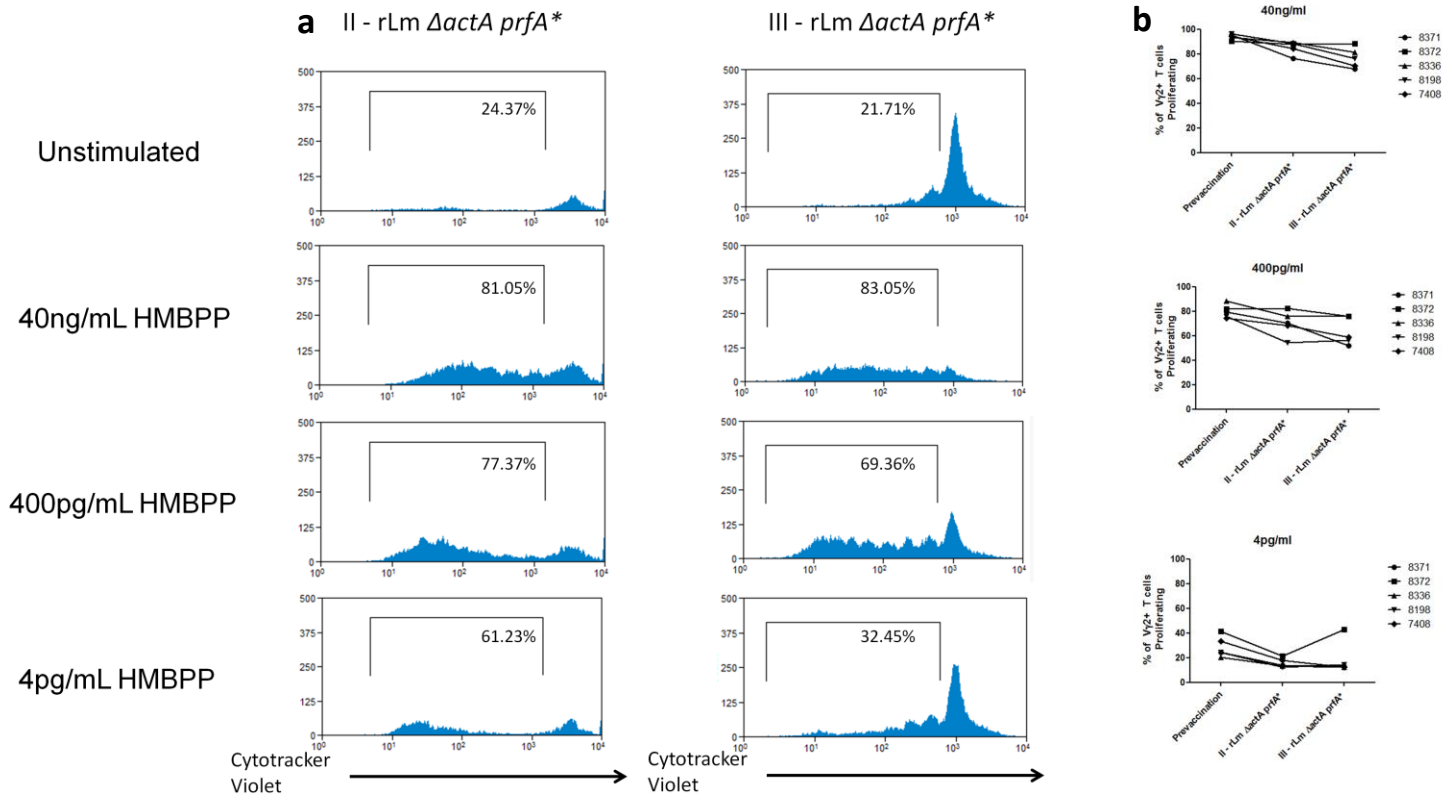


Figure 13. Decreased sensitivity of Vy2V62 T cells from *Listeria monocytogenes* $\Delta actA prfA^*$ vaccinated macaques to ex vivo phosphoantigen restimulation

A) Representative flow histograms of Vy2+ T cell proliferation following ex vivo stimulation of PBMCs from macaques after the second (II) or third (III) administration of HMBPP producing *Listeria*-based vaccine strain $\Delta actA prfA^*$. PBMCs derived from naïve or vaccinated macaques were stained with CytoTracker Violet dye (Invitrogen) and stimulated with the indicated concentrations of HMBPP in the presence of media containing 100U/mL rhIL-2 for 7 days. After 7 day culture, cells were washed, stained with CD3 and Vy2 fluorescent antibodies, fixed, and analyzed by flow cytometry. Proliferating cells were determined by CytoTracker Violet dye dilution. Percentages on the histogram represent the percentages of proliferating cells as determined through CytoTracker Violet dye dilution. B) Graphs of proliferative responses of individual Rhesus macaques prior to *Listeria*-based vaccination (pre-vaccination), after administration of 2 doses of the HMBPP producing *Listeria*-based vaccine through intratracheal inoculation (II), and after third (III) dose of the vaccine. Macaques were challenged with the indicated concentrations of HMBPP for 7 days in the presence of 100U/mL IL-2 and percentages of proliferating Vy2+ T cells were determined as described previously.

Listeria-based vaccines show decreased proliferative responses to both high and low dose phosphoantigen restimulation. Interestingly, a decreased proliferative response to low dose phosphoantigen is more pronounced than high dose stimulation, suggesting that repeated stimulation of V γ 2V δ 2 T cells with potent phosphoantigens may result in decreased sensitivity of V γ 2V δ 2 T cells to subsequent phosphoantigen exposure. By contrast, macaques receiving the HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ vaccine showed strong proliferative responses to HMBPP that were preserved after repeated vaccine exposure (Figure 14A). In addition, while the percentage and total number of V γ 2V δ 2 T cells in *ex vivo* PBMC cultures of macaques vaccinated with HMBPP-producing *Listeria* remained the same or decreased in number following 7 day culture in most cases, V γ 2V δ 2 T cell percentage and absolute numbers in *ex vivo* PBMC cultures of macaques vaccinated with HMBPP-deficient *Listeria* increased significantly in number (Figure 14B) suggesting that V γ 2V δ 2 T cells activated *in vivo* with HMBPP may undergo activation-induced cell death when re-stimulated *ex vivo*, resulting in fewer total cells after 7 days.

We next examined whether V γ 2V δ 2 T cells from macaques vaccinated with HMBPP producing *Listeria*-based vaccines would show decreased cytokine production following HMBPP stimulation. Surprisingly, we see a significant decrease in TNF α production from V γ 2+ T cells from macaques vaccinated with 3 doses of the *Listeria*-based vaccine compared to macaques vaccinated with 2 doses (Figure 15A). Similar decreases in IFN γ secretion were seen in these macaques (data not shown), suggesting that V γ 2V δ 2 T cells from macaques vaccinated with 3 significantly less amounts of Th1 cytokines. Although V γ 2V δ 2 T cells from macaques repeatedly

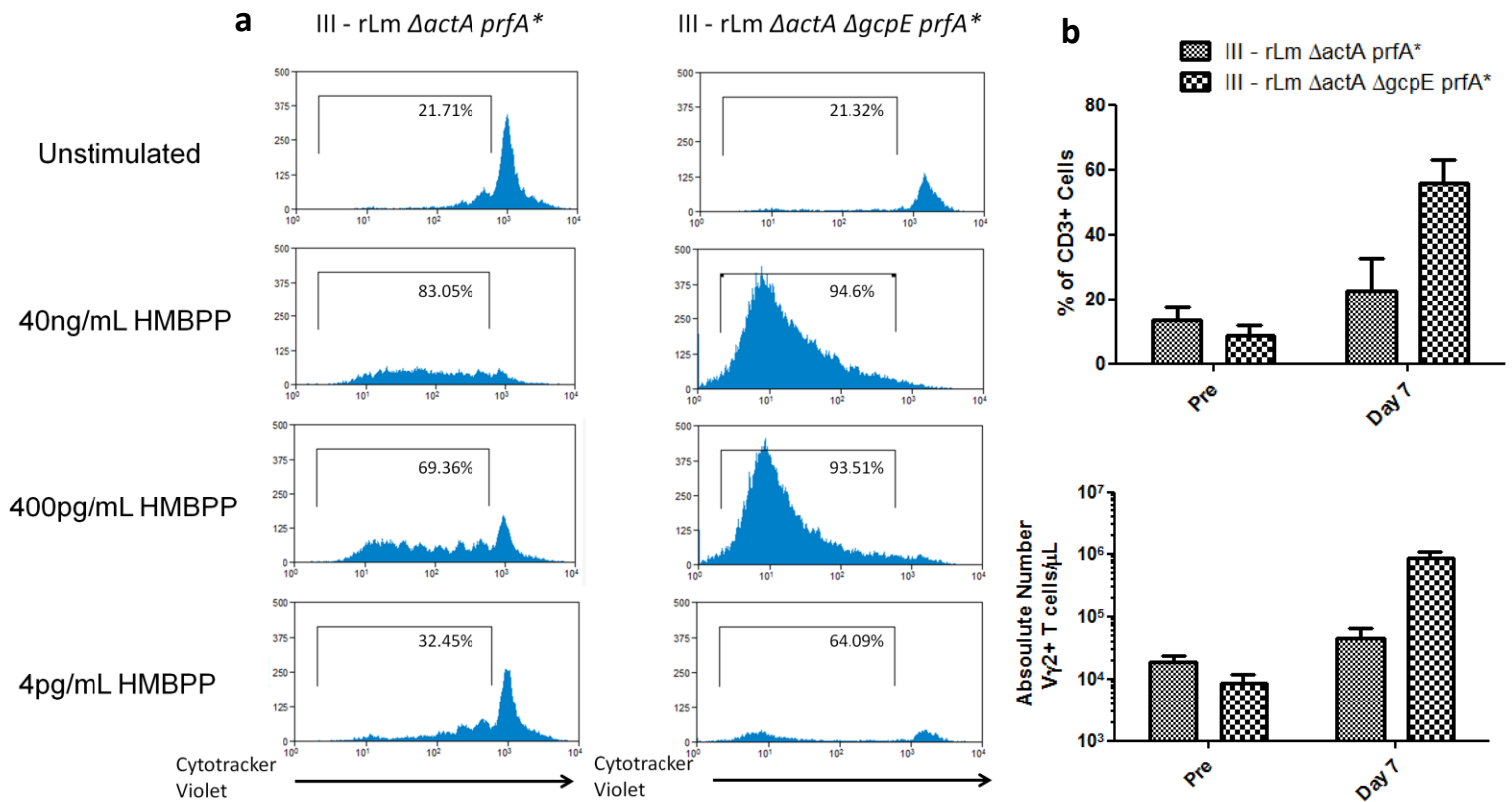


Figure 14. ***Ex vivo* proliferative responses of Vy2V δ 2 T cells maintained in *Listeria monocytogenes* $\Delta actA$ $\Delta gcpE$ *prfA** vaccinated macaques**

A) Representative flow histograms of Vy2+ T cell proliferation following ex vivo stimulation of PBMCs from macaques after the third (III) administration of the indicated *Listeria*-based vaccine strain. B) Percentages (top) and absolute number (bottom) of Vy2+ T cells in *ex vivo* cultures of PBMCs derived from macaques vaccinated 3 doses (III) of HMBPP producing or HMBPP deficient *Listeria*-based vaccine strains before and after restimulation with 40ng/mL HMBPP (top), Percentages of Vy2+ T cells in PBMCs prior to restimulation were determined by staining 0.5x10⁶ PBMCs for CD3 and Vy2 immediately following PBMC isolation.

doses of the *Listeria*-based Tuberculosis vaccine are less sensitive to HMBPP and secrete

challenged with HMBPP synthase-deficient *Listeria*-based Tuberculosis vaccines also show

decreases in sensitivity to HMBPP stimulation, these generally remain more responsive to low

dose HMBPP stimulation than macaques repeatedly challenged with the HMBPP producing

Listeria-based vaccine (Figure 15B), suggesting that these cells may respond to a lower amount

of phosphoantigen exposure *in vivo*.

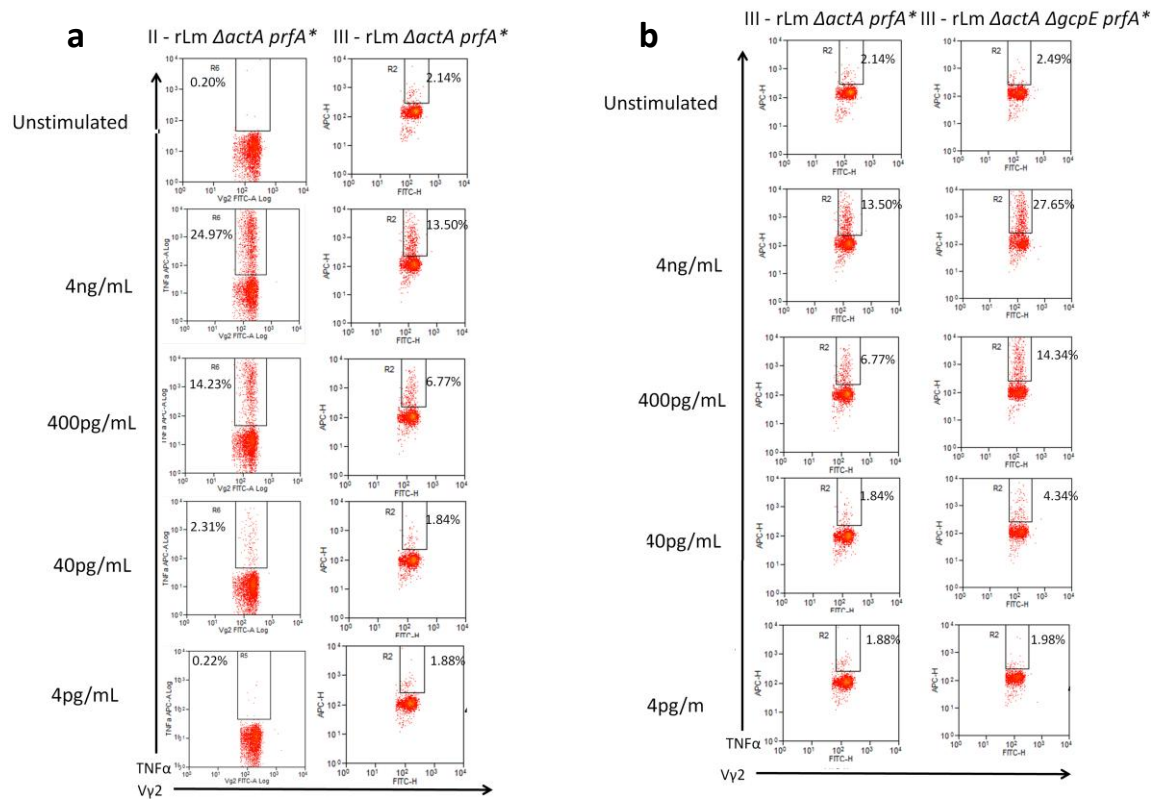


Figure 15. Cytokine production of Vy2V δ 2 T cells from macaques repeatedly vaccinated with *Listeria monocytogenes* Δ actA prfA* after ex vivo phosphoantigen restimulation A) Representative flow histograms of Vy2+ T cell TNF α production by PBMCs from macaques after the second (II) or third (III) administration of the HMBPP producing *Listeria*-based vaccine strain. Flow histograms were gated on CD3+ lymphocytes. Percentages represent the percentages of Vy2+ T cells that stain positive for TNF α . B) Representative flow histograms of Vy2+ T cell TNF α production by PBMCs from macaques after the third (III) administration of the HMBPP producing (left) or HMBPP deficient (right) *Listeria*-based vaccine strain.

CHAPTER 5

Investigating the contribution of HMBPP production on *Listeria monocytogenes*-based vaccine efficacy

5.1. Rationale

To date, all vaccination efforts in clinical trials for controlling *Mycobacterium tuberculosis* have included the use of administering a *Mycobacterium* such as the *Mycobacterium bovis* BCG vaccine to infants followed by a construct designed to boost vaccine-induced immunity generated by the initial vaccine^{46,47}. Many other laboratories searching for replacement vaccines for the BCG vaccine look to other species of the *Mycobacterium* or genetically attenuated forms of *Mycobacterium tuberculosis* itself¹¹⁹⁻¹²¹. All of these organisms express the MEP pathway of isoprenoid biosynthesis and induce strong expansion of V γ 2V δ 2 T cells in hosts containing these cells⁵⁶. Our work examining the consequences of repeated exposure to the HMBPP producing *Listeria monocytogenes*-based vaccine delivery system suggests that priming hosts with an HMBPP producing organism and providing booster doses with an organism that expresses HMBPP may have a detrimental effect to the anti-microbial activity of these cells. Indeed, our studies demonstrate that the V γ 2V δ 2 T cells in macaques stimulated repeatedly with HMBPP-producing *Listeria*-based Tuberculosis vaccines so hyporesponsiveness and decreased cytokine secretion following HMBPP re-exposure in comparison to cells taken from naïve macaques. Interestingly, the *Listeria monocytogenes* strain lacking the enzyme necessary for HMBPP production is still capable of stimulating V γ 2V δ 2 T cell activation and memory develop, although expansion occurs to a significantly attenuated magnitude. V γ 2V δ 2 T cells

derived from macaques receiving this HMBPP-deficient *Listeria*-based Tuberculosis vaccine maintain high sensitivity to HMBPP following repeated administration of the vaccine. In our current study, we examine the consequence of *Mycobacterium tuberculosis* challenge of macaques vaccinated with three doses of the HMBPP producing and HMBPP deficient *Listeria* based Tuberculosis vaccines to compare the impact that V γ 2V δ 2 T cell hyporesponsiveness induced by excess HMBPP exposure may have had on vaccine efficacy.

5.2. Tuberculosis-induced Pathology and Bacterial Burden Decreased by HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ -based vaccine but not HMBPP producing *Listeria* $\Delta actA prfA^*$ -based vaccine

To determine the impact of vaccine-induced immune responses against the *Listeria*-based Tuberculosis vaccines, Rhesus macaques were vaccinated 3 times with HMBPP producing *Listeria* $\Delta actA prfA^*$ -based vaccine expressing Tuberculosis antigens ESAT6 and Ag85B or HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ -based vaccine expressing the same antigens according to the schedule indicated in figure 8. Macaques were challenged with 80 CFU of *Mycobacterium tuberculosis* Erdman strain in the right caudal lung lobe. Macaques were monitored for an 8 week period for respiratory distress, lethargy and weight loss. Throughout the 8 week period, 1 naïve macaque and 1 macaque vaccinated with the *Listeria* $\Delta actA prfA^*$ -based vaccine reached the humane endpoint criteria of 20% weight loss during week 8 and were euthanized. 8 weeks following infection, all macaques were euthanized to compare the severity of gross pathology following infection. All naïve Rhesus macaques infected with *M.tb.* Erdman presented with coalescing granulomas and extensive damage extensive damage to

right caudal lung lobe as well as granulomatous lesions in all lung lobes. While macaques vaccinated with the HMBPP-producing *Listeria* $\Delta actA$ *prfA**-based vaccine showed extensive damage to the right caudal lung lobe of similar severity to naïve Rhesus macaques, gross pathologic damage was restricted to the right caudal, or right caudal and right medial lung lobes in these macaques. Surprisingly, Rhesus macaques vaccinated with the HMPP deficient *Listeria* $\Delta actA$ $\Delta gcpE$ *prfA**-based vaccine showed significantly attenuated lung pathology in the right caudal lung lobe and restriction of pulmonary lesions to the right lung lobes in all cases (Figure 16). Furthermore, while naïve macaques showed extensive extrapulmonary granulomas in the spleen, liver, and kidneys, vaccinated macaques demonstrated a significant decrease in extrapulmonary gross lesions (Table IV). Collectively, these data demonstrate macaques vaccinated with the *Listeria*-based Tuberculosis vaccine deficient for HMBPP production exhibit a more significant protection against *M.tb.*-induced pathology and dissemination than the HMBPP producing *Listeria*-based Tuberculosis vaccine.

Organ Involved	rLm x 1	rLm x 3	rLm $\Delta gcpE$ x 3	Control
Spleen	1/5 (20%)	1/6 (16.7%)	2/6 (33.3%)	8/12 (66.7%)
Liver	1/5 (20%)	2/6 (33.3%)	3/6 (50%)	9/12 (75%)
Kidney	2/5 (40%)	1/6 (16.7%)	2/6 (33.3%)	3/12 (25%)

Table IV. Limited Extrapulmonary Dissemination in Vaccinated Macaques
Number and percentages of Rhesus macaques with granulomas detected on gross examination after necropsy

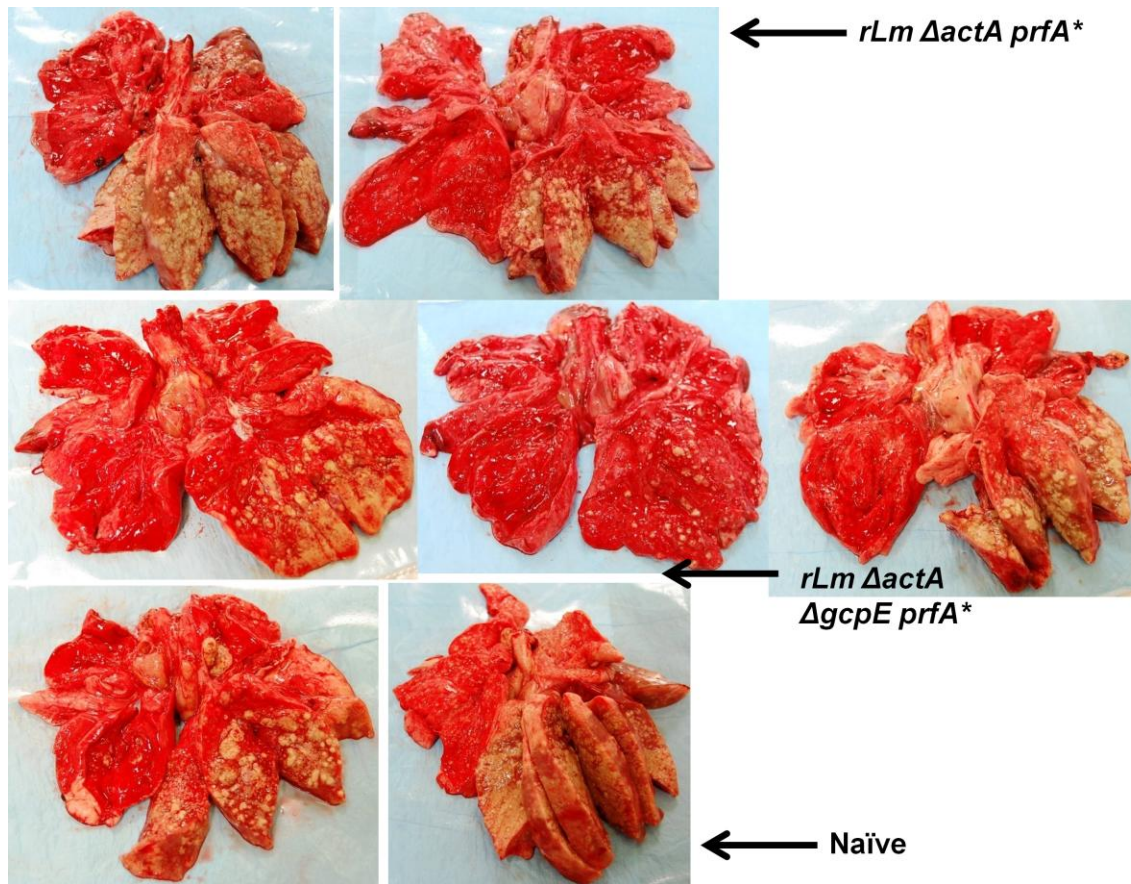


Figure 16. Gross pathology and bacterial burden of Rhesus macaques vaccinated with 3 doses of HMBPP producing *Listeria*-based Tuberculosis vaccine or HMBPP deficient *Listeria*-based Tuberculosis vaccine.

Representative images of lungs vaccinated with 3 doses of HMBPP producing *Listeria*-based Tuberculosis vaccine *rLm ΔactA prfA** (top), HMBPP deficient *Listeria*-based Tuberculosis vaccine *rLm ΔactA ΔgcpE prfA** (middle) or naïve macaques (bottom). Macaques were euthanized 8 weeks following Tuberculosis infection in the right caudal lobe. Lungs were harvested and each lobe was cut open and examined for gross granulomatous lesions in the lung parenchyma.

5.3. Decreased Bacterial Burden in lungs of macaques vaccinated by HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ -based vaccine but not HMBPP producing *Listeria* $\Delta actA prfA^*$ -based vaccine

To determine the impact of the *Listeria*-based vaccines on *M.tb.* bacillary load in the lungs throughout the pulmonary Tuberculosis infection of macaques, Bronchoalveolar Lavage was performed on macaques, and BALF was placed on 7H11 media to determine CFU counts in macaque lungs. While BAL CFU counts remained low throughout the 6 weeks macaques vaccinated with the HMBPP deficient *Listeria*-based Tuberculosis vaccine were studied, naïve macaques and macaques vaccinated with the HMBPP producing *Listeria*-based vaccine showed accumulation of *M.tb.* bacilli in the BALF of macaques starting at week 4 post-infection (Figure 17A). To determine bacillary load in lungs, tissue samples taken from the right caudal, right medial, right cranial, and left caudal lobes of the lungs of necropsied macaques were homogenized CFU counts retrieved from tissue sections in each lobe were determined. Macaques vaccinated with the HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ -based vaccine also demonstrated significantly fewer decreased bacterial burdens in tissue homogenates generated from each of the lung lobes (Figure 17B).

5.4. PPD-specific $\alpha\beta$ T cell responses decreased in macaques vaccinated with HMBPP producing *Listeria* $\Delta actA prfA^*$ -based vaccine and HMBPP deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ -based vaccine

Many previous studies examining correlates of *M.tb.*-induced severity have established that hyperactivation of the adaptive immune system can result in severe immunopathology and extensive lung damage in *M.tb.*-infected individuals. Vaccine efficacy of BCG and other

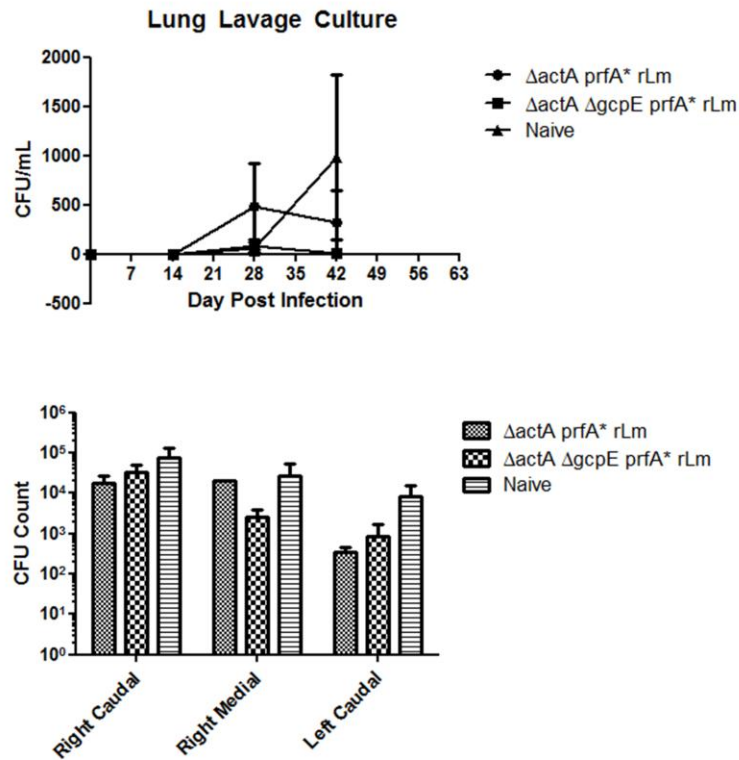


Figure 17. **Bacterial burden of Rhesus macaques vaccinated with 3 doses of HMBPP producing *Listeria*-based Tuberculosis vaccine or HMBPP deficient *Listeria*-based Tuberculosis vaccine.**

A) Bacterial burden of naïve and vaccinated macaques obtained from bronchoalveolar lavage (BAL) fluid. B) CFU counts from tissue homogenization and plating of serial dilutions of 1 cm³ section of tissue derived from the indicated lung lobes after gross pathologic analysis of necropsied macaques.

Tuberculosis vaccine regimens have been correlated with a decreased PPD-specific $\alpha\beta$ T cell response. To determine whether the increased pathology seen in the macaques vaccinated with the HMBPP-deficient *Listeria*-based vaccine was due to an increased PPD-specific $\alpha\beta$ T cell response following infection, we examined the pulmonary PPD-specific $\alpha\beta$ T cell responses of macaques throughout infection. By week 4, all macaques exhibited a decrease in weight and an increase in erythrocyte sedimentation rate (data not shown), suggesting an ongoing inflammatory response in all macaques by this timepoint. At week 4, macaques vaccinated with either the HMBPP producing or HMBPP deficient *Listeria*-based vaccines demonstrate decreased pulmonary PPD-specific Th1 responses compared to naïve macaques (Figure 18 top left and top middle histograms). By week 6, however, pulmonary PPD-specific PPD-specific Th1 responses are high in all groups, reaching 45-50% of all $\alpha\beta$ T cells in the pulmonary compartment. These data suggest that both the HMBPP producing or HMBPP deficient *Listeria*-based vaccines downmodulate PPD-specific $\alpha\beta$ T cells early on in Tuberculosis infection to a similar degree, but neither are incapable of preventing severe immunopathology and dissemination later in infection in the current model.

5.5. Pulmonary and Circulating V γ 2V δ 2 T cells in macaques vaccinated with HMBPP producing *Listeria* Δ actA prfA*-based vaccine decrease throughout *M.tb.* challenge

Macaques vaccinated multiple times with the HMBPP producing *Listeria*-based Tuberculosis vaccine demonstrated decreased sensitivity to phosphoantigen restimulation. We sought to determine whether there was a significant difference in the kinetics or activity of V γ 2V δ 2 T cells

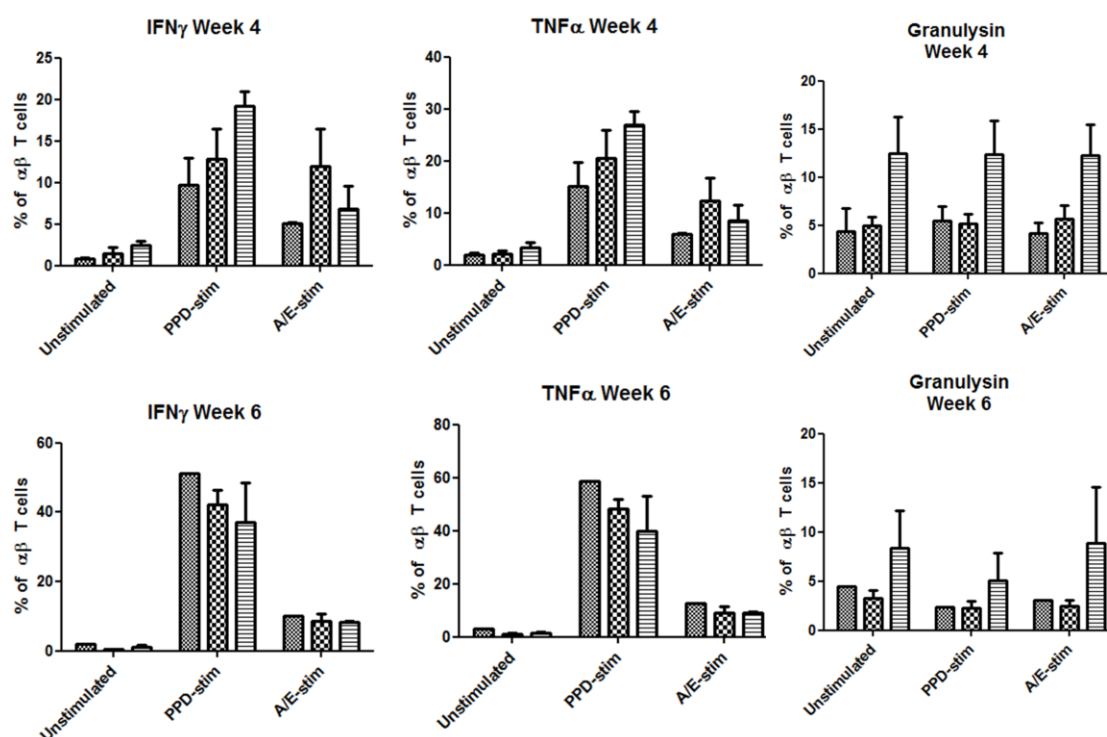


Figure 18. Antigen-specific $\alpha\beta$ T cell responses throughout *M.tb.* Challenge
 Histograms of percentages of IFN γ , TNF α or Granulysin-producing $\alpha\beta$ T cells in the pulmonary compartment during Week 4 (top) or Week 6 (bottom) post-infection. PBMCs derived from naïve or vaccinated *M.tb.*-infected macaques were stimulated with CD28 and CD49d in the absence (Unstimulated) or presence of 1 μ g/mL *M.bovis* PPD (PPD-stim) or pooled peptides derived from ESAT6 and Ag85B (A/E-stim) for 6 hours and percentages of $\alpha\beta$ T cells expressing Th1 or Cytolytic cytokines were determined by FACS analysis.

following Tuberculosis challenge of these vaccinated macaques. The percentage and absolute number of circulating V γ 2V δ 2 T cells were significantly higher in macaques vaccinated with the HMBPP producing *Listeria*-based Tuberculosis vaccine than in macaques vaccinated with the HMBPP deficient *Listeria*-based Tuberculosis vaccine prior to infection (Figure 19A). Macaques vaccinated with the HMBPP deficient *Listeria*-based Tuberculosis vaccine had significantly higher levels of circulating V γ 2V δ 2 T cells than naïve macaques. Surprisingly, macaques vaccinated with HMBPP producing *Listeria*-based Tuberculosis vaccine showed a progressive decrease in circulating V γ 2V δ 2 T cell percentage and absolute number throughout infection with significant decreases in circulating V γ 2V δ 2 T cell numbers occurring as early as 14 days post Tuberculosis challenge. By contrast, macaques vaccinated with the HMBPP deficient *Listeria*-based Tuberculosis vaccine saw increases in circulating V γ 2V δ 2 T cells throughout infection. Naïve macaques also showed a significant increase in circulating V γ 2V δ 2 T cells during infection, although this increase in circulating V γ 2V δ 2 T cells occurs later in infection than in macaques vaccinated with the HMBPP deficient *Listeria*-based vaccine.

Similar to circulating V γ 2V δ 2 T cells, pulmonary V γ 2V δ 2 T cells in vaccinated macaques were more abundant than in naïve macaques, and macaques vaccinated with the HMBPP-producing vaccine show decreases in percentage throughout *M.tb.* infection, although these decreases were less robust than changes seen in blood. Collectively, these data indicate a high representation of circulating and pulmonary V γ 2V δ 2 T cells in macaques that received the HMBPP producing *Listeria*-based Tuberculosis vaccine that decreases throughout *M.tb.* infection, while macaques that received the HMBPP deficient *Listeria*-based Tuberculosis vaccine show a lower level of V γ 2V δ 2 T cells pre-infection that increases throughout infection.

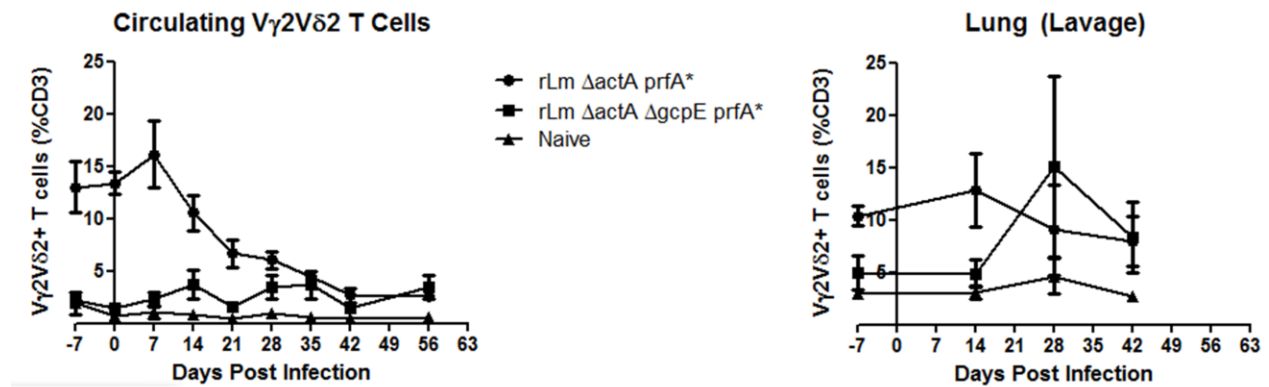


Figure 19. **Pulmonary and Circulating V γ 2V δ 2 T cells decrease throughout *M.tb.* infection of HMBPP producing *Listeria*-based Tuberculosis Vaccinated Macaques**
 Graphs of V γ 2V δ 2 T cell percentage in Blood (A) or BAL (B) of naïve or vaccinated Rhesus macaques throughout *M.tb.* infection. PBMCs or BAL lymphocytes were isolated from macaques at the indicated times, stained for antibodies against CD3 and V γ 2, and analyzed by FACS.

5.6. Increased apoptosis in V γ 2V δ 2 T cells derived from macaques vaccinated with HMBPP producing *Listeria* Δ actA prfA*-based vaccine during M.tb. infection

Because persistent antigen presence and repeated activation of antigen-specific T cells has been associated with activation-induced cell death in a variety of models, we examined whether the decrease in circulating V γ 2V δ 2 T cells throughout infection of the macaques vaccinated with HMBPP producing *Listeria*-based Tuberculosis vaccine was due to an increase in cell death. Because V γ 2V δ 2 T cells increase following infection until week 2, and subsequently in activated caspase-3 in V γ 2V δ 2 T cells. While PBMCs harvested from macaques vaccinated with the HMBPP producing *Listeria*-based Tuberculosis vaccine showed low levels of activated caspase-3 in all groups, the majority of pulmonary V γ 2V δ 2 T cells from macaques vaccinated with the HMBPP producing *Listeria*-based Tuberculosis vaccine expressed activated caspase-3 (Figure 20). Activated caspase-3+ V γ 2V δ 2 T cells were less abundant in naïve macaques and macaques vaccinated with the HMBPP-deficient *Listeria*-based vaccine, suggesting that these cells are less likely to undergo activation-induced cell death *in vivo*. *Ex vivo* antigen restimulation of the peripheral blood mononuclear cells also results in a dramatically increased percentage of apoptotic V γ 2V δ 2 T cells as opposed to naïve macaques or macaques vaccinated with HMBPP deficient *Listeria*-based vaccine. Collectively, these data suggest that repeated phosphoantigen stimulation through *Listeria*-mediated HMBPP production leads to increased activation-induced cell death following antigen re-exposure. This activation-induced cell death may decrease the ability of macaques to

limit *Mycobacterium tuberculosis* growth early in infection. This hypothesis will be discussed further in Chapter 6.

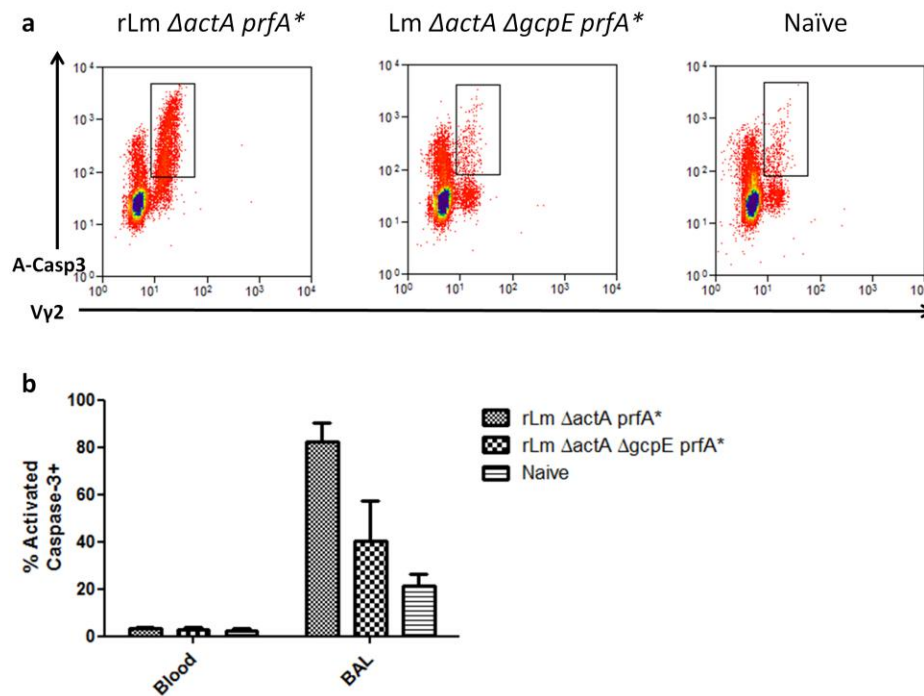


Figure 20. High Activated Caspase-3+ expression from Vγ2Vδ2 T cells of macaques vaccinated with HMBPP producing Listeria-based Tuberculosis Vaccinated Macaques

A) Representative histograms of pulmonary Vγ2+ T cells expressing activated caspase-3. BAL cells were harvested from macaques on day 14 following *M.tb.* infection, fixed with Foxp3 Cytofix/Cytoperm (Biolegend) and stained for antibodies against CD3, Vγ2 and activated Caspase-3. Histograms generated from FACS plots gated on CD3+ lymphocytes. B) Bar blots of Activated Caspase-3+ Vγ2+ T cells in blood (left) and BAL (right) of naïve or vaccinated rhesus macaques on day 14 following *M.tb.* infection. Percentages indicate percent of total CD3+ Vγ2+ T cells that stain positive for activated caspase-3.

CHAPTER 6

Discussion

6.1. In vivo expansion of V γ 2V δ 2 T cells after intratracheal administration of attenuated *Listeria monocytogenes* Δ actA prfA*

The current study is the first to examine the safety and immunogenicity of a *Listeria*-based vaccine vector into the lungs of macaques. Contrary to the attenuated strains of *Listeria monocytogenes* deficient for alanine production¹²², our particular strain of *Listeria monocytogenes* used is replication competent in the absence of any particular supplementation, suggesting that bacilli will be able to complete multiple rounds of replication *in vivo* without supplementation, but virulence will be decreased due to the inability of the organism to spread from cell to cell through actin nucleation and motility^{72,73}. Macaques showed no signs of clinical distress following the vaccination, and developed robust V γ 2V δ 2 T cell activation and accumulation in the pulmonary compartments. Interestingly, the percentage of V γ 2V δ 2 T cells that accumulate in the pulmonary compartment following pulmonary vaccination was significantly higher than the levels seen following systemic administration of our attenuated *Listeria* vector (Figure 8 and Reference 61). Additionally, significant increases in the percentage and number of circulating V γ 2V δ 2 T cells are seen despite an inability to detect *Listeria* bacilli in the blood of macaques vaccinated through the intratracheal route. This suggests that V γ 2V δ 2 T cells may become activated in the pulmonary compartment or the hilar and tracheobronchial lymph nodes, and subsequently recirculate in the blood stream. Indeed, increases in activated V γ 2V δ 2 T cells were seen in the small and large intestine of 1 rhesus

macaque euthanized following vaccination with the attenuated *Listeria* based vector (data not shown), suggesting that these V γ 2V δ 2 T cells activated through pulmonary exposure to phosphoantigens may recirculate and traffic to other mucosal environments. Similarly, cynomolgus macaques colonized with *Helicobacter pylori* infection have been shown to be more resistant to developing active Tuberculosis infection when exposed to low dose *Mycobacterium tuberculosis* Erdman¹²³. Because *Helicobacter pylori* infection in macaques results in a significant increase in V γ 2V δ 2 T cells¹²⁴, the activation of these V γ 2V δ 2 T cells in cynomolgus macaques in the GI tract by *H.pylori* could result in increased pulmonary resistance against *M.tb.* through recirculation and trafficking out to the pulmonary compartment. Our current data provides further support for the hypothesis that activated V γ 2V δ 2 T cells significantly contribute to minimizing pulmonary damage following infection with *Mycobacterium tuberculosis* and possibly other HMBPP-producing pulmonary pathogens.

6.2. In vivo stimulation of V γ 2V δ 2 T cells through Mevalonate or MEP Pathway from *Listeria monocytogenes*

This study provides the first *in vivo* comparison of HMBPP-mediated V γ 2V δ 2 T cell expansion, differentiation, and memory formation following exposure to IPP-mediated V γ 2V δ 2 T cell stimulation in the context of a bacterial infection. *In vivo* expansion and effector-function maturation of V γ 2V δ 2 T cells during bacterial infections can be mediated by HMBPP or IPP^{125,126, 127,128}. It has been postulated that other speculative microbial antigens also contribute to immune expansion of $\gamma\delta$ T cells^{125,126}. Cytokines alone during infection have also been implicated for activation/expansion of V γ 2V δ 2 T cells⁵⁹. These fundamental immunologic questions have not been addressed in humans and animal models, and now the current study is

starting to dissect them in nonhuman primates using genetically-modified *Listeria monocytogenes*. Our *Listeria* $\Delta actA$ **$\Delta gcpE$** *prfA** mutant provides a unique opportunity to investigate the impact of IPP in *Listeria* on V γ 2V δ 2 T-cell immune responses, as *gcpE* knock out in *Listeria* $\Delta actA$ **$\Delta gcpE$** *prfA** would not further attenuate *Listeria* replication and infectivity *in vivo* while losing the ability to produce HMBPP, and therefore allow us to compare parental *Listeria* $\Delta actA$ *prfA** for studies comparing HMBPP and IPP-driven immune biology and responses of V γ 2V δ 2 T cells.

HMBPP-synthase-deficient *Listeria* $\Delta actA$ **$\Delta gcpE$** *prfA** showed a significant loss of the ability to induce *in vivo* expansion of V γ 2V δ 2 T cells in macaques compared to the parental strain. Such a loss was most striking in the circulation during the primary pulmonary exposure to $\Delta actA$ *Listeria* **$\Delta gcpE$** *prfA**, suggesting that HMBPP is needed to fully expand V γ 2V δ 2 T cells in primary *Listeria* infection of naïve macaques. Moderate increases in percentages of circulating V γ 2V δ 2 T cells were seen following secondary pulmonary exposure to HMBPP-deficient *Listeria* $\Delta actA$ **$\Delta gcpE$** *prfA**. These results suggest that IPP produced by *Listeria* or *Listeria*-mediated IPP induction in host cells contribute to activating V γ 2V δ 2 T cells, and that IPP-primed V γ 2V δ 2 T cells could undergo enhanced expansion after the secondary exposure to HMBPP-deficient *Listeria* $\Delta actA$ **$\Delta gcpE$** *prfA**. This notion is supported by the published observation that orthopoxvirus incapable of producing HMBPP appears to prime and activate V γ 2V δ 2 T cells perhaps through “cellular stress” production of IPP by infected cells^{129 130}. Such “cellular stress”-induced production of IPP stimulating V γ 2V δ 2 T cells has also been reported in the setting of flu and oncogenesis^{58,91}.

The current study also extends previous work by examining the role of microbial HMBPP in pulmonary V γ 2V δ 2 T cell responses during infections. Earlier studies have shown that infections with HMBPP-producing bacteria including *Listeria*, BCG and Mtb^{59,62,64} could remarkably activate and expand V γ 2V δ 2 T cells, and these activated $\gamma\delta$ T cells could readily traffic to and accumulate in the pulmonary compartment. Now the results from the current study suggest that bacterially-derived HMBPP and IPP delivered through the pulmonary mucosa greatly contributes to the pulmonary accumulation of V γ 2V δ 2 T cells after *Listeria* exposure. In addition, an increase in circulating V γ 2V δ 2 T cells occurs in response to pulmonary delivery of *Listeria*. The accumulation of V γ 2V δ 2 T cells in the blood likely occurs as a result of migration and recirculation of these clonally-expanded $\gamma\delta$ T cells, as $\Delta actA prfA^*$ *Listeria* is remarkably attenuated and we did not detect recoverable CFU of *Listeria* bacteria or large numbers of neutrophils/macrophages in BAL fluid or blood (data not shown). Since clonal expansion of V γ 2V δ 2 T cells is accommodated in lymphoid tissues instead of lung tissues, the increased numbers of V γ 2V δ 2 T cells in the pulmonary compartment may result from *Listeria*-driven expansion of these cells in peripheral lymphoid tissues including tracheal or nasal-associated lymphoid tissue (NALT) after intra-tracheal *Listeria* exposure. Our results consist with highly immunogenic features of $\Delta actA prfA^*$ *Listeria* as seen during intranasal immunization of mice⁷⁶, and implicate the utility of potential immunization for eliciting anti-microbial immune responses of V γ 2V δ 2 T effector cells in the pulmonary compartment^{64 131 61,132}.

Interestingly, the current study showed the predominant CD28- memory phenotype of V γ 2V δ 2 T cells in macaques re-exposed to *Listeria* $\Delta actA prfA^*$ while animals exposed to HMBPP-deficient *Listeria* $\Delta actA \Delta gcpE prfA^*$ maintained higher frequencies of CD28+ memory V γ 2V δ 2 T

cells. These results suggest that HMBPP in *Listeria* is a major driver polarizing cells toward a late-stage memory phenotype⁹⁸. While immunological memory is a hallmark of adaptive immune responses, accumulating evidence implicates that V γ 2V δ 2 T cells demonstrate adaptive features with immunologic memory-like responses in infections^{56,59,62,129}. Many previous studies have determined that CD28 signaling helps to mediate a more pronounced expansion of memory T cells following re-exposure, suggesting V γ 2V δ 2 T cells activated through an IPP-dependent mechanism may retain a greater capacity to expand and respond to subsequent antigen exposure. Indeed, V γ 2V δ 2 T cell exhaustion has been demonstrated by repeated exposure to potent synthetic phosphoantigen stimulation when pure compounds have been administered in combination with IL-2. Stimulation of V γ 2V δ 2 T cells using an IPP-producing bacterium or viral pathogen may serve as a mechanism through which V γ 2V δ 2 T cell exhaustion can be avoided or prevented. Given that immune memory often confers protection against re-infection, our finding provides rationale to manipulate HMBPP or IPP-specific $\gamma\delta$ T cells for immune intervention.

6.3. *V γ 2V δ 2 T cell hyporesponsiveness and exhaustion following repeated phosphoantigens exposure in HMBPP producing *Listeria* Δ actA prfA**

The current study demonstrates a potential detrimental effect of repeated vaccination with HMBPP-producing organisms on the host immune response of human and non-human primates due to the desensitization of V γ 2V δ 2 T cells to phosphoantigens. In response to repeated administration of a HMBPP-producing *Listeria*-based vaccine expressing *M.tb.* antigens ESAT6 and Ag85B, we see a decrease in the magnitude of expansion in the pulmonary and circulating compartments, decreased proliferative responses to antigenic stimulation, and decreased

cytokine production following antigen re-exposure. Clinical efforts to prevent spread of Tuberculosis involve the use of live *Mycobacterium bovis* BCG administration at doses that range from $1-8 \times 10^8$ CFU¹⁰⁷. As a result, significant phosphoantigen delivery occurs as a result of the initial BCG vaccination. Studies are ongoing to find a good candidate for a second agent, either viral or bacterial vector-delivered *M.tb.* antigens or subunit vaccines, to boost BCG-induced immunity⁴⁶. Our data suggests that high dose administration of a vector containing HMBPP as a booster agent may result in significant exhaustion of V γ 2V δ 2 T cells. Indeed, our preliminary data from Tuberculosis challenge of these macaques demonstrates a decrease in V γ 2V δ 2 T cell number throughout Tuberculosis infection in macaques repeatedly given Listeria-based vaccine vectors that express the potent phosphoantigen HMBPP, and that elimination of HMBPP production decreases activation-induced cell death and increases phosphoantigen-induced proliferation of these cells. Many human cohort studies have determined that revaccination with BCG does not increase protection against Tuberculosis¹³³. Repeated BCG at the high doses given may result in a similar exhaustion of V γ 2V δ 2 T cells as we have seen in our experiments. Our previous studies using purified synthetic phosphoantigen, Picostim, and IL-2 to treat cynomolgus macaques infected with *Mycobacterium tuberculosis* demonstrated significant protective effect of this compound in minimizing Tuberculosis-induced pathology and spread (Chen et. al. manuscript in preparation). In the previous studies, Picostim and IL-2 were given three times throughout tuberculosis infection at 2 week intervals following infection. Interestingly, while the first administration of Picostim and IL-2 caused a significant increase in pulmonary and circulating V γ 2V δ 2 T cells, subsequent treatments showed either diminished V γ 2V δ 2 T cell expansion or no V γ 2V δ 2 T cell expansion following treatment.

Previous studies using potent synthetic phosphoantigen BrHPP and IL-2 in healthy cynomolgus macaques showed that when the same dose of BrHPP and IL-2 is given to healthy macaques at 4 week or 8 week intervals, a expansion of V γ 2V δ 2 T cells occurs, followed by a sharp contraction in cell number. Peak V γ 2V δ 2 T cell expansion decreases in magnitude after each administration, and by dose 4, there is no significant increase in V γ 2V δ 2 T cells above baseline⁹⁵. Interestingly, a single treatment of macaques infected with *Yersinia pestis* was sufficient to significantly decrease pulmonary pathology associated with pneumonic plague, but was insufficient to prevent spread of pneumonic plague to other organs or prevent mortality⁶¹. Collectively, previous studies as well as our current data suggest that single high dose or repeated low dose phosphoantigen exposure appears to be beneficial to macaques, while repeated high dose potent phosphoantigen exposure seems to be detrimental to the host.

In addition to vaccine-mediated phosphoantigen exposure, many bacterial and protozoan pathogens use the MEP pathway for isoprenoid production and make HMBPP⁹². As a result, repeated infection with these microorganisms may also result in V γ 2V δ 2 T cell hyporesponsiveness, anergy, and activation-induced cell death. Indeed, studies examining fetal blood, neonates, children, adults, and the elderly have shown a progressive increase in V γ 2V δ 2 T cells represented in the blood as an individual develops^{134,135}. By adulthood, most V γ 2V δ 2 T cells are no longer naïve, suggesting that humans encounter many sources of V γ 2V δ 2 T cells throughout their lifetime¹¹⁸. Future studies examining the effect of V γ 2V δ 2 T cell exhaustion on vaccine efficacy will combine our previous experience of vaccine construction and phosphoantigen treatment of macaques to induce V γ 2V δ 2 T cell exhaustion through repeated

phosphoantigen exposure in macaques vaccinated with *Listeria*-based vaccines or the BCG vaccine to determine the impact of V γ 2V δ 2 T cell exhaustion on the efficacy of these constructs.

Our data suggests that repeated exposure to potent phosphoantigens results in decreased sensitivity of V γ 2V δ 2 T cells to infection. Due to the low infectious dose necessary for Tuberculosis infection, a high sensitivity to phosphoantigen would be beneficial for allowing rapid host detection of *Mycobacterium tuberculosis* by V γ 2V δ 2 T cells. While the exact mechanism through which V γ 2V δ 2 T cells contribute to decreasing *M.tb.* pathology is not well understood, activated V γ 2V δ 2 T cells are potent producers of Th1 cytokines IFN γ and TNF α ⁵⁹. IFN γ and TNF α are known to work in a synergistic manner to limit the intracellular growth of *M.tb.* inside macrophages and spread of *M.tb.* throughout the lung and extrapulmonary sites. In addition, V γ 2V δ 2 T cells produce a number of cytokines and chemokines that could allow for recruitment of other cell types to the site of infection⁵⁶. Indeed, preliminary data from our *Listeria*-based vaccine studies show an increase in CD3 lymphocyte number in the lung at 2-4 weeks following Tuberculosis challenge of macaques, and these CD3- lymphocytes express abundant amounts cytolytic molecules such as Granulysin and Perforin. These CD3- lymphocytes may have been recruited directly or indirectly by V γ 2V δ 2 T cells and could contribute greatly to limiting *M.tb.* infection. Further studies are needed to determine the chemoattractant molecules produced by V γ 2V δ 2 T cells upon activation as well as the phenotype and function of the CD3- lymphocytes that appear in *M.tb.*-infected lungs of macaques.

6.4. Phosphoantigen production in *Listeria*-based Tuberculosis Vaccine Efficacy

In Chapter 5, we examined the pathologic and immunologic consequences of *M.tb.* infection of rhesus macaques and the impact of *Listeria*-based Tuberculosis vaccines to minimize pathology. Our study was the first to challenge Rhesus macaques with the 80 CFU dose of the *Mycobacterium tuberculosis* Erdman strain. Surprisingly, despite the selection of a low dose inoculum, naïve macaques developed a rapidly progressive and widely disseminating Tuberculosis infection. In all naïve macaques, the lobe of inoculation showed extensive damage, and in some cases edema and adherence to the diaphragm. Dissemination to other organs occurred in all cases of naïve animals, and macaques demonstrated weight loss up to 20% of total bodyweight within an 8 week period. A 2-dose homologous vaccine-boost regimen using the HMBPP producing *Listeria*-based vaccine was previously shown to cause significant decreases in pulmonary pathology against *M.tb.* infection in a cynomolgus macaque model of infection. By contrast, our 3-dose homologous vaccine boost regimen was largely ineffective in decreasing pulmonary pathology in Rhesus macaques challenged, but was effective in minimizing extrapulmonary dissemination. It is possible that differences in vaccine efficacy may be due to differences in the animal models. Active pulmonary Tuberculosis is typically induced in cynomolgus macaques through inoculation with 500 CFU *M.tb.* Erdman³. Active pulmonary Tuberculosis in cynomolgus macaques rarely results in pulmonary dissemination, and the pulmonary pathology of Tuberculosis is much less severe than that seen in rhesus macaques⁴. A previous study comparing BCG vaccine efficacy in cynomolgus macaques and rhesus macaques showed a robust protection of pathology in BCG-vaccinated cynomolgus macaques, but a much less robust level of protection seen in BCG-vaccinated rhesus macaques¹³⁶.

In addition to the difference in animal models, our current vaccination schedule consisted in a 3-dose homologous vaccine-boost regimen. Our studies described in chapters 4 and 5 have elucidated significant detrimental consequences to V γ 2V δ 2 T cell sensitivity and increased apoptosis of V γ 2V δ 2 T cells following infection, which could contribute to a decrease in the level of protection seen in our 3-dose vaccine regimen. Our comparison of HMBPP producing *Listeria*-based Tuberculosis Vaccine to HMBPP deficient *Listeria*-based Tuberculosis Vaccine efficacy in the 3-dose vaccine regimen model allowed us to compare the impact of repeated high dose phosphoantigens exposure on the vaccine-elicited response. Interestingly, we saw significant decreases in pulmonary pathology and bacterial burden in the HMBPP deficient *Listeria*-based Tuberculosis Vaccine compared to naïve macaques. Because a loss of HMBPP synthetic ability of the *Listeria*-based Tuberculosis vaccine abrogated the vaccine-induced exhaustion of V γ 2V δ 2 T cells seen in the HMBPP producing *Listeria*-based Tuberculosis vaccine, this vector could potentially be used to boost BCG vaccine-induced responses without causing V γ 2V δ 2 T cell exhaustion and death.

6.5. Pattern-specific Immunity to *Mycobacterium tuberculosis*

V γ 2V δ 2 T cells represent a class of pattern recognizing T cells with high proliferative capacity and potent responses to *Mycobacterium tuberculosis* that could potentially be harnessed as immunotherapeutic agents against *M.tb.* strains that have developed resistance to current antibiotic treatment. Similar studies examining a class of lipid recognizing T cells, the CD1d-restricted NKT cells, showed that NKT cell activation through α -galactosylceramide corresponded with a decrease in bacterial burden and pathology in mice infected with *M.tb*¹³⁷.

In vitro studies showed that NKT cells could respond to *M.tb.*-infected macrophages and inhibit the intracellular growth of *M.tb.*¹³⁸. High dose or repeated administration of α -galactosylceramide resulted in decreased response of CD1d-restricted NKT cells *in vitro* and *in vivo*^{139,140}, reflecting a similar pattern of high dose antigen-related exhaustion and death seen with the V γ 2V δ 2 T cell subset. Although the specific lipid antigens that can be detected by CD1d-restricted NKT cells are only recently being characterized, increasing numbers of studies have identified lipids derived from various bacterial species that can be loaded into CD1d molecules and presented to CD1d-restricted NKT cells^{141,142}. As a result, it is possible that decreased BCG vaccine efficacy and overall decreased host T cell response to *Mycobacterium tuberculosis* may be due to repeated exposure to non-protein antigens resulting in exhaustion, anergy, and death of innate-like T cells responsible for early control of *Mycobacterium tuberculosis*.

Collectively, our work points out a subset of pattern recognizing cells whose exhaustion as a consequence of aging and repeated exposure to pathogens may contribute to the wide variation in vaccine efficacy seen both in terms of geography and vaccination age. Interventions that restore the sensitivity of these pattern-recognizing T cells or adoptive transfer of responsive cells remain possible avenues for decreasing *M.tb.* burden.

6.6. Pitfalls and Considerations

A major pitfall of our current study design is that we were unable to analyze the expansion, memory formation, and functional hyporesponsiveness of the $\alpha\beta$ T cell responses generated from the *Listeria*-based vaccines. As a result, we are unable to conclusively determine whether

repeated vaccination of our macaques with *Listeria*-based Tuberculosis vaccines expressing ESAT6 and Ag85B resulted in the exhaustion of the ESAT6 and Ag85B-specific peptide responses of CD4 and CD8 T cells along with the phosphoantigen specific V γ 2V δ 2 T cell responses. Our lab has over 10 years of experience in using MHC class II tetramer technology to study peptide-specific responses generated in response to infection or vaccination of macaques, but the particular MHC alleles necessary for the use of this tetramer technology were not present in the macaques used in this study¹⁴³. Due to the significant genetic heterogeneity of macaques used in research, identification and recruitment of macaques with specific MHC alleles would require extensive screening of macaques. Our lab continues to expand the repertoire of MHC class II tetramers we have available as well as screen macaques for detailed studies of CD4 and CD8 T cell dynamics following vaccination and Tuberculosis infection of macaques in future studies.

CITED LITERATURE

1. Iademarco, M.F. & Koh, W.J. 2011: the year in review. Part II: tuberculosis and lung disease. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* **16**, 1291-1299 (2012).
2. Zumla, A., Raviglione, M., Hafner, R. & von Reyn, C.F. Tuberculosis. *The New England journal of medicine* **368**, 745-755 (2013).
3. Kaushal, D., Mehra, S., Didier, P.J. & Lackner, A.A. The non-human primate model of tuberculosis. *Journal of medical primatology* **41**, 191-201 (2012).
4. Gormus, B.J., Blanchard, J.L., Alvarez, X.H. & Didier, P.J. Evidence for a rhesus monkey model of asymptomatic tuberculosis. *Journal of medical primatology* **33**, 134-145 (2004).
5. Azad, A.K., Sadee, W. & Schlesinger, L.S. Innate immune gene polymorphisms in tuberculosis. *Infection and immunity* **80**, 3343-3359 (2012).
6. Stein, C.M. Genetic epidemiology of tuberculosis susceptibility: impact of study design. *PLoS pathogens* **7**, e1001189 (2011).
7. Nicol, M.P. & Wilkinson, R.J. The clinical consequences of strain diversity in *Mycobacterium tuberculosis*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102**, 955-965 (2008).
8. Druszczyńska, M., Kowalewicz-Kulbat, M., Fol, M., Włodarczyk, M. & Rudnicka, W. Latent *M. tuberculosis* infection--pathogenesis, diagnosis, treatment and prevention strategies. *Polish journal of microbiology / Polskie Towarzystwo Mikrobiologow = The Polish Society of Microbiologists* **61**, 3-10 (2012).

9. Ernst, J.D. The immunological life cycle of tuberculosis. *Nature reviews. Immunology* **12**, 581-591 (2012).
10. McNerney, R., *et al.* Tuberculosis diagnostics and biomarkers: needs, challenges, recent advances, and opportunities. *The Journal of infectious diseases* **205 Suppl 2**, S147-158 (2012).
11. Harris, J. & Keane, J. How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clinical and experimental immunology* **161**, 1-9 (2010).
12. Diedrich, C.R. & Flynn, J.L. HIV-1/mycobacterium tuberculosis coinfection immunology: how does HIV-1 exacerbate tuberculosis? *Infection and immunity* **79**, 1407-1417 (2011).
13. Lin, P.L., Plessner, H.L., Voitenok, N.N. & Flynn, J.L. Tumor necrosis factor and tuberculosis. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **12**, 22-25 (2007).
14. Grosset, J.H., Singer, T.G. & Bishai, W.R. New drugs for the treatment of tuberculosis: hope and reality. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* **16**, 1005-1014 (2012).
15. Uyei, J., Coetzee, D., Macinko, J. & Guttmacher, S. Integrated delivery of HIV and tuberculosis services in sub-Saharan Africa: a systematic review. *The Lancet infectious diseases* **11**, 855-867 (2011).
16. Pritchard, A.J., Hayward, A.C., Monk, P.N. & Neal, K.R. Risk factors for drug resistant tuberculosis in Leicestershire--poor adherence to treatment remains an important cause of resistance. *Epidemiology and infection* **130**, 481-483 (2003).
17. M'Imunya J, M., Kredo, T. & Volmink, J. Patient education and counselling for promoting adherence to treatment for tuberculosis. *Cochrane database of systematic reviews* **5**, CD006591 (2012).

18. Cobelens, F.G. For whom the bell tolls: isoniazid preventive therapy and tuberculosis drug resistance. *Science translational medicine* **5**, 180fs112 (2013).
19. Abubakar, I., *et al.* Drug-resistant tuberculosis: time for visionary political leadership. *The Lancet infectious diseases* (2013).
20. Romano, M. & Huygen, K. An update on vaccines for tuberculosis - there is more to it than just waning of BCG efficacy with time. *Expert opinion on biological therapy* **12**, 1601-1610 (2012).
21. Stewart, J.H.t. & Levine, E.A. Role of bacillus Calmette-Guerin in the treatment of advanced melanoma. *Expert review of anticancer therapy* **11**, 1671-1676 (2011).
22. Kawai, K., Miyazaki, J., Joraku, A., Nishiyama, H. & Akaza, H. Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer: current understanding and perspectives on engineered BCG vaccine. *Cancer science* **104**, 22-27 (2013).
23. Koya, M.P., Simon, M.A. & Soloway, M.S. Complications of intravesical therapy for urothelial cancer of the bladder. *The Journal of urology* **175**, 2004-2010 (2006).
24. Bannister, C., Bennett, L., Carville, A. & Azzopardi, P. Evidence behind the WHO guidelines: hospital care for children: what is the evidence that BCG vaccination should not be used in HIV-infected children? *Journal of tropical pediatrics* **55**, 78-82 (2009).
25. Walzl, G., Ronacher, K., Hanekom, W., Scriba, T.J. & Zumla, A. Immunological biomarkers of tuberculosis. *Nature reviews. Immunology* **11**, 343-354 (2011).
26. Caruso, A.M., *et al.* Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *Journal of immunology* **162**, 5407-5416 (1999).
27. Lin, P.L., *et al.* CD4 T cell depletion exacerbates acute Mycobacterium tuberculosis while reactivation of latent infection is dependent on severity of tissue depletion in cynomolgus macaques. *AIDS research and human retroviruses* **28**, 1693-1702 (2012).

28. Salgame, P. Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection. *Current opinion in immunology* **17**, 374-380 (2005).
29. Lin, P.L., *et al.* Tumor necrosis factor neutralization results in disseminated disease in acute and latent *Mycobacterium tuberculosis* infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis and rheumatism* **62**, 340-350 (2010).
30. Quesniaux, V.F., *et al.* TNF in host resistance to tuberculosis infection. *Current directions in autoimmunity* **11**, 157-179 (2010).
31. Green, A.M., Difazio, R. & Flynn, J.L. IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during *Mycobacterium tuberculosis* infection. *Journal of immunology* **190**, 270-277 (2013).
32. Qiu, Z., *et al.* Multifunctional CD4 T cell responses in patients with active tuberculosis. *Scientific reports* **2**, 216 (2012).
33. Suni, M.A., Maino, V.C. & Maecker, H.T. Ex vivo analysis of T-cell function. *Current opinion in immunology* **17**, 434-440 (2005).
34. Thakur, A., Pedersen, L.E. & Jungersen, G. Immune markers and correlates of protection for vaccine induced immune responses. *Vaccine* **30**, 4907-4920 (2012).
35. Behar, S.M., Woodworth, J.S. & Wu, Y. Next generation: tuberculosis vaccines that elicit protective CD8+ T cells. *Expert review of vaccines* **6**, 441-456 (2007).
36. Behar, S.M. Antigen-specific CD8(+) T cells and protective immunity to tuberculosis. *Advances in experimental medicine and biology* **783**, 141-163 (2013).
37. Randhawa, P.S. Lymphocyte subsets in granulomas of human tuberculosis: an in situ immunofluorescence study using monoclonal antibodies. *Pathology* **22**, 153-155 (1990).
38. Flynn, J.L., Goldstein, M.M., Triebold, K.J., Koller, B. & Bloom, B.R. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis*

- infection. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 12013-12017 (1992).
39. Chen, C.Y., *et al.* A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS pathogens* **5**, e1000392 (2009).
 40. Dieli, F., *et al.* Granulysin-dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by Vgamma9/Vdelta2 T lymphocytes. *The Journal of infectious diseases* **184**, 1082-1085 (2001).
 41. Spencer, C.T., *et al.* Granzyme A produced by gamma(9)delta(2) T cells induces human macrophages to inhibit growth of an intracellular pathogen. *PLoS pathogens* **9**, e1003119 (2013).
 42. Stegelmann, F., *et al.* Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against *Mycobacterium tuberculosis*. *Journal of immunology* **175**, 7474-7483 (2005).
 43. Grode, L., *et al.* Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. *The Journal of clinical investigation* **115**, 2472-2479 (2005).
 44. Magalhaes, I., *et al.* rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. *PloS one* **3**, e3790 (2008).
 45. Kaufmann, S.H. & Hess, J. Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. *Immunology letters* **65**, 81-84 (1999).
 46. McShane, H. Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **366**, 2782-2789 (2011).

47. Mustafa Abu, S. & Al-Attayah, R. Tuberculosis: looking beyond BCG vaccines. *Journal of postgraduate medicine* **49**, 134-140 (2003).
48. Sorensen, A.L., Nagai, S., Houen, G., Andersen, P. & Andersen, A.B. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infection and immunity* **63**, 1710-1717 (1995).
49. Skjot, R.L., *et al.* Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infection and immunity* **68**, 214-220 (2000).
50. Frigui, W., *et al.* Control of *M. tuberculosis* ESAT-6 secretion and specific T cell recognition by PhoP. *PLoS pathogens* **4**, e33 (2008).
51. Li, A.H., *et al.* Contrasting transcriptional responses of a virulent and an attenuated strain of *Mycobacterium tuberculosis* infecting macrophages. *PloS one* **5**, e11066 (2010).
52. Hagedorn, M., Rohde, K.H., Russell, D.G. & Soldati, T. Infection by tubercular mycobacteria is spread by nonlytic ejection from their amoeba hosts. *Science* **323**, 1729-1733 (2009).
53. Kuzuyama, T. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Bioscience, biotechnology, and biochemistry* **66**, 1619-1627 (2002).
54. Tanaka, Y., *et al.* Nonpeptide ligands for human gamma delta T cells. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8175-8179 (1994).
55. Tanaka, Y., *et al.* Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* **375**, 155-158 (1995).
56. Morita, C.T., Jin, C., Sarikonda, G. & Wang, H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunological reviews* **215**, 59-76 (2007).

57. Caccamo, N., *et al.* Aminobisphosphonate-activated gammadelta T cells in immunotherapy of cancer: doubts no more. *Expert opinion on biological therapy* **8**, 875-883 (2008).
58. Jameson, J.M., Cruz, J., Costanzo, A., Terajima, M. & Ennis, F.A. A role for the mevalonate pathway in the induction of subtype cross-reactive immunity to influenza A virus by human gammadelta T lymphocytes. *Cellular immunology* **264**, 71-77 (2010).
59. Chen, Z.W. Immune biology of Ag-specific gammadelta T cells in infections. *Cellular and molecular life sciences : CMLS* **68**, 2409-2417 (2011).
60. Ali, Z., *et al.* Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven antimicrobial and cytotoxic responses of pulmonary and systemic Vgamma2Vdelta2 T cells in macaques. *Journal of immunology* **179**, 8287-8296 (2007).
61. Huang, D., *et al.* Antigen-specific Vgamma2Vdelta2 T effector cells confer homeostatic protection against pneumonic plaque lesions. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7553-7558 (2009).
62. Shen, Y., *et al.* Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science* **295**, 2255-2258 (2002).
63. Dieli, F., *et al.* Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. *The Journal of experimental medicine* **198**, 391-397 (2003).
64. Ryan-Payseur, B., *et al.* Multieffector-functional immune responses of HMBPP-specific Vgamma2Vdelta2 T cells in nonhuman primates inoculated with *Listeria monocytogenes* DeltaactA prfA*. *Journal of immunology* **189**, 1285-1293 (2012).
65. Goossens, P.L. & Milon, G. Induction of protective CD8+ T lymphocytes by an attenuated *Listeria monocytogenes* actA mutant. *International immunology* **4**, 1413-1418 (1992).
66. Tangney, M. & Gahan, C.G. *Listeria monocytogenes* as a vector for anti-cancer therapies. *Current gene therapy* **10**, 46-55 (2010).

67. Brockstedt, D.G. & Dubensky, T.W. Promises and challenges for the development of *Listeria monocytogenes*-based immunotherapies. *Expert review of vaccines* **7**, 1069-1084 (2008).
68. Liu, D. *Listeria*-based anti-infective vaccine strategies. *Recent patents on anti-infective drug discovery* **1**, 281-290 (2006).
69. Gray, M.J., Freitag, N.E. & Boor, K.J. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infection and immunity* **74**, 2505-2512 (2006).
70. Portnoy, D.A., Chakraborty, T., Goebel, W. & Cossart, P. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infection and immunity* **60**, 1263-1267 (1992).
71. Schnupf, P. & Portnoy, D.A. Listeriolysin O: a phagosome-specific lysin. *Microbes and infection / Institut Pasteur* **9**, 1176-1187 (2007).
72. Kocks, C., *et al.* *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. *Cell* **68**, 521-531 (1992).
73. Suarez, M., Gonzalez-Zorn, B., Vega, Y., Chico-Calero, I. & Vazquez-Boland, J.A. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. *Cellular microbiology* **3**, 853-864 (2001).
74. Thompson, R.J., Bouwer, H.G., Portnoy, D.A. & Frankel, F.R. Pathogenicity and immunogenicity of a *Listeria monocytogenes* strain that requires D-alanine for growth. *Infection and immunity* **66**, 3552-3561 (1998).
75. Miki, K., *et al.* Induction of protective cellular immunity against *Mycobacterium tuberculosis* by recombinant attenuated self-destructing *Listeria monocytogenes* strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infection and immunity* **72**, 2014-2021 (2004).

76. Qiu, J., *et al.* Intranasal vaccination with the recombinant *Listeria monocytogenes* DeltaactA prfA* mutant elicits robust systemic and pulmonary cellular responses and secretory mucosal IgA. *Clinical and vaccine immunology : CVI* **18**, 640-646 (2011).
77. Singh, R. & Paterson, Y. *Listeria monocytogenes* as a vector for tumor-associated antigens for cancer immunotherapy. *Expert review of vaccines* **5**, 541-552 (2006).
78. Le, D.T., Dubenksy, T.W., Jr. & Brockstedt, D.G. Clinical development of *Listeria monocytogenes*-based immunotherapies. *Seminars in oncology* **39**, 311-322 (2012).
79. Le, D.T., *et al.* A live-attenuated *Listeria* vaccine (ANZ-100) and a live-attenuated *Listeria* vaccine expressing mesothelin (CRS-207) for advanced cancers: phase I studies of safety and immune induction. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 858-868 (2012).
80. Radulovic, S., *et al.* Therapeutic cancer vaccines in cervical cancer: phase I study of Lovaxin-C. *Journal of B.U.ON. : official journal of the Balkan Union of Oncology* **14 Suppl 1**, S165-168 (2009).
81. Seavey, M.M., *et al.* A novel human Her-2/neu chimeric molecule expressed by *Listeria monocytogenes* can elicit potent HLA-A2 restricted CD8-positive T cell responses and impact the growth and spread of Her-2/neu-positive breast tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 924-932 (2009).
82. Nestler, U., *et al.* Expression of the reporter genes lacZ and EGFP in human glioblastoma cells using *Listeria monocytogenes* as vector for gene transfer. *Neurological research* **31**, 859-868 (2009).
83. Olino, K., *et al.* Tumor-associated antigen expressing *Listeria monocytogenes* induces effective primary and memory T-cell responses against hepatic colorectal cancer metastases. *Annals of surgical oncology* **19 Suppl 3**, S597-607 (2012).

84. Bouwer, H.G., Alberti-Segui, C., Montfort, M.J., Berkowitz, N.D. & Higgins, D.E. Directed antigen delivery as a vaccine strategy for an intracellular bacterial pathogen. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5102-5107 (2006).
85. Yan, L., *et al.* Selected prfA* mutations in recombinant attenuated *Listeria monocytogenes* strains augment expression of foreign immunogens and enhance vaccine-elicited humoral and cellular immune responses. *Infection and immunity* **76**, 3439-3450 (2008).
86. Begley, M., *et al.* Analysis of the isoprenoid biosynthesis pathways in *Listeria monocytogenes* reveals a role for the alternative 2-C-methyl-D-erythritol 4-phosphate pathway in murine infection. *Infection and immunity* **76**, 5392-5401 (2008).
87. Begley, M., *et al.* The interplay between classical and alternative isoprenoid biosynthesis controls gamma delta T cell bioactivity of *Listeria monocytogenes*. *FEBS letters* **561**, 99-104 (2004).
88. Qin, G., *et al.* Type 1 responses of human Vgamma9Vdelta2 T cells to influenza A viruses. *Journal of virology* **85**, 10109-10116 (2011).
89. Jomaa, H., *et al.* Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. *FEMS immunology and medical microbiology* **25**, 371-378 (1999).
90. Qin, G., *et al.* Phosphoantigen-expanded human gamma delta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *The Journal of infectious diseases* **200**, 858-865 (2009).
91. Gomes, A.Q., Martins, D.S. & Silva-Santos, B. Targeting gamma delta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. *Cancer research* **70**, 10024-10027 (2010).

92. Heuston, S., Begley, M., Gahan, C.G. & Hill, C. Isoprenoid biosynthesis in bacterial pathogens. *Microbiology* **158**, 1389-1401 (2012).
93. Puan, K.J., *et al.* Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. *International immunology* **19**, 657-673 (2007).
94. Singh, N., Cheve, G., Avery, M.A. & McCurdy, C.R. Targeting the methyl erythritol phosphate (MEP) pathway for novel antimalarial, antibacterial and herbicidal drug discovery: inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) enzyme. *Current pharmaceutical design* **13**, 1161-1177 (2007).
95. Sicard, H., *et al.* In vivo immunomanipulation of V gamma 9V delta 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model. *Journal of immunology* **175**, 5471-5480 (2005).
96. Gong, G., *et al.* Phosphoantigen-activated V gamma 2V delta 2 T cells antagonize IL-2-induced CD4+CD25+Foxp3+ T regulatory cells in mycobacterial infection. *Blood* **113**, 837-845 (2009).
97. Borowski, A.B., *et al.* Memory CD8+ T cells require CD28 costimulation. *Journal of immunology* **179**, 6494-6503 (2007).
98. Ness-Schwickerath, K.J., Jin, C. & Morita, C.T. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *Journal of immunology* **184**, 7268-7280 (2010).
99. Andersen, P. & Doherty, T.M. The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nature reviews. Microbiology* **3**, 656-662 (2005).
100. Nolz, J.C. & Harty, J.T. Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. *Advances in experimental medicine and biology* **780**, 69-83 (2011).
101. Rouanet, C. & Locht, C. Boosting BCG to protect against TB. *Expert review of respiratory medicine* **4**, 339-348 (2010).

102. Poorolajal, J., *et al.* Long-term protection provided by hepatitis B vaccine and need for booster dose: a meta-analysis. *Vaccine* **28**, 623-631 (2010).
103. Gidengil, C.A., Sandora, T.J. & Lee, G.M. Tetanus-diphtheria-acellular pertussis vaccination of adults in the USA. *Expert review of vaccines* **7**, 621-634 (2008).
104. Cortese, M.M., Baughman, A.L., Brown, K. & Srivastava, P. A "new age" in pertussis prevention new opportunities through adult vaccination. *American journal of preventive medicine* **32**, 177-185 (2007).
105. Deasy, A. & Read, R.C. Challenges for development of meningococcal vaccines in infants and children. *Expert review of vaccines* **10**, 335-343 (2011).
106. Goldstone, S.E. & Vuocolo, S. A prophylactic quadrivalent vaccine for the prevention of infection and disease related to HPV-6, -11, -16 and -18. *Expert review of vaccines* **11**, 395-406 (2012).
107. Barreto, M.L., Pereira, S.M. & Ferreira, A.A. BCG vaccine: efficacy and indications for vaccination and revaccination. *Jornal de pediatria* **82**, S45-54 (2006).
108. Leung, C.C., *et al.* Efficacy of the BCG revaccination programme in a cohort given BCG vaccination at birth in Hong Kong. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* **5**, 717-723 (2001).
109. Tala-Heikkila, M.M., Tuominen, J.E. & Tala, E.O. Bacillus Calmette-Guerin revaccination questionable with low tuberculosis incidence. *American journal of respiratory and critical care medicine* **157**, 1324-1327 (1998).
110. Roth, A.E., *et al.* Effect of revaccination with BCG in early childhood on mortality: randomised trial in Guinea-Bissau. *Bmj* **340**, c671 (2010).
111. Andersen, A., *et al.* The immunological effect of revaccination with Bacille Calmette-Guerin vaccine at 19 months of age. *Vaccine* **31**, 2137-2144 (2013).

112. Green, D.R., Droin, N. & Pinkoski, M. Activation-induced cell death in T cells. *Immunological reviews* **193**, 70-81 (2003).
113. Brenner, D., Krammer, P.H. & Arnold, R. Concepts of activated T cell death. *Critical reviews in oncology/hematology* **66**, 52-64 (2008).
114. Kim, P.S. & Ahmed, R. Features of responding T cells in cancer and chronic infection. *Current opinion in immunology* **22**, 223-230 (2010).
115. Selliah, N., *et al.* T cell signaling and apoptosis in HIV disease. *Immunologic research* **27**, 247-260 (2003).
116. Spengler, U. & Nattermann, J. Immunopathogenesis in hepatitis C virus cirrhosis. *Clinical science* **112**, 141-155 (2007).
117. Hailemichael, Y., *et al.* Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. *Nature medicine* **19**, 465-472 (2013).
118. De Rosa, S.C., *et al.* Ontogeny of gamma delta T cells in humans. *Journal of immunology* **172**, 1637-1645 (2004).
119. Jensen, K., *et al.* A recombinant attenuated Mycobacterium tuberculosis vaccine strain is safe in immunosuppressed simian immunodeficiency virus-infected infant macaques. *Clinical and vaccine immunology : CVI* **19**, 1170-1181 (2012).
120. Saikolappan, S., *et al.* The fbpA/sapM double knock out strain of Mycobacterium tuberculosis is highly attenuated and immunogenic in macrophages. *PloS one* **7**, e36198 (2012).
121. Nambiar, J.K., *et al.* Protective immunity afforded by attenuated, PhoP-deficient Mycobacterium tuberculosis is associated with sustained generation of CD4+ T-cell memory. *European journal of immunology* **42**, 385-392 (2012).

122. Li, Z., Zhao, X., Higgins, D.E. & Frankel, F.R. Conditional lethality yields a new vaccine strain of *Listeria monocytogenes* for the induction of cell-mediated immunity. *Infection and immunity* **73**, 5065-5073 (2005).
123. Perry, S., *et al.* Infection with *Helicobacter pylori* is associated with protection against tuberculosis. *PloS one* **5**, e8804 (2010).
124. Romi, B., *et al.* *Helicobacter pylori* induces activation of human peripheral gammadelta+ T lymphocytes. *PloS one* **6**, e19324 (2011).
125. Born, W.K., Kemal Aydintug, M. & O'Brien, R.L. Diversity of gammadelta T-cell antigens. *Cell Mol Immunol* (2012).
126. Li, L. & Wu, C.Y. CD4+ CD25+ Treg cells inhibit human memory gammadelta T cells to produce IFN-gamma in response to M tuberculosis antigen ESAT-6. *Blood* **111**, 5629-5636 (2008).
127. Wesch, D., Peters, C., Oberg, H.H., Pietschmann, K. & Kabelitz, D. Modulation of gammadelta T cell responses by TLR ligands. *Cellular and molecular life sciences : CMLS* **68**, 2357-2370 (2011).
128. Rincon-Orozco, B., *et al.* Activation of V gamma 9V delta 2 T cells by NKG2D. *J Immunol* **175**, 2144-2151 (2005).
129. Shao, L., *et al.* Expansion, reexpansion, and recall-like expansion of Vgamma2Vdelta2 T cells in smallpox vaccination and monkeypox virus infection. *Journal of virology* **83**, 11959-11965 (2009).
130. Joseph, B. & Goebel, W. Life of *Listeria monocytogenes* in the host cells' cytosol. *Microbes and infection / Institut Pasteur* **9**, 1188-1195 (2007).
131. Chen, Z.W. Immune regulation of gammadelta T cell responses in mycobacterial infections. *Clinical immunology* **116**, 202-207 (2005).

132. Tu, W., *et al.* The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a gammadelta T cell population in humanized mice. *The Journal of experimental medicine* **208**, 1511-1522 (2011).
133. Rodrigues, L.C., *et al.* Effect of BCG revaccination on incidence of tuberculosis in school-aged children in Brazil: the BCG-REVAC cluster-randomised trial. *Lancet* **366**, 1290-1295 (2005).
134. Tsuyuguchi, I., Kawasumi, H., Ueta, C., Yano, I. & Kishimoto, S. Increase of T-cell receptor gamma/delta-bearing T cells in cord blood of newborn babies obtained by in vitro stimulation with mycobacterial cord factor. *Infection and immunity* **59**, 3053-3059 (1991).
135. Engelmann, I., Santamaria, A., Kremsner, P.G. & Luty, A.J. Activation status of cord blood gamma delta T cells reflects in utero exposure to Plasmodium falciparum antigen. *The Journal of infectious diseases* **191**, 1612-1622 (2005).
136. Langermans, J.A., *et al.* Divergent effect of bacillus Calmette-Guerin (BCG) vaccination on Mycobacterium tuberculosis infection in highly related macaque species: implications for primate models in tuberculosis vaccine research. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11497-11502 (2001).
137. Chackerian, A., Alt, J., Perera, V. & Behar, S.M. Activation of NKT cells protects mice from tuberculosis. *Infection and immunity* **70**, 6302-6309 (2002).
138. Gansert, J.L., *et al.* Human NKT cells express granulysin and exhibit antimycobacterial activity. *Journal of immunology* **170**, 3154-3161 (2003).
139. Parekh, V.V., *et al.* Glycolipid antigen induces long-term natural killer T cell anergy in mice. *The Journal of clinical investigation* **115**, 2572-2583 (2005).
140. Courtney, A.N., *et al.* Intranasal but not intravenous delivery of the adjuvant alpha-galactosylceramide permits repeated stimulation of natural killer T cells in the lung. *European journal of immunology* **41**, 3312-3322 (2011).

141. Cohen, N.R., Garg, S. & Brenner, M.B. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. *Advances in immunology* **102**, 1-94 (2009).
142. Venkataswamy, M.M. & Porcelli, S.A. Lipid and glycolipid antigens of CD1d-restricted natural killer T cells. *Seminars in immunology* **22**, 68-78 (2010).
143. Wei, H., *et al.* DR*W201/P65 tetramer visualization of epitope-specific CD4 T-cell during M. tuberculosis infection and its resting memory pool after BCG vaccination. *PloS one* **4**, e6905 (2009).

APPENDIX A – OACIB APPROVAL LETTERS

UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

February 5, 2010

Zheng Chen
Microbiology & Immunology
M/C 790

Dear Dr. Chen:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **1/19/2010**. *The protocol was not initiated until final clarifications were reviewed and approved on 2/3/2010. The protocol is approved for a period of 3 years with annual continuation.*

Title of Application: Listeria-Based Vaccination of Macaques for SIV/SHIV Infection

ACC Number: 2009253

Initial Approval Period: 2/3/2010 to 1/19/2011

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH (Subcontract from U of WI)	Non-Peptide HIV Vaccine			Matched to subcontract
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RC2 CA149023	Funded	200906395	PS1	Miroslav Malkovsky (U of WI), Zheng Chen (UIC-PI on subcontract)

UIC

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,


Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/mbb
cc: BRL, ACC File, James Frencher, PAF 200906395



March 24, 2011

Zheng Chen
Microbiology & Immunology
M/C 790

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Chen:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 1/20/2011. *The protocol was not initiated until final clarifications were reviewed and approved on 3/23/2011. The protocol is approved for a period of 3 years with annual continuation.*

Title of Application: Analysis of Virulence of actA gcpE Double-knockout *Listeria Monocytogenes*

ACC Number: 10-228

Initial Approval Period: 3/23/2011 to 1/20/2012

Current Funding: *Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "What Investigators Need to Know about the Use of Animals" (<http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf>) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/mbb
cc: BRL, ACC File, James Frencher



January 27, 2012

Zheng Chen
Microbiology & Immunology
M/C 790

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Chen:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on *01/27/2012*.

Title of Application: IL-2/Treg Based Immunity to TB and AIDS Related TB

ACC Number: 10-122

Modification Number: 04

Nature of Modification: *Addition of ART treatment to animals added under mod #3. This will allow original control group to serve as the control for these animals and for comparison across all groups. ART treatment will be as described in original protocol.*

Protocol Approved: 07/21/2010

Current Approval Period: 07/20/2011 to 07/20/2012. *Protocol is eligible for 1 additional year of renewal prior to expiration and resubmission.*

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*
Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL064560 (years 12-16)	Funded	201001989	UIC	Zheng Chen

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee
BM/ss

cc: BRL, ACC File, Crystal Ying Chen, Dan Huang



March 22, 2012

Zheng Chen
Microbiology & Immunology
M/C 790

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Chen:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on 03/20/2012.

Title of Application: IL-2/Treg Based Immunity to TB and AIDS Related TB

ACC Number: 10-122

Modification Number: 05

Nature of Modification: *Change in study design to focus on the effect of Listeria-based vaccines on TB infection and not HIV-related TB reactivation. Addition of 8 cynos without prior vaccination as controls.*

Protocol Approved: 07/21/2010

Current Approval Period: 07/20/2011 to 07/20/2012. *Protocol is eligible for 1 additional year of renewal prior to expiration and resubmission.*

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL064560 (years 12-16)	Funded	201001989	UIC	Zheng Chen

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee
BM/ss

cc: BRL, ACC File, Crystal Ying Chen, Dan Huang

Phone (312) 996-1972 • Fax (312) 996-9088



July 30, 2012

Zheng Chen
Microbiology & Immunology
M/C 790

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Chen:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed in accordance with the Animal Care Policies of the University of Illinois at Chicago on *07/30/2012*.

Title of Application: IL-2/Treg Based Immunity to TB and AIDS Related TB

ACC Number: 10-122

Modification Number: 10-122-07

Nature of Modification: *PI is requesting to add another booster injection for Listeria TB antigen vaccines to boost Th17 immune response prior to the start of challenge.*

Protocol Approved: 7/21/10

Current Approval Period: 7/20/12-7/20/13.

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL064560 (years 12-16)	Funded	2010-01989	UIC	Zheng Chen

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/ss

cc: BRL, ACC File, Crystal Ying Chen, Dan Huang



October 17, 2012

Zheng Chen
Microbiology & Immunology
M/C 790

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Chen:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10/16/12**.

Title of Application: IL-2/Treg Based Immunity to TB and AIDS Related TB

ACC Number: 10-122

Modification Number: 10-122-08

Nature of Modification: *Request for reduction of M.tb Erdman dose to 80 CFU.*

Protocol Approved: 7/21/10

Current Approval Period: 7/20/12-7/20/13.

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
ROI HL064560 (years 12-16)	Funded	2010-01989	UIC	Zheng Chen

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/ss

cc: BRL, ACC File, Crystal Ying Chen, Dan Huang

Phone (312) 996-1972 • Fax (312) 996-9088



Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

August 5, 2013

Zheng Chen
Microbiology & Immunology
M/C 790

Dear Dr. Chen:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 7/16/2013. *The protocol was not initiated until final clarifications were reviewed and approved on 8/5/2013. The protocol is approved for a period of 3 years with annual continuation.*

Title of Application: Vg2-Vd2 T Cell Exhaustion in TB Vaccines

ACC Number: 13-122

Initial Approval Period: 8/5/2013 to 7/16/2014

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

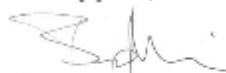
Funding Agency	Funding Title			Portion of Proposal Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Other
				Linked with 13-105, 13-128 and 13-129
Funding Number	Current Status	UIC PAF NO.	Performance Site	Funding PI
ROI HL064560 (years 12-16)	Funded	201001989	UIC	Zheng Chen

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding

sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (<http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf>) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



Bradley Merrill, PhD
Chair, Animal Care Committee

BM/mbb

cc: BRL, ACC File, James Frencher



Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

March 3, 2014

Zheng Chen
Microbiology & Immunology
M/C 790

Dear Dr. Chen:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on 3/3/14.

Title of Application: Vg2-Vd2 T Cell Exhaustion in TB Vaccines

ACC Number: 13-122

Modification Number: 2

Nature of Modification: *Addition of control group (6 animals) to ensure that non-specific peptide-specific T cells stimulated by Listeria monocytogenes administration are not providing significant contribution to the protective response seen. 1 dose of Listeria based vaccine strain (del) actA(del)gcpE prfA* that does not express any TB antigens and will be given through IT inoculation in order to activate listeria protein-specific T cells and challenge these animals to see if these cells would contribute to protection against M.tb challenge (challenged on week 12). Blood, BAL, and rectal biopsies will be collected periodically and study endpoint is week 20.*

Protocol Approved: 8/5/2013



Current Approval Period: 8/5/2013 to 7/16/2014. Protocol is eligible for 2 additional years of renewal prior to expiration and resubmission.

Current Funding: Portions of this protocol are supported by the funding sources indicated in the table below.
Number of funding sources: 1

Funding Agency	Funding Title			Portion of Funding Matched
NIH	IL-2/Treg Based Immunity to TB and AIDS Related TB			Other
				Linked with 13-105, 13-128 and 13-129
Funding Number	Current Status	UIC PAF NO.	Performance Site	Funding PI
ROI HL064560 (years 12-16)	Funded	201001989	UIC	Zheng Chen

Phone (312) 996-1972 • Fax (312) 996-9088

APPENDIX B – COPYRIGHT PERMISSION REQUEST FORMS

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Description of the desired material (use extra pages if necessary): Republish figures 1-5 of this text as part of the thesis of James Frencher (first author)

Journal/publication in which the material

is to be republished (please use full title): Thesis Dissertation: Role of Isoprenoid Biosynthesis in Listeria-based Tuberculosis Vaccine Efficacy.

Company/organization

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Education and Training

<u>Degree</u>	<u>Years</u>	<u>Institution</u>	<u>Discipline</u>
B.S.	2005-2007	University of Michigan Ann Arbor, MI	Microbiology GPA: 3.3 MCAT: 35
M.S.	2007-2008	University of Michigan Ann Arbor, MI	Molecular and Cellular Biology
M.D./Ph.D.	2008-2015	University of Illinois College of Medicine Chicago, Illinois	Ph.D. in Immunology USMLE Step 1: 251

Professional Experience

- 2008-2015 **PhD Thesis Research:** Laboratory of Zheng W. Chen M.D. Ph.D. at the University Of Illinois College Of Medicine at Chicago
My PhD research project employed a multi-faceted approach to examining the impact of repeated stimulation of the immune system by bacterial-associated molecular patterns on the development and maintenance of vaccine-induced immune responses in non-human primate models of Mycobacterium tuberculosis infection. In addition, I planned and participated in projects examining the prognostic utility of platelet parameters in HIV-related fatal malaria infections and assisted in the development of a macaque model for HIV-related TB infection.
- 2007 **Graduate Research Assistant:** Laboratory of David Irani, M.D. at the Holtom-Garrett Neuroimmunology Laboratory at the University of Michigan.
My research in the lab of Dr. David Irani examined the mechanism of neuroprotection conferred by the the FDA approved, highly lipophilic ARB candesartan on in a mouse model of neurovirulent West Nile Virus.
- 2005-2006 **Undergraduate Research:** Laboratory of Alfred Chang M.D.. at the University of Michigan Medical Center Department of Surgery.
As an undergraduate I was awarded the Cancer Center Undergraduate Research Fellowship, which provided me institutional funding to carry out an independent research project. My research examined the role of co-stimulatory molecules on the ability of T cells to develop strong anti-tumor responses.

RESEARCH ACTIVITIES

Original Peer Reviewed Publications

1. **J. Frencher**, A.Qaqish, L. Shen, Z. Zhang, D. Huang, C. Chen, R. Wang, A. Rizzo, Z. Chen. "Repeated phosphoantigen exposure reduces efficacy of Listeria-based T-cell targeting vaccine" (manuscript in preparation)
2. **J. Frencher**, B Ryan-Payseur, Z. Ali, D. Huang, C. Chen, R. Wang, A. Rizzo, Z. Chen "Severe Malarial Anemia and Fatal HIV-associated Malaria infection Associated with Accumulation of Large Dysfunctional Platelets" (manuscript in preparation)
3. **J. Frencher**, A.Qaqish, L. Shen, Z. Zhang, D. Huang, C. Chen, R. Wang, Z. Chen. "Combined peptide Ag-phosphoantigen immunization of rhesus macaques by respiratory route induced detectable protection against tuberculosis" (manuscript in preparation)
4. H. Shen, Y. Wang, C. Chen, **J. Frencher**, D. Huang, B. Ryan-Payseur, Z. Chen. "Th17-related cytokines induce in vitro recall-like expansion and effector function of HMBPP-specific V γ 2V δ 2 T cells after infections" Eur J Immunol. 2015 Feb;45(2):442-51.
5. **J. Frencher**, H. Shen, L. Yan, A. Rizzo, N. Freitag, L. Shen, Z. Chen. Infection with HMBPP-deficient IPP-producing Listeria mutant alters pulmonary and systemic immune responses, effector functions, and memory polarization of V γ 2V δ 2 T cells. J Leukoc Biol. 2014 Dec;96(6):957-67.
6. **J. Frencher**, B. Ryan-Pasyeur, D. Huang, R. Wang, P. McMullen, N. Letvin, W. Collins, N. Freitag, M. Malkovsky, C. Chen, L. Shen, Z. Chen "SHIV Antigen Immunization Alters Patterns of Immune Responses to SHIV/malaria Co-infection and Protects against Life-threatening SHIV-related Malaria." J Infect Dis. 2013 Jul 15;208(2):260-70.
7. B. Ryan-Pasyeur, **J. Frencher**, L. Shen, C. Chen, D. Huang, Z. Chen. "Multieffector-functional immune responses of HMBPP-specific V γ 2V δ 2 T cells in nonhuman primates inoculated with Listeria monocytogenes Δ actA prfA*." J. Immunol 2012 Aug 1;189(3):1285-93.
8. J. Qiu, L. Yan, J. Chen, C. Chen, L. Shen, N. Letvin, B. Haynes, N. Freitag, L. Rong, **J. Frencher**, D. Huang, X. Wang, Z.Chen "Intranasal vaccination with the recombinant Listeria monocytogenes Δ actA prfA* mutant elicits robust systemic and pulmonary cellular responses and secretory mucosal IgA." Clin Vaccine Immunol. 2011 Apr;18(4):640-6.

Invited Talks

- 2013 UIC MSTP Student Grand Rounds. "Kawasaki Disease – New insights in pathophysiology and treatment mechanism". May 2nd, 2013.
- 2013 Microbiology and Immunology Department Seminar Series, University of Illinois College of Medicine: "The Role of HMBPP on TB-induced Pathology". February 21st, 2013.
- 2012 Biological Research Laboratory Seminar Series, University of Illinois College of Medicine: "Macaques as a Model organism for Infectious Diseases". October 25th, 2012.
- 2012 International Gamma Delta T Cell Conference, Freiburg Germany "Knock out of HMBPP Synthase in recombinant Listeria vaccine vector reduced the ability of the vaccine to induce V γ 2V δ 2 T-cell expansion and elicit Th17 and Th22 $\alpha\beta$ T cell responses in macaques" June 1, 2012
- 2011 Biological Research Laboratory Seminar Series, University of Illinois College of Medicine: "Modeling HIV-related malaria in Rhesus macaques". December 22nd, 2011.

EDUCATION ACTIVITIES

Teaching

- 2013-2015 Adjunct Professor in Biology – Malcolm X College
1. Hold Course Lectures for various courses including Anatomy and Physiology, Cell Biology and Medical Terminology
 2. Prepare, administer and grade examinations, quizzes, presentations,
 3. hold office hours
 4. Counsel students on academic achievement, difficulty, and career development
 5. Write letters of recommendation
- 2011-2012 Urban Health Program Teaching Assistant - UIC College of Applied Health Sciences
1. Conduct review lectures in Physiology to applied health science undergraduates at UIC
 2. Hold tutoring sessions for individuals or small groups who were in danger of failing physiology
- 2009-2013 Urban Health Program Peer Educator – UIC College of Medicine

1. Conduct review lectures in Biochemistry, Histology, Physiology, Microbiology, Immunology, and Step 1 Preparation to first and second year medical students at UIC
2. Tutor individuals and small groups of medical students preparing for USMLE step1

2007-2008 Graduate Student Instructor – University of Michigan

1. Hold discussions sections for undergraduate students in the courses of Introductory Biology, Genetics, and Molecular and Cell Biology Lab
2. Prepare, administer and Grade Examinations
3. Hold office hours for struggling students

2005-2006 Science Learning Center Tutor – University of Michigan

1. Tutor small groups of undergraduate students in the subjects of Chemistry and Biology

Mentoring

2013 Zhouan (Alvin) Zhang – Graduate Student in Laboratory of Zheng W. Chen M.D. Ph.D.

2011 Arwa Qaqish – Graduate Student in Laboratory of Zheng W. Chen, M.D. Ph.D.

ORGANIZATIONAL ACTIVITIES

Professional Societies

2014 Society for Interventional Radiology (SIR)

2013 American Physician Scientist Association (APSA)

2012 American College of Physicians (ACP)

2011 Infectious Disease Society for America (IDSA)

2011 Association for Immunologists (AAI)

2011 American Society for Microbiology (ASM)

2008 American Medical Association (AMA)

Leadership Positions

2012-2014 MSTP Admissions Committee Member

2010-2012 Gold Humanism Honor Society Selection Committee Member

2008-2010 Chicago Medical Student Counsel – M3/M4 Subcommittee Member

2008-2010 Medical School Class of 2012 Class Board Vice President

RECOGNITION**Honors and Awards**

2014 UIC Urban Health Program Jose Celso Barbosa Scholarship Award

2013, 2014 UIC Medical Scientist Training Program Travel Award

2012 International Gamma Delta T Cell Conference Travel Award

2011 University of Illinois College of Medicine Research Day Gold Prize Winner

2006 University of Michigan Cancer Center Research Fellowship