Adoptively Transferred Vγ2Vδ2 T cells Protect against the Dissemination of *M. tuberculosis* in Macaques

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THESIS

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This work is dedicated to my loving parents, Mahmoud and Samirah Qaqish, who taught me to do my best and to always hope for the best.

I also dedicate this thesis to my dear husband Zaid for being by my side throughout this journey.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

MTB	*Mycobacterium tuberculosis*
TB	Tuberculosis
BCG	*Mycobacterium bovis*-Bacillus Calmette Guerin
HIV	Human Immune Deficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
γδ	Gamma Delta
Vγ2Vδ2	V gamma 2 V delta 2
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP	Isopentenyl Pyrophosphate
IL-2	Interleukin-2
PAg	Phosphoantigen
PBMCs	Peripheral Blood Mononuclear Cells
BAL	Broncho-Alveolar Lavage
BALF	Broncho-Alveolar Lavage Fluid
PBL	Peripheral Blood Leukocytes
CFU	Colony Forming Unit
AM	Alveolar Macrophage
MΦ	Macrophage
IFN-γ	Interferon gamma
CD	Cluster of Differentiation
Th1	T helper 1
WHO	World Health Organization
TNF-α	Tumor Necrosis Factor
DCs	Dendritic Cells
PRR	Pathogen Recognition Receptor
APCs	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
ABP	Aminobisphosphonate
AICD	Activation Induced Cell Death
MDR-TB	Multi-Drug Resistant Tuberculosis
XDR-TB	Extensively Drug Resistant Tuberculosis

LIST OF ABBREVIATIONS (continued)

DCs	Dendritic Cells
BTN3A1	Butyrophilin 3A1
ICS	Intracellular Cytokine Staining
PBS	Phosphate Buffered Saline
BSL-3	Biosafety Level 3
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
Cy7	Cyanine 7
APC	Allophycocyanin
PB	Pacific Blue
AF700	Alexa-Fluor 700
RBC	Red Blood Cell
RPMI	Rosewell Park Memorial Institute Media
R10A	RPMI with 10% fetal bovine serum and antibiotics
IPS	International Primatological Society
EDTA	Ethylenediaminetetraacetic acid
IV	Intravenous
IM	Intramuscular
PMA	Phorbol Myristate Acetate
FBS	Fetal Bovine Serum

SUMMARY

Scientific Background: Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), is one of the oldest, most expanded and most lethal diseases in human history. Once thought to be controlled by the BCG vaccine, an attenuated form of Mycobacteria, TB re-emerged due to the development of drug resistant strains and the spread of HIV/AIDS. The vaccine has also proved to be non-protective for adults when compared to children. There is a pressing need to develop a new protective vaccine, and this cannot be done without a deep understanding of the immune response against MTB.

A main focus of our lab is the Vγ2Vδ2 T cells, a major human γδ T-cell subset that exists only in primates and not in other animals. These cells recognize phosphate rich compounds, such as HMBPP, essentially generated by a variety of microbes including MTB and, IPP, produced by cancer cells and some virally infected cells.

Problem and Aims: In a previous attempt to understand the function of Vγ2Vδ2 T cells in immunity against TB, we have used HMBPP and IL-2 to expand these cells in vivo in the context of a non-human primate TB infection model. Results from this study suggested that Vγ2Vδ2 T cells confer high protection against the disease. However, this protection could not be attributed solely to Vγ2Vδ2 cells, as IL-2 also expands other immune cells. Hence, we aimed at studying the exact role that Vγ2Vδ2 play in fighting TB using a cleaner experiment where autologous ex-vivo expanded Vγ2Vδ2 T cells are

adoptively transferred into macaques after TB infection.

Despite the discovery of γδ T cells 30 years ago, there is still no definitive in vivo evidence indicating that phosphoantigen (PAg) specific Vγ2Vδ2 T cells can protect against MTB infection in human. Here, we aimed at exploring the role of Vγ2Vδ2 T cells against TB using the adoptive cell transfer approach and a TB infection macaque model.

Approach: Peripheral blood mononuclear cells (PBMCs) were collected from cynomologus macaques and frozen down over time. Prior to the adoptive transfer, PBMCs were thawed and used for in vitro Vγ2Vδ2 T cell expansion using a standard protocol. Autologous Vγ2Vδ2 T cells were transferred to TB infected macaques on early time points after infection. Animals were evaluated for immune responses and infection status over time after adoptive transfer. At 8 weeks after infection, macaques were subjected to complete necropsy for evaluation of gross pathology and bacterial burdens.

Results: Outcomes of this study came to support a protective role of Vγ2Vδ2 T cells against TB. Infused Vγ2Vδ2 cells started to show up in the bronchoalveolar lavages (BALs) of monkeys 6 hours after infusion, peaked at 24-48 hours and were still detectable at 7 days. Along the study, the Vγ2Vδ2 group showed significantly reduced weight loss and lymphocytopenia compared

to the control peripheral blood lymphocytes (PBL) group. Bacterial CFUs in the BALs were significantly lower in the Vγ2Vδ2 group. Upon necropsy, macaques who took Vγ2Vδ2 T cells showed significantly reduced bacterial dissemination to lung lobes other than the primarily infected right caudal lobe. Reduced dissemination was manifested by the absence of, or highly reduced granulomatous lesions as well as significantly less bacterial CFUs compared to the PBL controls. Reduced dissemination also applied for extra-pulmonary TB manifestation.

Conclusions: We are the first to expand Vγ2Vδ2 T cells from macaques and adoptively transfer them into a TB infection model. This study generated the first evidence that Vγ2Vδ2 T cells confer protection against MTB and can give new insights for the design of new vaccines against the disease.

CHAPTER I

Introduction

A. Life cycle of *Mycobacterium Tuberculosis*

The life cycle of *Mycobacterium tuberculosis* (MTB) starts with actively infected individuals. Aerosols exhaled by those individuals form a main (or soul) source of disease transmission in humans. Once inhaled by surrounding people, aerosols are carried down to the lung alveoli, where bacteria are released to start infecting an array of cells; with a preference for alveolar macrophages (AMs) (1). Initially, an innate immune response is evoked; recruiting inflammatory cells to come to the rescue (2). An adaptive immune response gets launched only later (approximately 2 weeks), after the spread of MTB to the closest lymph nodes (3-5). In the lymph nodes, dendritic cells present bacterial antigens to T cells leading to their activation, differentiation and expansion. The "now" effector T cells migrate to the site of infection, where, in combination with other cells, participate in the formation of granulomas. Granulomas are "organized structures that contain macrophages (MΦs), lymphocytes and fibroblasts" (6). Within the granuloma, T cells (mainly CD4 T cells) secrete interferon-ɣ (IFN-ɣ), which in turn activates MΦs to help restrict the dispersal and replication of MTB (6).

Despite the fact that the human system can control and clear most bacterial infections, it cannot lead to clearance of MTB. Because of that, most infected individuals harbor the bacteria but under control, showing no clinical symptoms of disease in a state known as “latent infection” (7). In fact, up to one third of the world is latently infected with tuberculosis (TB); forming an enormous reservoir of potential infectious bacteria. Studies have shown that 5–10% of latently infected individuals do develop active disease during their lives (8).

Upon reactivation, actively infected individuals transmit the disease through coughing of aerosol containing the infectious pathogen. Figure 1.1 shows a schematic flow of the life cycle of MTB.

B. Evolution of *Mycobacterium tuberculosis*

TB is an ancient disease that is widely spread and continuously expanding all over the world. MTB accompanied humans for thousands and thousands of years. Around 2,500 years ago, Hippocrates, the ancient Greek physician, reported that "phthisis", which means consumption, was the most prevalent and deadly malady thereby (9). Interestingly, there are evidence that MTB lived in humans before the era of Hippocrates, around 4,000 years ago, as “tubercular decay” was found in skulls and spines of Egyptian mummies (10).

With time, through genomic adaptation, MTB learned to live inseparably from humans as a specialized pathogen. For a long time, it has been thought that mycobacteria were first introduced to the human population as a result of

contact with *Mycobacterium bovis* infecting farm animals. However, new genetic analysis studies have proved that MTB is more closely related to *Mycobacterium africanum* than *Mycobacterium bovis* indicating that MTB strains emerged and co-evoluted about 70,000 years ago when anatomically modern humans migrated out of Africa. Along time, increases in human population caused the expansion of the pathogen that seemed to adapt to both low and high population densities (11).

C. Tuberculosis (TB) epidemiology

MTB was discovered by Robert Koch in 1882 (12). In his “Nobel Prize” speech, Koch stated terrifying facts about the disease saying: "If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings die from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more." (13)

Despite the fact that a huge first step towards fighting MTB was taken by koch’s discovery to the causative agent of the fatal disease in 1882, it took a significant amount of time to reduce morbidity and mortality of TB in the world. The use of antibiotic treatments along with education of the population and the development of the *Mycobacterium bovis*-Calmette Guerin (BCG) vaccine altogether helped in controlling the disease over the years. Hence, significant progress in the treatment and control of TB infection was achieved.

However, starting from the 1980s and 1990s, rates of tuberculosis increased dramatically, especially in the developing world, mainly due to HIV/AIDS pandemics, development of drug resistance, and limited public health infrastructure (14). While rates have stabilized, they remain extremely high (15).

Despite obvious dangers, therapies have been employed based on assumptions of low rates of drug resistance (16). Reports of high rates of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) in recent years have alerted for new forms of TB, with new challenges and greater problems (17-19).

In 2013, the World Health Organization (WHO) revealed 1.5 million annual deaths caused by the disease with approximately one third of the world's population being latently infected and prone to reactivation. Figure 1.2 shows the relative spread and prevalence of TB in the world.

D. Treatment of tuberculosis

After Koch's discovery of MTB, many attempts were made over the years to find a compound that could stop the growth of TB bacteria. It took decades to find a drug that can effectively kill MTB and still be minimally poisonous to humans. In 1943, the antibiotic streptomycin was discovered and later proved to be effective against tuberculosis. Unfortunately, early afterwards, it became evident that MTB was becoming resistant to streptomycin. Another drug called para-aminosalicylic acid was developed in 1943. When used together with

streptomycin, development of drug resistance was highly reduced. In 1952, another drug called isoniazid was developed and also found its place in TB therapy (20). Thus, in the decade between 1944 and 1954, the three drugs; streptomycin, para-aminosalicylic acid, and isoniazid became available. When taken in combination, and for a sufficient length of time that could reach up to 6 months, the prognosis for a patient with TB disease changed from dismal to the expectation of cure (20) .

In 1980s and 1990s, the incidence of TB started to re-increase causing more deaths every year. HIV-AIDS, the administration of corticosteroids and tumor necrosis factor (TNF) inhibitors for treatment of allergies and autoimmune diseases and the use of chemotherapy for treatment of cancer; all create highly immunosuppressive host environments that causes the reactivation of latent TB. Adherence to treatment courses is becoming very hard due to lengthy treatment periods, side effects and toxicities of the multi-drug cocktails, and drug-drug interaction problems (especially in cases of HIV coinfection). This incomplete treatment is causing a tremendous increase in the development of multi-drug resistant strains making TB eradication even harder (21).

Because different kinds of bacillary populations coexist in the host, ranging from actively growing to latent, TB treatment using drugs is very challenging and potentially leading to resistance. Thus, up to this time, the cornerstone for any treatment of tuberculosis is still multidrug therapy. At least two drugs are given at the same time to prevent the emergence of drug resistance. Sometimes

patients are treated with up to four different antibacterial drugs, and for periods of a minimum of 6 to 24 months (22).

Because of all problems related to TB treatment, the best hope for decreasing the burden of the disease is by creating an effective vaccine. For that, a deep understanding of the immune response against MTB is crucial.

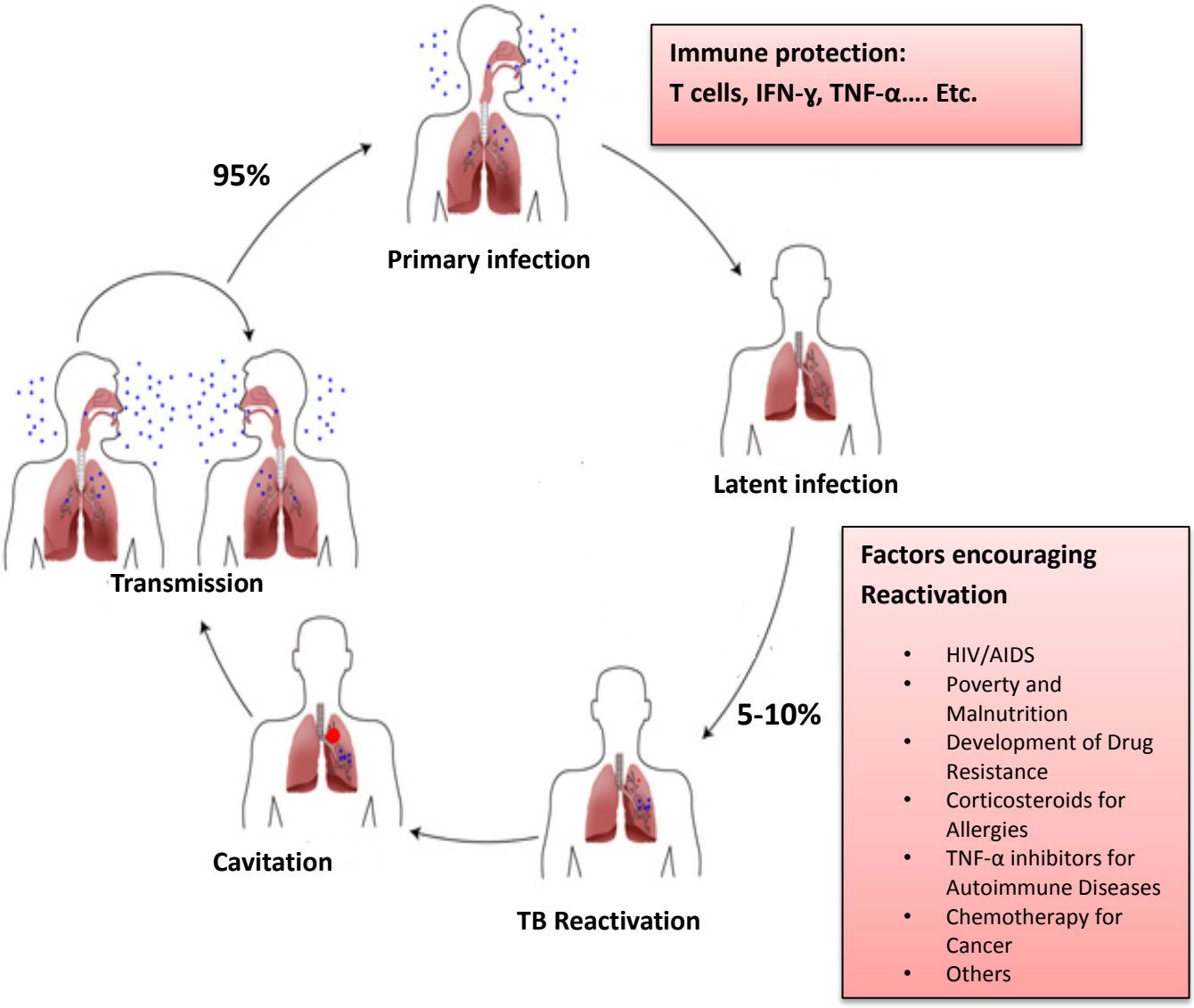

Figure 1.1: Life Cycle of *Mycobacterium tuberculosis*. HIV/AIDS: Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome, IFN-ɣ: Interferon-ɣ, TNF-α: Tumor Necrosis Factor-α, TB: tuberculosis. Adopted from (23). Appendix C shows permission of use.

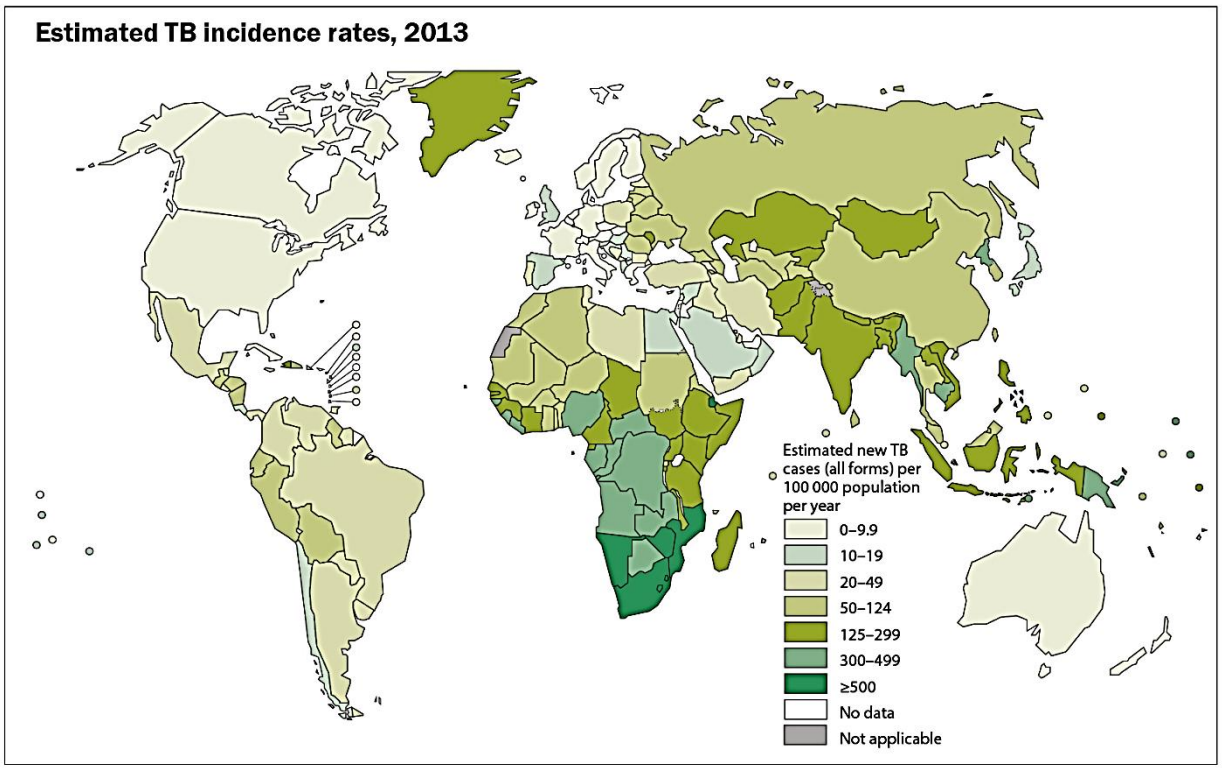

Figure 1.2: Estimated Tuberculosis Incidence Rate of 2013. Estimated new tuberculosis cases per 100,000 population per year. Adopted from the WHO Global Tuberculosis Report 2014. (http://www.who.int/tb/publications/global_report/en/)

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E. Innate immune response against *Mycobacterium tuberculosis*

MTB is transmitted through the inhalation of aerosols generated by the coughing and sneezing of actively infected individuals (24,25). These aerosols are taken into the alveolar spaces of the lungs where the pathogen is actively taken up by professional phagocytic cells, primarily resident AMs, where they live and replicate (26,27). MTB can also actively infect and replicate within dendritic cells (DCs), monocytes, neutrophils and alveolar type II epithelial cells (28-30).

MΦs possess various mechanisms to help eliminate mycobacteria. These include the generation of toxic reactive oxygen species, increasing the acidity of phagosomes and permitting phagosome/lysosome fusion (26). In addition, MΦs can restrict the release of intracellular mycobacteria by undergoing autophagy; "a cellular process through which cytoplasmic components, including organelles and intracellular pathogens, are sequestered in a double-membrane-bound autophagosome and delivered to the lysosome for degradation" (31). Autophagy leads to the maturation of phagosomes by increasing their acidity; which in turn help kill harbored mycobacteria (32). Once inside the MΦ, MTB becomes highly resistant to clearance by phagocytosis. This is mediated by a variety of immune evasion mechanisms for the pathogen own advantage. MTB inhibit the trafficking and maturation of the phagosomes where they reside; allowing them to escape digestion and killing by lysosomal enzymes (32-35). MTB has the ability to detoxify toxic oxygen and nitrogen radicals produced by

phagocytic cells (33-35). Several virulence mechanisms of MTB help to optimize bacterial spread from cell to cell. For example, MTB induces the infected cells to die through necrosis, freeing intracellular bacteria from compartment constrains and allowing them to infect freshly recruited phagocytic cells and, subsequently, expanding into a larger population (36). MTB possesses multiple mechanisms for inhibiting host cell apoptosis (37-39); allowing prolonged survival of infected cells and more bacterial expansion. In addition, inhibition of apoptosis leads to inhibition of autophagy and hence, inhibition of powerful degradative phagocytosis of bacteria (39).

Early phases of innate immune response to MTB infection involve continuous recruitment of innate cells such as neutrophils, monocytes, interstitial MΦs and DCs. Once in the lungs, these cells get infected and housed by the growing mycobacteria. Usually, in most infectious diseases, innate cells recruited to the site of infection help kill and eliminate the invading pathogen. However, this is not the case in TB where mycobacteria take advantage of the innate phagocytic cells accumulating early on in the lungs, and rapidly expand to spread into a larger population of cellular niches (36).

Infection of the AM occurs through receptor-mediated phagocytosis. Upon entry, recognition of MTB is mediated through the engagement of pattern recognition receptors (PRRs). Interestingly, the earliest of these interactions which signals through the mannose receptor, induces an anti-inflammatory program; delaying the recruitment innate immune cells to the lungs (40).

Normally, when an inflammatory response is initiated; DCs are rapidly recruited to the lungs (41). In the case of MTB infection, major influx of APCs into the lung is delayed for the first 5–7 days following infection (4).

The early mobilization of APCs to the lung is critical to the timely control of an MTB infection. This delay impairs the downstream adaptive immune activation and bacterial control (40).

F. The adaptive immune response against *Mycobacterium tuberculosis*

DCs carrying MTB and MTB antigens from the site of infection migrate to the draining lymph nodes to present antigens to T cells and initiate MTB specific adaptive immune responses. As mentioned earlier, MTB induces a delay in the priming phase of the adaptive immune response that leads to the establishment of bacterial persistence. Generally, in case of TB, it takes 2 to 3 weeks after infection for an adaptive immune response to launch.

A successful priming of the adaptive immune response by DCs triggers the expansion and phenotypic and functional maturation of MTB specific T cells, which enter the circulation and home to the site of infection. Mature MTB specific T cells enhance the phagocytic activity of infected MΦs and induce the production of toxic reactive oxygen and nitrogen species. In addition, MTB specific T cells produce an array of cytotoxic/cytolytic cytokines capable of killing infected cells as well as directly killing mycobacteria; in certain instances (42).

At the end, more importantly, an effective T cell response aids the formation and maturation of the granuloma, considered necessary for containment of infection (42).

1. The role of CD4+ T helper 1 (Th1) Cells

CD4 + Th1 T cells have long been recognized for their immune protective role against TB. Interestingly, the WHO estimates the risk of developing TB to be between 20 and 37 times greater in people living with HIV than among those without HIV infection due to the loss of CD4+ T cells (43).

Th1 cells are characterized by the production of their signature cytokine, IFN-γ. The differentiation of Th1 cells requires the cytokine IL-12 secreted by activated MΦs infected with MTB. Humans carrying mutations in IL-12 or the IFN-γ receptor have shown higher susceptibility to mycobacterial disease. (44) Similar results have been obtained from studies using mice defective in IFN-ɣ production. Interestingly, recent studies have suggested that rapid MTB specific Th1 cell depletion may contribute to the early onset of TB in individuals with latent *M. tuberculosis* reactivation (45).

IFN-ɣ functions to enhance the phagocytic activity of infected MΦs, mainly, by the induction of synthesis of reactive nitrogen and oxygen species. It also enhances the antigen presenting capacity of infected cells by inducing the upregulation of MHC II and necessary co-stimulatory molecules. In addition, it induces production of chemokine attractants important for the recruitment of

more Th1 cells to the site of infection. Futhermore, IFN-ɣ inhibits IL-17 production by T cells, thus, limiting pathogenic neutrophil recruitment and associated necrotic inflammation (44).

In addition to IFN-ɣ, Th1 cells produce TNF-α and IL-2. TNF-α proved to be essential for the control of MTB infection after it was noticed that TNF-α blockade used to treat inflammatory autoimmune diseases caused reactivation of TB in patients (46). Indeed, mice deficient in this cytokine are the most susceptible to MTB infection. The most striking characteristic of TNF deficiency is poor phagocyte activation; which leads to a deficiency in chemokine mediated recruitment of cells important for granuloma formation (47).

2. The role of CD8+ T cells

Typically, CD4+ T cells respond to exogenous antigens presented by MHC class II, after being engulfed and processed along the endocytic pathway of the APCs. On the other hand, CD8+ T cells respond to endogenous antigens present in the cytosol of cells, after being degraded by the proteasome and coupled for presentation to MHC class I. In some cases, MHC class I can present antigens that enter the endocytic pathway, a process known as cross-presentation. Following macrophage infection, MTB survives and replicates in the phagosome. In addition to cross-presentation, several mechanisms have been proposed to explain how bacterial antigens traffic from the phagosome to the cytoplasm where they can enter the class I MHC processing pathway to be presented to CD8+ T cells. Ultimately, mycobacterial antigens do enter the

class I MHC pathway, since measurable CD8+ T cell responses are evoked by infection in both humans and experimental animals (48,49).

In 1992, Flynn and colleagues showed that mice lacking proper MHC I presentation succumb rapidly following IV infection. These data were the first truly convincing evidence that CD8+ T cells were required for optimum resistance to TB (50). Moreover, antibody-mediated depletion of CD8+ T cells in vivo has shown to increase host susceptibility, while adoptive transfer of purified CD8+ T cells enhanced host resistance (51-53).

CD8+ T cells elicited by MTB infection are cytolytic in vivo. MTB specific cytolytic activity is detected within 4 weeks of MTB infection and persists for at least 9 months. In vitro studies has shown that CD8+ T cells acquire the ability to lyse infected MΦs, and the lysis is dependent upon granule exocytosis. The strong cytolytic activity characterizing CD8 + T cells is mediated through the production of the killing cytokines perforin, granulysin and granzymes A and B; all playing different roles in target cell killing. For example, perforin makes holes in the membranes of target cells allowing proteolytic cytokines, such as granzyme B to enter the cell (51-54).

CD8+ T cells are are also major producers of IFN-γ, TNF-α, and IL-2. Interestingly, Tascon et al. showed that the ability of adoptively transferred naïve CD8+ T cells to protect T cell-deficient recipients required IFN-ɣ (51-54).

I. Gamma Delta T cells

Since their discovery in 1986, the function of this subset of T cells has been intensely investigated and is now starting to be resolved (55).

Gamma Delta (γδ) T cells represent a population of non-conventional T cells that are identified by the expression of the γδ TCR instead of the αβ TCR (56). These cells evolved early in vertebrate phylogeny and are found in primitive cartilaginous fish along with B cells and αβ T cells. γδ T cells do not respond to peptide antigens presented by major histocompatibility complex (MHC) class I and class II molecules that stimulate the majority of CD8 and CD4 αβ T cells, respectively.

In secondary lymphoid organs they account for only 2%-3% of all $CD3^+$cells and the highest abundance of these cells is seen in the gut mucosa (57).

Human γδ T cells can be divided into three main populations based on δ chain expression. Cells expressing the δ1 chain mainly populate mucosal surfaces, specifically the intraepithelial layer. They function in preserving the integrity of the epithelial tissue especially when exposed to damage caused by infection or transformation (58). They also respond to stress induced antigens expressed on epithelial cells (59). The second population express the Vδ3 chain and forms only ~0.2% of circulating T cells. Vδ3 T cells are prominent in the liver and expand in certain cases of chronic viral infection and in cases of leukemia (60). The third and major population of γδ T cells expresses the Vδ2 chains. They represent the majority of circulating γδ T cells in healthy human adults,

comprising 50%-90% of the peripheral γδ T cell population (61). These are known for their importance in microbial infections and are the major subject of this thesis. Following is a review of the main knowledge about these cells.

1. Vγ2Vδ2 T cells

The Vδ2 chain pairs almost exclusively with Vγ9 (also termed Vγ2). The Vγ2Vδ2 pairing is present only in humans and nonhuman primates but not in rodents or lower animals (61). These cells respond to self-nonpeptide and foreign nonpeptide phosphoantigens (PAgs) presented by a newly discovered antigen presenting molecule; butyrophilin 3A1 (BTN3A1) (62-64).

Vγ2Vδ2 T cells are considered both innate and adaptive immune cells as they share hallmarks of both. From the innate side, these cells recognize conserved microbial entities and rapidly respond and exert functions such as cytokine production. And, from the adaptive side, after antigen recognition, Vγ2Vδ2 T cells start to proliferate and expand to produce memory cells that can, in turn, rapidly respond upon re-exposure to antigen and exert function. Immunological memory is a hallmark of the adaptive immunity and the basis of any successful vaccination.

2. Antigens recognized by Vγ2Vδ2 T cells

The natural antigens recognized by Vγ2Vδ2 T cells contain critical phosphate moieties. Early studies demonstrated that Vγ2Vδ2 T cells are strongly activated by a variety of killed microorganisms including bacteria such as MTB (65) and parasites such as *Plasmodium falciparum* (66). Later, antigens have been

identified as (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP), and isopentenyl diphosphate (IPP), central intermediate metabolites in isoprenoid synthesis (67) (Figure 1.3).

Isoprenoids are essential compounds that play a variety of roles in cellular metabolism and in the synthesis of a large number of natural products. Diverse compounds, such as cholesterol, steroid hormones, rubber, and various vitamins, are derived from this pathway (68). The ubiquitous nature of this pathway provided a partial explanation for the broad reactivity of Vγ2Vδ2 T cells for both prokaryotic and eukaryotic pathogens.

HMBPP is a key intermediate produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid synthesis. The MEP pathway is found in most Eubacteria, apicomplexan protozoa, cyanobacteria, and plant chloroplasts. In contrast, human cells utilize the mevalonate pathway for isoprenoid biosynthesis (69), which also activates Vγ2Vδ2 T cells *in vitro* following presentation by professional antigen presenting cells or tumor cells, but only at concentrations not achieved physiologically; unless in transformed cells such as cancer and some virally infected cells (70,71).

Interestingly, the intracellular levels of IPP can be manipulated by the therapeutically administered drugs. aminobisphosphonates (ABPs) such as pamidronate and zoledronic acid, which are in clinical use for osteoporosis and hypercalcemia of malignancies. These can enhance intracellular levels of IPP by

the inhibition of farnesyl diphosphate synthase (FPPS) (71,72), contributing to the activation and expansion of human Vγ9Vδ2 T cells (Figures 1.3 and 1.4).

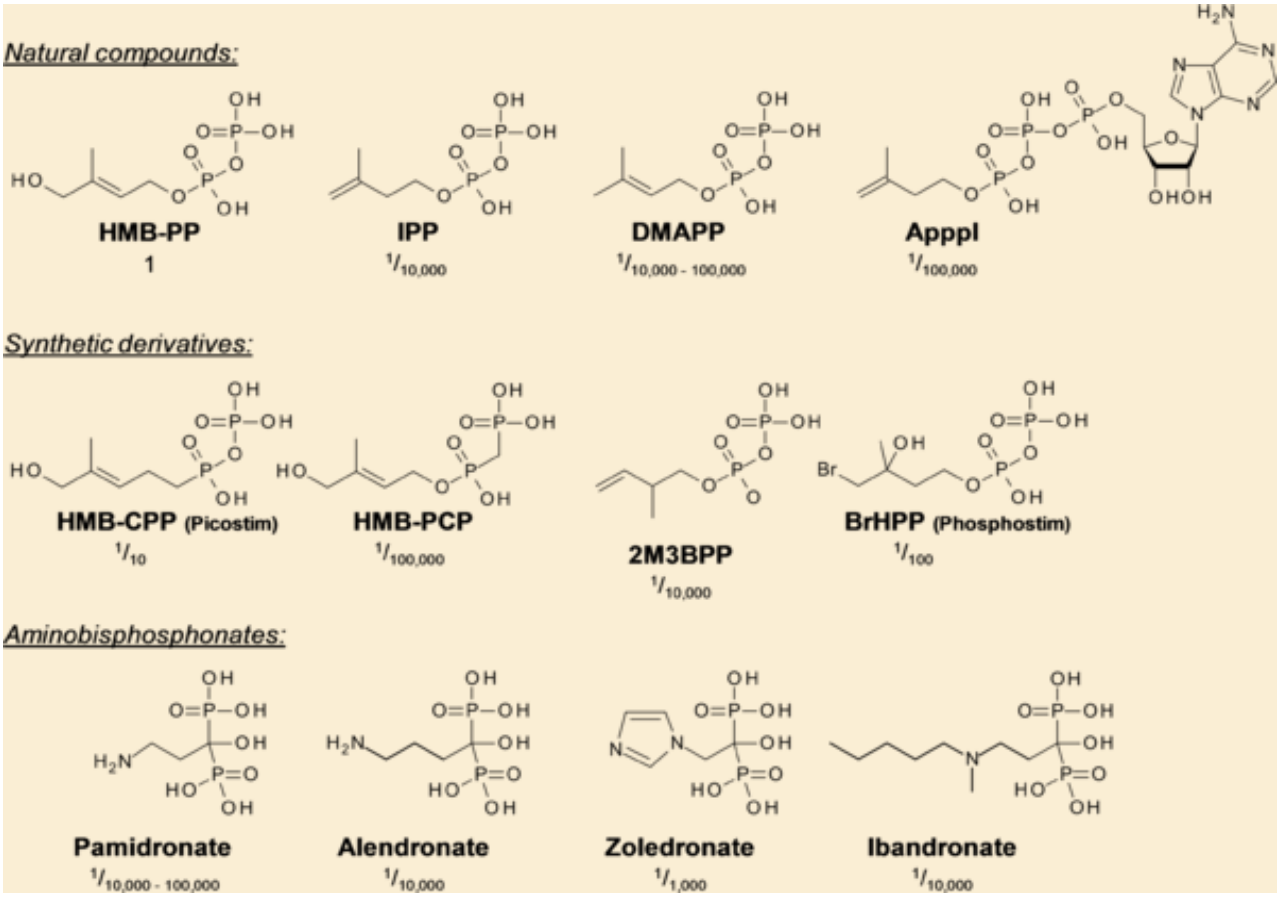


Figure 1.3: Natural and synthetic compounds with their approximate bioactivities on human Vγ2Vδ2 T cells, as compared with HMBPP whose bioactivity has been set to 1. Adopted from (55). Permission of use is shown in Appendix D.

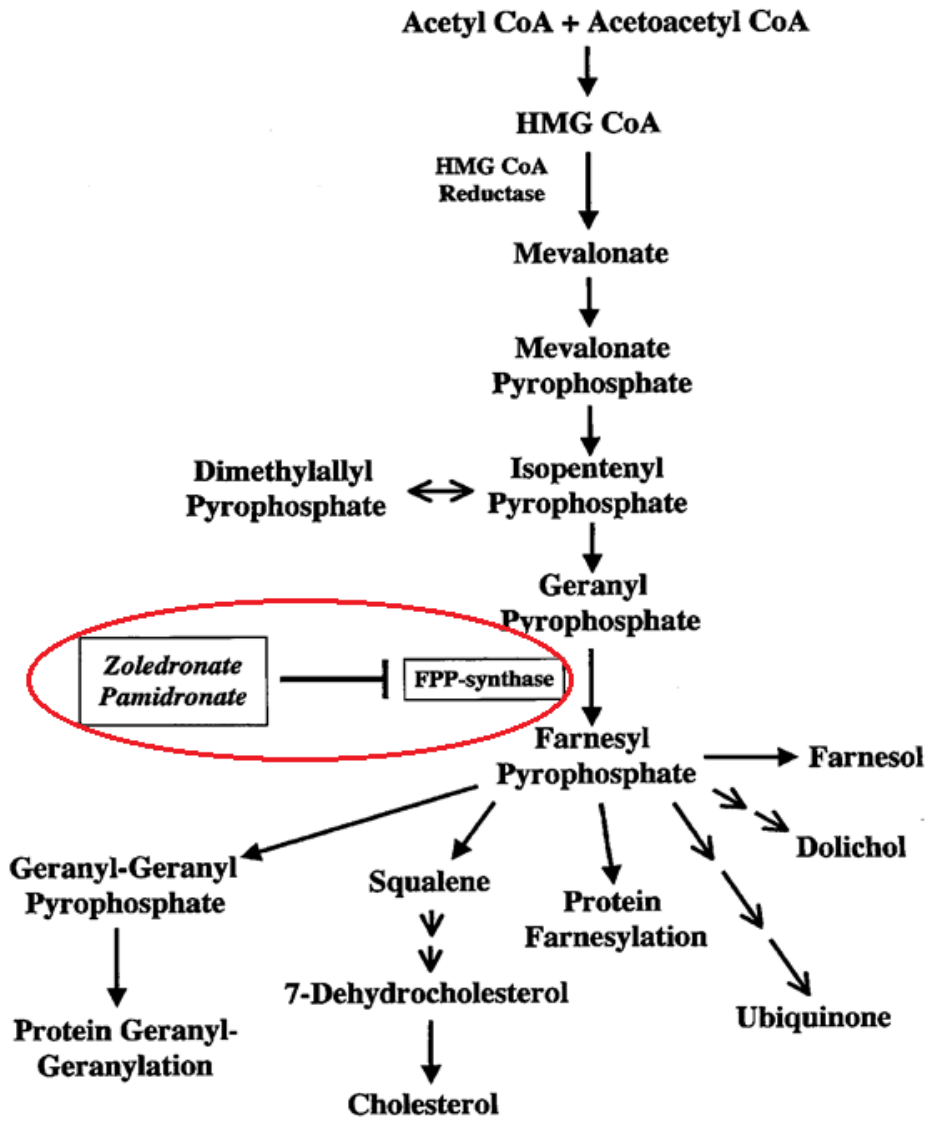


Figure 1.4: Mevalonate pathway and downstream steps in isoprenoid biosynthesis**.** The aminobisphophonate Zoledroninc Acid inhibits the enzyme farnesyl diphosphate synthase (FPP); leading to the accumulation of isopenetenyl pyrophosphate (IPP) recognized by Vγ2Vδ2 T cells. Adopted from (70). Permission of use shown in Appendix E.

3. Antigen presentation to Vγ2Vδ2 T cells

Vγ2Vδ2 T cells recognize host and microbe derived phosphoantigens (PAgs) in the presence of antigen presenting cells, but the molecular basis for PAg recognition has long remained a major unanswered question in the biology of Vγ2Vδ2 T cells. Many studies consistently agreed with the existence of a PAg presenting molecule. The small size of PAgs, the requirement for cell-cell contact, the findings that PAgs fail to bind directly to the soluble Vγ2Vδ2 TCR and the fact that the putative antigen binding groove of the Vγ2Vδ2 TCR is much larger than that occupied by the PAg alone have suggested that PAgs activate lymphocytes by forming complexes with specialized antigen presenting molecules (73).

Antigen internalization and processing proved to be not required for activation of Vγ2Vδ2 T cells with PAgs. PAgs can be presented by non-professional antigen-presenting cells, including Vγ2Vδ2 T cells themselves. Cells lacking the expression of the β microglobin chain; an essential part of the major histocompatibility complex (MHC) class I and MHC class I like antigen presenting molecules were still able to present PAgs to Vγ2Vδ2 T cells. All that indicated that none of the known human antigen presenting molecules including MHCI and MHCI related molecules are involved in PAg presentation. Moreover, these findings suggested a ubiquitous nature of the antigen presenting molecule (74). Interestingly, some evidence indicates that the presentation of PAgs is species specific, which excludes the possibility of

antigen presentation by a highly homologous antigen presenting molecule expressed in different animal species.

In the September 2013 issue of Nature Immunology, through the use of a genetic approach, De Libero and colleagues identified butyrophilin BTN3A1, a ubiquitously expressed and nonpolymorphic molecule, as the molecule that presents antigen to Vγ2Vδ2 T cells (62). BTN3A1 belongs to a family of immunoglobulin-like molecules with immunomodulatory functions (75). Studies of the binding of IPP or HMBPP to BTN3A1 showed that the two molecules formed stable complexes, with the half-life of BTN3A1-HMBPP longer than that of BTN3A1-IPP. Comparison of the binding kinetics of PAgs with BTN3A1 showed faster association kinetics and slower dissociation kinetics in the case HMBPP compared to IPP, explaining the well recognized higher 'potency' of HMBPP in stimulating Vγ2Vδ2 T cells.

4. Remarkable plasticity of activated Vγ2Vδ2 T cells

The Vγ2Vδ2 subset displays remarkable functional plasticity upon TCR activation (76). Such plasticity was initially demonstrated in vitro through the activation of Vγ2Vδ2 T cells using IPP in the presence of different cytokine milieus. An abundance of IL-12 generates IFN-γ secreting Th1-like cells whereas an abundance of IL-4 generates IL-4-producing Th2-like cells (77). An IFN-γ/TNF-α secreting Th1-like phenotype is also generated following activation with HMBPP plus IL-2 (78). By contrast, HMBPP activation of Vγ2Vδ2 cells in the presence of IL-21 promotes a follicular helper (TFH) like phenotype that is

homes to lymph node germinal centers to function in B cell help (79). Vɣ2Vδ2 T cells have also been reported to express Foxp3 and to display regulatory activity after IPP activation in the presence of IL-15 and transforming growth factor-β (TGF-β) (80). In addition, after 18–24 hours of IPP stimulation alone, tonsillar Vɣ2Vδ2 T cells showed a considerable overexpression of antigen-presenting cell-like activity, with accompanying surface expression of MHC-II, CD80, CD86, CD40 and CD54 (81).

The recognition of phosphorylated antigens allows Vɣ2Vδ2 T cells to mediate potent antimicrobial immune responses or promote the destruction of transformed host cells that upregulate IPP production (68)

CHAPTER II

Materials and Methods

A. Bacterial Strains

The *Mycobacterium tuberculosis* (MTB) used in this study is of the Erdman Strain. This was a gift from Dr William Jacobs (Albert Einstein College of Medicine). MTB Erdman strain and all samples derived from MTB infected macaques were placed on Middlebrook 7H11 media plates.

Mycobacterium bovis Karlson and Lessel (ATCC® 35748™); strain TMC 1108 [BCG Pasteur SM-R] was used to infect monocytes for the inhibition of bacterial intracellular growth assay.

B. Challenge of Cynomolgus Macaques with *Mycobacterium tuberculosis*

Cynomolgus 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 lobe, and 500 CFUs of MTB Erdman strain was administered in 3 mL of saline followed by 3 mL bolus of air to ensure full inoculum was administered to the macaques. Macaques were monitored for clinical signs of pulmonary TB 3 times weekly following infection and weighted once weekly to determine the extent of weight loss following infection.

C. Gross pathologic analysis of tuberculosis induced lesions

Eight weeks following infection, macaques were euthanized with pentobarbital 37.5 mg/kg IV) and immediately necropsied in a biological safety cabinet within a biosafety level 3 (BSL-3) facility. A specialized pathologist and assistants performed a blinded gross pathologic evaluation using standard procedures. Each step was documented and reported in details and pictures were taken. Organs including lung lobes, bronchial, mesenteric, axillary and inguinal lymph nodes, tonsils, liver spleen, kidneys, intestines and others were collected and labeled. Multiple tissue specimens were collected from all organs with gross lesions and also from organs showing no apparent lesions. For apparently infected organs, presence, number, location, size, distribution and consistency were recorded. A standard scoring system (82) was used to calculate gross pathology scores for TB lesions in lungs of infected macaques as follows: "For each of lung lobes, granuloma prevalence was scored 0–4 for (i) no visible granulomas, (ii) 1–3 visible granulomas, (iii) 4–10 visible granulomas, (iv) >10 visible granulomas, and (v) miliary pattern of granulomas, respectively. Granuloma size was scored 0–3 for (i) none present, (ii) <1–2 mm, (iii) 3–4 mm, and (iv) >4 mm, respectively. Pulmonary consolidation or atelectasis as viewed from organ exterior and cut surfaces were scored 0–2 for (i) absent, (ii) present focally in one lobe, and (iii) focally extensive within a 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 (i) visible but not enlarged, (ii) visibly enlarged unilaterally (≤2 cm), (iii) visibly enlarged bilaterally (≤2 cm), (iv) visibly enlarged unilaterally or bilaterally >2 cm, respectively. Tuberculosis lesions in hilar lymph nodes were scored 0–4 for (i) no granulomas visible on capsular or cut surface, (ii) focal or multifocal, circumscribed, non-coalescing granulomas, <2 mm, (iii) coalescing solid or caseating granulomas occupying<50% of nodal architecture, (iv) coalescing solid or caseating granulomas occupying >50% of nodal architecture, with residual nodal components still recognizable, and (v) complete granulomatous nodal effacement and caseation, respectively. One score was also given for tuberculosis-associated changes in other thoracic nodes. The tuberculosis lesions in each of extrathoracic organs were scored similarly as each lung lobe". All scoring of infected lungs and other tissues was performed in a completely blinded fashion.

D. Determining bacterial colony forming unit (CFU) counts in organ tissue homogenates.

One cubic centimeter tissue sections harvested from right caudal, right middle, 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 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 made using a homogenizer (PRO

200, PRO Scientific INC, CT) and diluting the homogenate in sterile PBS+0.05% Tween-80. 5-fold serial dilutions of samples were plated on Middlebrook 7H11 plate (BD).

E. Peripheral blood mononuclear cell (PBMC) isolation.

Freshly collected EDTA anti-coagulated blood was centrifuged and the buffy coat was removed and diluted in phosphate buffered saline (PBS). Diluted buffy coats were layered over Ficoll-Paque Plus (Amersham, Piscataway, NJ) and centrifuged to separate PBMCs from red blood cells (RBCs) and granylocytes. 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 hemocytometer.

F. Bronchoalveolar Lavage (BAL) and Bronchoalveolar Lavage Fluid (BALF) collection.

BAL was performed as previously described. Briefly, Macaques were sedated with Ketamine (10 mg/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 10 mg/kg of solution was placed in the lungs of macaques, and the recovery rate was greater than 50% in all cases.

G. Phenotyping of peripheral blood mononuclear cells and bronchoalveolar lavage lymphocytes from Macaques.

For cell-surface staining, lymphocytes were stained with up to 6 antibodies (conjugated to FITC, PE, APC, PB, PECy7, AF-700) for 15 min. 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).

H. Intracellular Cytokine Staining of Mononuclear Cells.

Intracellular cytokine staining (ICS) was performed as previously described (83). Briefly, 0.5×10^6 cells plus mAbs CD28 (1µg/ml) and CD49d (1µg/ml) were incubated with or without 50ng/mL HMBPP in 100µl 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 for 15 min, cells were permeabilized for 45 min (Cytofix/cytoperm, BD) and stained 45 min for intracellular cytokines.

I. Ethics statement

The use of macaques and experimental procedures were approved by Institutional Animal Care and Use Committee and Biosafety Committee

(Protocol A 13-128 (Appendix A)), University of Illinois College of Medicine at Chicago (UIC), and we followed the national and international guidelines [International Primatological Society (IPS) International Guidelines for the acquisition, care and breeding of nonhuman primates] regarding “The use of non-human primates in research”

J. Inhibition of *Mycobacteria* growth assay

The THP1 human monocyte cell line was cultured in RPMI-1640 supplemented with 10 % heat inactivated fetal calf serum. Cells were seeded at 2 × 10^5cells per well in 24 well plates and differentiated to macrophages by stimulation with 20 ng/ml phorbol myristate acetate (PMA) for 18 to 20 hours. Cells were subsequently washed with sterile medium and infected with either BCG at a dose of 10 or 20 bacteria per cell. After 3 hours of incubation at 37°C, supernatants were aspirated and each well was washed three times to remove non-ingested mycobacteria and medium was replaced with 10% FBS containing RPMI-1640 supplemented with 10µg/mL gentamicin to kill all extracellular bacteria. 5mM EDTA in PBS

Ex vivo expanded macaque Vδ2Vδ2 T cells were used as effector cells. aliquoted into wells containing target cells at the indicated ratios. The total volume of target effector:target cell mixture was 200 µl in all cases. At 24 hours following co-culture, cells were centrifuged, medium was removed, and cells were lysed using sterile water solution to release intracellular bacteria. Solutions were

serially diluted and placed on Middlebrook 7H10 agar plates to determine CFU counts of BCG after 3 weeks of incubation at 37°C-5% CO_2

Percentages of mycobacterial growth inhibition were determined as follows:

% inhibition = 100 - [100×(CFU in the presence of Vδ2Vδ2 T cells /CFU in the absence of Vδ2Vδ2 T cells)].

K. Collection of cells for adoptive transfer

Monkeys of assigned to receive Vɣ2Vδ2 T cells or peripheral blood lymphocytes (PBLs) were bled 4 times before the onset of the study in order to collect and freeze enough PBLs for the expansion/generation of cells to be infused back into the monkeys. 10% of the total monkey blood volume was collected weekly for 4 weeks. Blood was drawn from monkeys' cephalic or saphenous veins under anesthesia (Ketamine 10 mg/kg IM). This has been shown to be well-tolerated by macaques in past studies. The macaques maintained hematocrit and hemoglobin levels within the normal range with this blood collection schedule. Cell blood counts were ran at each collection time point as to make sure the animals were not anemic (hematocrit < 25%). If these limits were met, sample collection was delayed until the animal has returned to normal red blood cell parameters.

After the series of blood collections described above, monkeys had to rest for at least two weeks before the start of the actual experiment.

L. Adoptive transfer of autologous cells into macaques

For the IV administration of cells, monkeys were given 10 mg/kg ketamine IM for sedation. An IV catheter was placed in the cephalic or saphenous vein. After cell infusion, the catheter was flushed with ~2 ml sterile saline, and then removed.

M. Ex vivo expansion of Vγ9Vδ2 T cells

Fresh, frozen or a mix of both fresh and frozen PBMCs were used as a starting cell population for the ex vivo expansion of Vγ9Vδ2 T cells. Frozen PBMCs were thawed quickly after removal from liquid Nitrogen. Cryo vials were immediately placed in a 37°C water bath until thawed completely. Freezing medium was quickly washed out of cells and cells were washed 3-4 times with R10A. Cells were then counted using trypan blue exclusion to determine exact number of living cells.

PBMCs were resuspended in RPMI 1640 medium with 10% FBS, 1X antibiotics/antimycotics (Life Technologies; 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 2.5 µg/mL of Fungizone) and 5 µM Zoledronic Acid (Sigma Aldrich).

On day zero, cells were resuspended in culture medium at $2x10^6$ cells/ml and seeded in U shaped wells 96 well plates at 100µL/well. Plates were placed in a humidified 37°C, 5% CO_2 incubator. On day 3, a 100µL of human recombinant IL-2 (1000 IU/ml of R10A) were added to each well. On day 6, depending on the color of the medium and cell density, more IL-2 was added and cells were split. The culture was maintained at a cell density of 0.5-2 x 10^6 cells/ml. Fresh

medium containing human IL-2 (1000 IU/ml) only (without Zometa) was added every 2-3 days, and cells were split as needed. Cells were harvested on day 10-12 and the frequency, phenotype, and functions of γδ T cells was determined by flow cytometry.

N. Statistical analysis

Statistical analysis was done using paired or unpaired two-tailed Student's t test using Graphpad software (Prism, La Jolla, CA). Similar trends were seen using non-parametric analysis. Data compared were based on percentage, unless otherwise stated.

Chapter III

Ex vivo Expanded Macaque Vγ2Vδ2 T Cells Using Zoledronate and IL-2 Show Th1-Like Effector Functions and Inhibit the Intracellular Growth of Mycobacteria

A. Introduction and Rationale:

Vγ2Vδ2 T cells are only present in humans and non-human primates (68,84). Because of their ability to recognize and kill/lyse various types of tumor cells, these cells have been a major attraction in the field of cancer immunotherapy. It is believed that expanding these cells in vivo in cancer patients could help with cancer treatment (68,85).

In vivo expansion of Vγ2Vδ2 T cells is achieved by treating patients with aminobisphosphonates (ABPs) and IL-2 injections (85). The extent of Vγ2Vδ2 T cell expansion has proved to correlate well with the objective anti-tumor response in patients. However, several problems arose with such ABP/IL-2 treatment. Given the ubiquitous nature of the antigen presenting molecule, the IPP that accumulate in cells after ABP treatment can be presented to Vγ2Vδ2 T cells by any cell type and not only by antigen presenting cells. This robust and continuous activation of Vγ2Vδ2 T cells can induce cell anergy, immune exhaustion, and/or activation induced cell death (AICD). Even one intravenous

ABP injection in the absence of exogenous IL-2 probably leads to loss of Vγ2Vδ2T-cell function (68,85).

Since the vast majority of Vγ2Vδ2 T cells (approximately 98% in adults) are memory cells, there is a limited extent to which these cells can respond and expand after activation. The activation of Vγ2Vδ2 T cells by non-professional APCs that lack the proper costimulatory signals and/or cytokines has the tendency to lead to incomplete activation and AICD (68,76). This outcome appears to happen in monkeys because intravenous immunization can only be performed 2–4 times before there is no further Vγ2Vδ2 T-cell response (86). Similarly, patients actively treated with ABP without IL-2 ended up having low numbers of Vγ2Vδ2 T cells that couldn't be expanded in vitro in response to HMBPP and IL-2. Interestingly, recovery of proliferative responses in the patients appeared to be very slow, as minor Vγ2Vδ2 T-cell expansions were seen 6 months after cessation of therapy (68).

Because of the many problems accompanying the in vivo expansion of Vγ2Vδ2 T cells mentioned above, the use of these cells for cancer immunotherapy has been redirected to the adoptive transfer approach (87-89). Given the fact that Vγ2Vδ2 T cells comprise only 1-5% of the total T cell population in human blood, it was essential to find an efficient way to activate and expand these cells in vitro in order to obtain high enough numbers of functional cells for adoptive transfer. Of the many ways listed in the literature, ex vivo expansion of Vγ2Vδ2 T cells for cancer therapy was mainly achieved by culturing patient

derived autologous peripheral blood mononucleated cells (PBMCs) with phosphoantigens or ABPs and IL-2.

Our lab has been studying the role of Vɣ2Vδ2 T cells in immunity against TB in macaques for around 15 years. It is now very well established that these cells respond to MTB phosphoantigens by secreting various anti TB cytokines such as IFN-ɣ, TNF-α, granulysin, granzymes and perforin and expand accordingly. Despite the many studies performed, there is no direct evidence for an immune protection function of these cells against TB (84,86,90). So far, there are no depleting antibodies for Vɣ2Vδ2 T cells in the market. And, the IL-2 used along with phosphoantigens for the in-vivo expansion of these cells in TB infected macaques expands other types of TB specific CD4 and CD8 T cells (84,91). Hence, the protective clinical outcomes obtained after this in vivo expansion of Vɣ2Vδ2 T cells cannot be attributable to Vɣ2Vδ2 T cells exclusively.

From here we sought to use the adoptive transfer of ex vivo expanded autologous Vɣ2Vδ2 T cells into TB infected macaques as a more direct way to explore their exact role in immunity against TB. And, since no one has previously performed expansion of these cells from monkeys, it was our goal to expand macaque Vɣ2Vδ2 T cells and explore their various phenotypic and functional abilities afterwards.

B. Results and Discussion:

1. Ex vivo expansion of macaque Vγ2Vδ2 T cells using zoledronic acid and Interleukin-2.

Our lab had some experience expanding Vγ2Vδ2 T cells from macaques, but that was small scale/short term expansion for the purpose of testing the potential phosphoantigen containment of different pathogen lysates and their capability of activating/expanding Vγ2Vδ2 T cells. In those instances, synthetic HMBPP was used as a positive control. Basically, monkey PBMCs were cultured in medium containing HMBPP (40ng/mL) and 3 days later, IL-2 was added to the culture. On day 7, cells were harvested and analyzed for the expansion of Vγ2Vδ2 T cells by flow cytometry.

Looking back at the literature, we found that human protocols for the ex vivo expansion of human Vγ2Vδ2 T cells mainly use ABPs such as zoledronate to activate Vγ2Vδ2 T cells at the beginning of the culture (87-89,92). The main reason for that is that these ABPs are commercially available FDA approved drugs. Another reason is that ABPs lead to the accumulation of IPP in cells and IPP is known to have a much lower potential to activate Vγ2Vδ2 T cells compared to HMBPP; HMBPP is a 10,000X more potent (93).

From our experience with in vivo expansion of Vγ2Vδ2 T cells in monkeys, HMBPP producing *Listeria* led to robust activation/expansion of Vγ2Vδ2 T cells compared to IPP only producing Listeria but that was accompanied by a rapid exhaustion of cells inducing anergy and AICD (94) (unpublished data). Since

cell functionality and viability is crucial at the end of any expansion protocol, we have decided to adopt the human protocol and use zoledronic acid (also known as zoledronate) for more delicate cell activation.

In large scale expansions, the starting population of cells is large and the ending population is at least 2 fold larger. For that, the adequate selection of culture vessels, the adequate maintenance of cell density, the maintenance of contamination free cultures and the availability of enough incubator space become a real challenge. In human trials, 24 well plates are commonly used and PBMCs were cultured at $1X10^6$ cells /mL/well (92). So, for example, if the starting population of cells is 10^9, around 42 plates are needed for culture initiation and this number is expected to at least double at the end of the expansion. We have tried to adopt this protocol, but cell activation and expansion obtained were very weak. So, we decided to go back to our original small scale expansion protocol and use U shaped wells-96 well plates instead and $2X10^5$ PBMCs/100µL/well.

Interestingly, the overall cell activation and expansion improved significantly both in terms of final numbers and percentages of Vɣ2Vδ2 T cells obtained at the end (data not shown). Since the number of Vɣ2Vδ2 T cells and antigen presenting cells is low compared to other cells in PBMCs, we believe that accumulation of cells in the bottom of a U shaped well significantly increases the chances of interaction between antigen presenting cells (mainly monocytes) and Vɣ2Vδ2 T cells, allowing more activation to occur.

Another modification we have made is the time point of IL-2 addition. Human protocols add IL-2 to the culture in the very beginning along with zoledronic acid (87-89,92). Despite the fact that the total number of cells obtained at the end of the culture is higher, purity and percentage of Vγ2Vδ2 T cells is affected by the expansion of non Vγ2Vδ2 T cells that might have been already activated in the body at the time of blood collection and PBMCs isolation. One possibility of IL-2 driven non-Vγ2Vδ2 expansion is that of T regulatory cells. These highly express CD25, the IL-2 receptor (95). We believe that allowing those cells to rest for 3 days after isolation decreases their capability of expansion. In addition, since monkeys are not pathogen free, any kind of infection at the time of blood collection might produce infection specific activated T cells that can expand rapidly in response to IL-2.

From our observation of cells in culture, clusters of activated cells that are beginning to expand start to show up as early as 24 hours after the culture onset. And, these clusters continue to increase in size with time despite the lack of exogenous IL-2. These clusters represent actively dividing clones of T cells. We believe that most of these are Vγ2Vδ2 T cells that got activated by APCs presenting IPP and started expansion by some sort of endogenous cytokine secretion such as IL-2, IL-7 and IL-15. We find this to be interesting and worth further investigation.

In human protocols the expansion culture continues for 14 days before being harvested to be adoptively transferred to patients. We have seen that, in most

cases, cells stop dividing and start to die by day 13. Since it is extremely important to transfer living and functional cells to the monkeys in order to evaluate their role in immunity against TB, we decided to end expansion cultures anywhere between days 10 and 12 to guarantee a successful adoptive transfer with healthy living functioning cells, especially because apoptotic cells can cause induction of an immunosuppressive response inside the body (96).

2. Monkeys respond differently to ex vivo Vγ2Vδ2 T cell expansion.

We have screened PBMCs from 60 monkeys for their response to the Vγ2Vδ2 T cell expansion protocol. And we have based the success of expansion on total number of cells yielded and percentage of Vγ2Vδ2 T cells at the end of the culture. As shown in Figure 3.1, monkeys differed greatly in terms of percentages of Vγ2Vδ2 T cells yielded as well as with fold expansion. This variation was equally observed in both male and female monkeys.

We were expecting to find a difference between age groups since most Vγ2Vδ2 T cells in adults are known to have a memory phenotype; meaning that these cells have already been activated and expanded in the body as a result of the many bacterial infections that one can encounter in a lifespan. We found that not to hold true in monkeys. All different age groups showed the same tendency of Vγ2Vδ2 T cell expansion (Figure 3.1). It might be that monkeys are

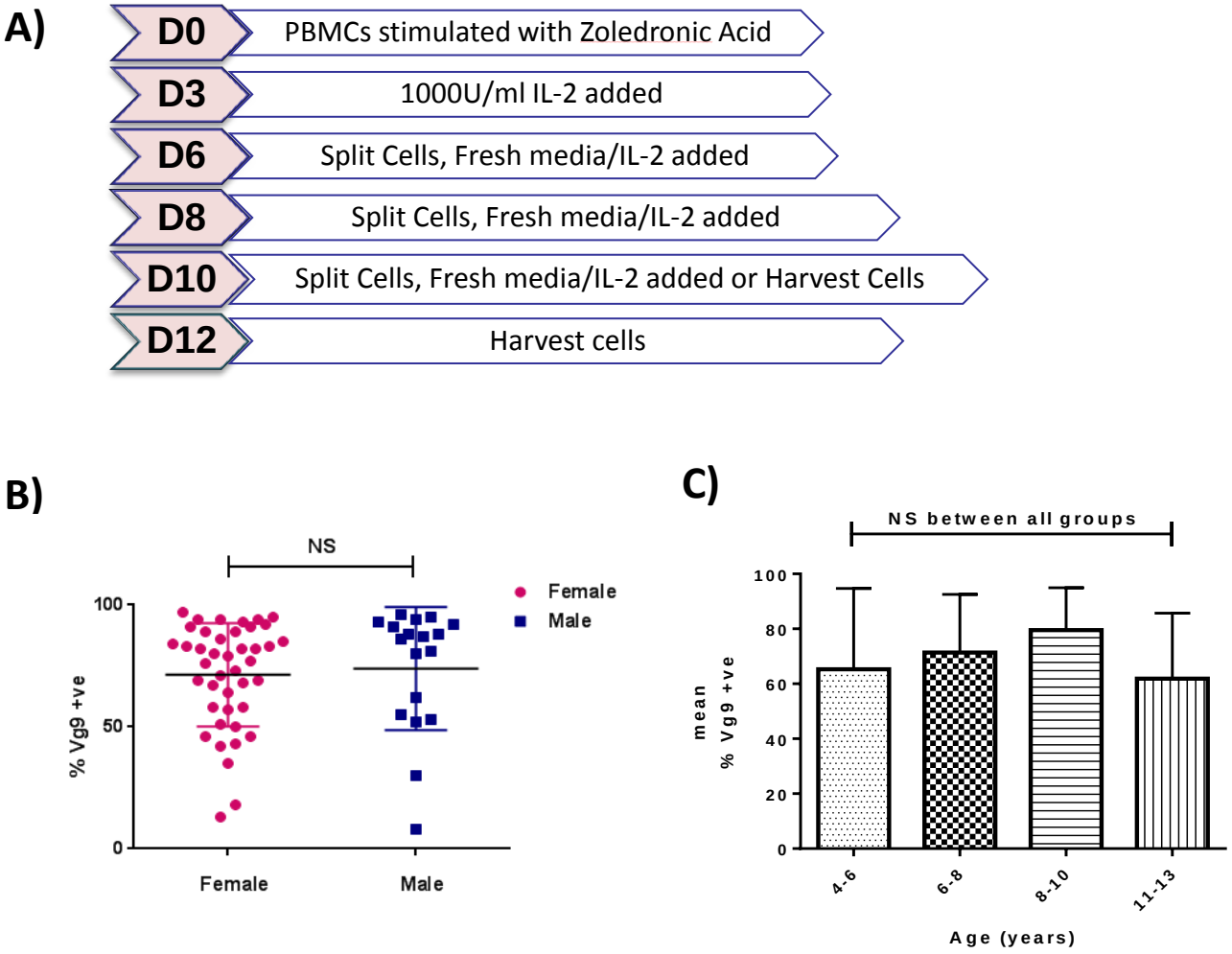


Figure 3.1: Monkeys respond differently to ex vivo Vɣ2Vδ2 T cell expansion. A) Steps of ex vivo expansion of macaque Vɣ2Vδ2 T cell using zoledronic acid and IL-2. B) Male and Female macaques respond similarly to the expansion protocol of Vɣ2Vδ2 T cells. C) Macaques of all age groups respond similarly to the Vɣ2Vδ2 T cell expansion protocol. D: day, IL-2: interleukin-2, NS: not significant.

different than humans in this aspect, since these monkeys originally came from non-human primate colonies raised in the wild or outdoors making them susceptible to different kinds of infection. The case is different for humans, especially in civilized parts of the world where sanitization/hygiene measures are taken into consideration when raising children all the way to adulthood.

3. No correlation was found between extent (fold) and purity (%) of ex vivo expansion of macaque Vγ2Vδ2 T cells.

Looking at the correlation between fold expansion and purity (percentage) of the Vγ2Vδ2 T cells obtained at the end, we found that all expansions that gave cell purities less than 40% also gave a cell yield less or equal to a 1 fold. Cell purities in this category were lower than the >1-2 fold yield category and significantly lower ($P<0.05$) than all other categories with more than a 2 fold yield (Figure 3.2).

Looking at individual purity values in categories with >2 fold yields, not all monkeys showed high purity such as 90% or higher. This indicates that, despite the addition of IL-2 on day 3 of culture initiation, some non- Vγ2Vδ2 CD3+ T cells are still expanding to some extent that can be as much as ~50% (Figure 3.2). As mentioned earlier, we expect these expanding cells to be either CD25 high T regulatory cells (Tregs), autoreactive T cells that get activated and respond to self antigens present in the PBMCs or T cells that were already highly activated in vivo in response to certain acute or chronic infections from which antigens are still presented in circulating antigen presenting cells.

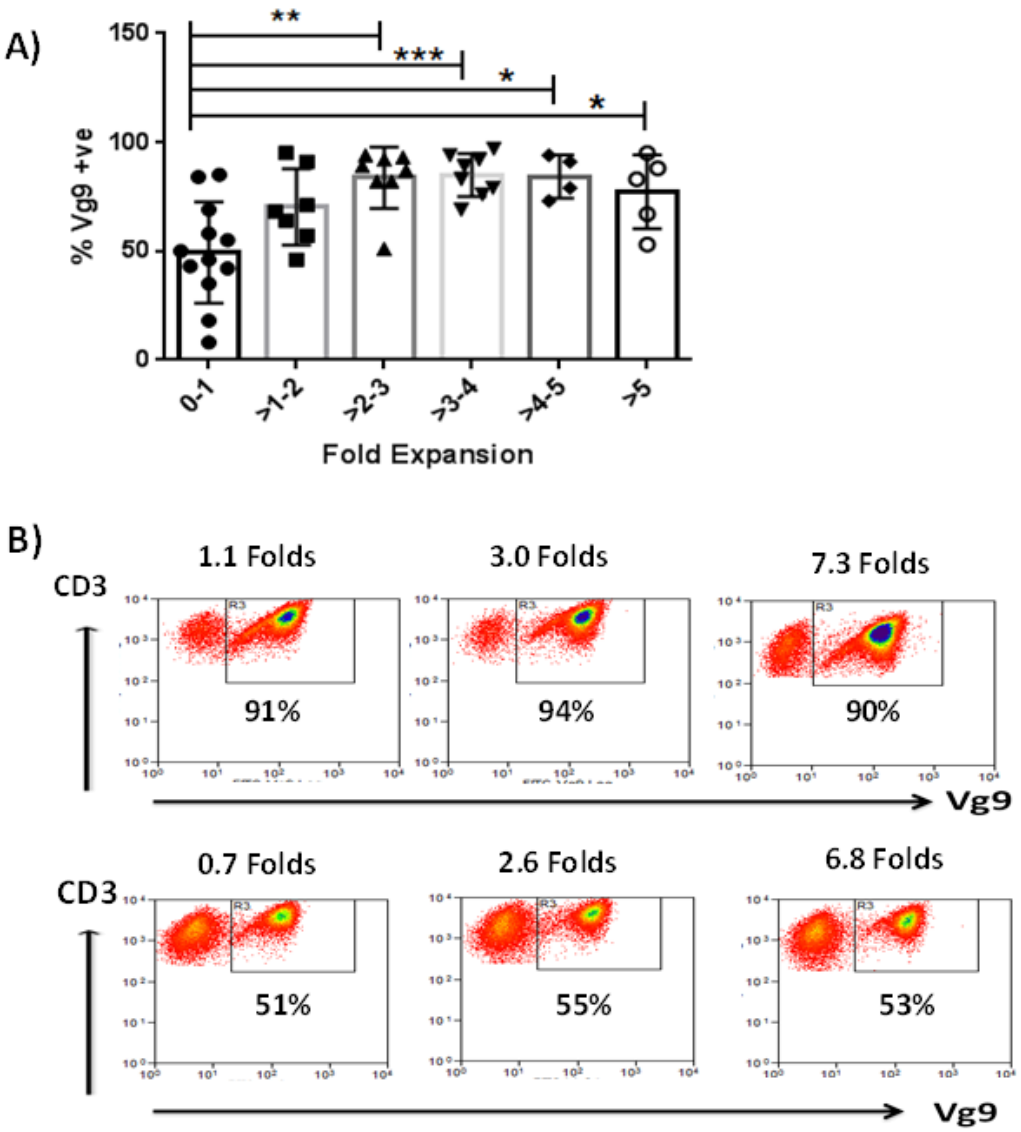


Figure 3:2: No correlation was found between extent (fold) and purity (%) of ex vivo expanded macaque Vγ2Vδ2 T cells. A) The relation between extent of expansion (folds) and cell purity (% of Vγ9 +ve of CD3+ cells) yielded at the end of expansion culture B) representative flow histograms showing examples of variable cell yields (folds) in respect to high cell purity (up panels) and low cell purity (down panels). * means statistically significant (P<0,05)

Another probability that could be thought of is the presence of a T cell population other than Vɣ2Vδ2 T cells that can recognize, respond and expand to phosphoantigen stimulation. Whether other subsets of gamma/delta T cells such as the Vδ1 subset respond to phosphoantigen is not known. This can be very interesting and can open a new field of investigation for a new aspect of immunity.

All of these questions are worth investigation. Answers to these questions can help improving the process of Vɣ2Vδ2 T cell expansion, especially in terms of cell purity yielded at the end. Having high percentages of Tregs or autoreactive T cells can certainly affect the outcomes of cancer immunotherapy and can cause autoimmunity. Nonetheless, it can affect outcomes of investigations that study the role(s) of these cells in immunity against infections.

4. Non Vɣ2Vδ2 T cells yielded after expansion are composed of both CD4 and CD8 T cells; and very few are T regulatory cells.

In a primary investigation, we inspected the identity of non-Vɣ2Vδ2 T cells present in the population of cells yielded after expansion. As Figure 3.3 shows, these cells represent a mix of both CD8+ and CD4+ T cells. Looking at the forkhead box protein 3 (FoxP3) expression, a marker for Tregs (95), low percentages (~5%) of non-Vɣ2Vδ2 T cells stained positive for FoxP3.

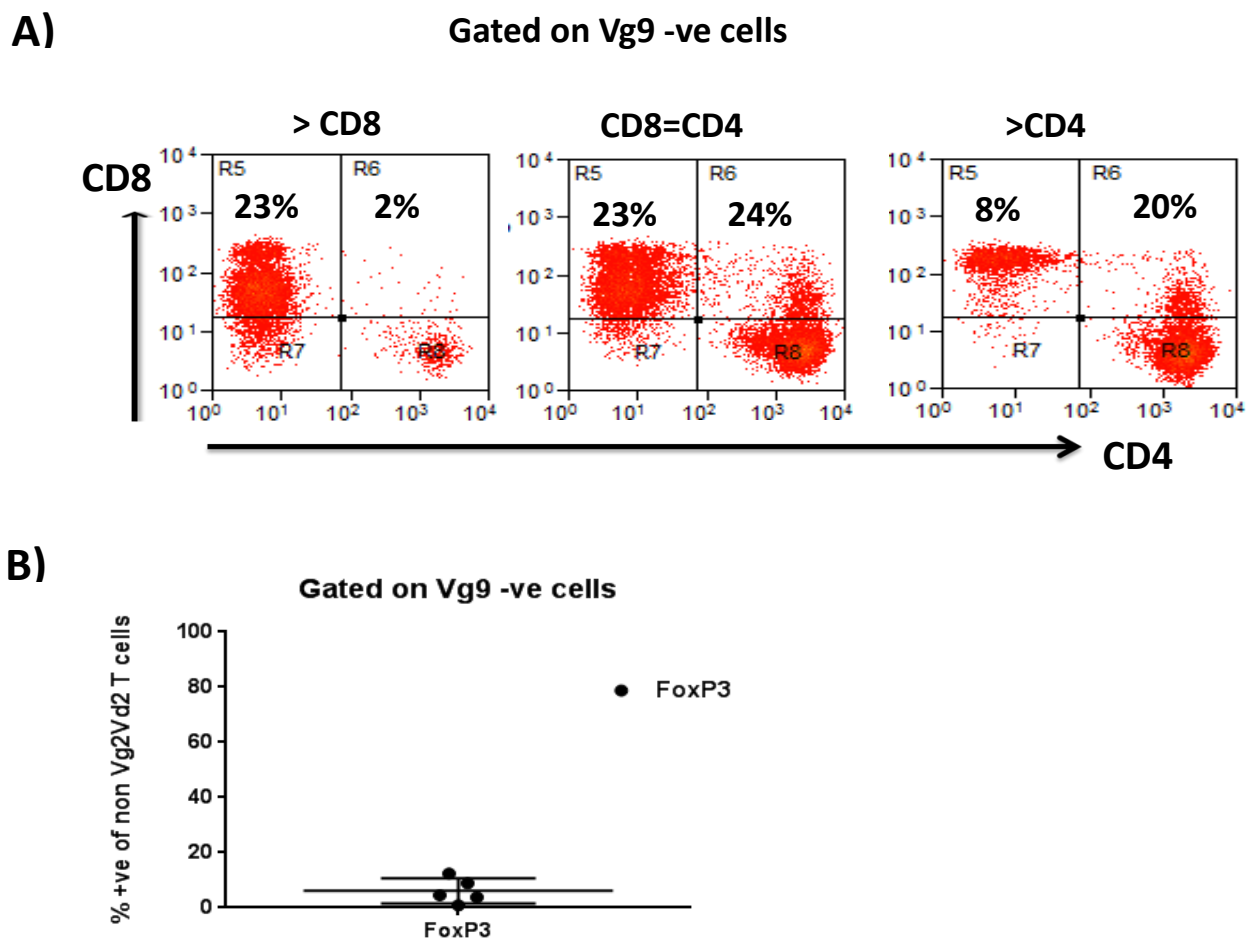


Figure 3.3: A) Non Vɣ2Vδ2 T cells yielded after expansion are composed of both CD4 and CD8 T cells; and B) very few are T regulatory cells. FoxP3: forkhead box protein 3

5. Ex vivo expanding Vγ2Vδ2 T cells show both effector and central memory phenotypes with a polarization to the effector memory.

We believe that the memory status of Vγ2Vδ2 T cells to be infused back into monkeys can impact the role they might play in immunity against TB infection. It is very well established that naïve (Tnaive) cells take longer to respond after antigen recognition but have a very high potency to differentiate and expand into both effector (TEM) and central memory (TCM) cells, with a polarization to the central memory category. TCM cells exert less function, like cytokine secretion and killing, upon antigen recognition but have a high potency to proliferate and expand. On the other hand, effector memory cells have a very high potential of exerting effector functions but have a reduced ability to divide and expand. Upon repetitive stimulation, effector memory cells become terminally differentiated cells that display robust cytotoxic potential by expressing killing cytokines in abundance and very little IFN-γ. However, these cells become unresponsive to further TCR stimulation and have no proliferative capacity (68,76,97).

Another difference between different categories of memory cells is their tissue homing preferences. While TCM prefer to stay in circulation and home back to the lymph nodes, TEM can be detected in the circulation and show a preference for homing to inflamed tissues, but very rarely to lymph nodes (97).

Human Vγ2Vδ2 T cells can be classified using surface expression of CD45RA, CD27 and CD28. CD45RA+CD27+ are Tnaive cells, CD45RA-CD27+CD28+ are

TCM and CD45RA-CD27+CD28- are TEM. Terminally differentiated TEM cells become CD45RA+ve again (TEMRA) cells to be CD45RA+CD27- (68,76,97).

From our expansions, after 10-12 days of culture, all resulting Vγ2Vδ2 T cells expressed the memory CD45RA-CD27+ phenotype. No naïve cells have been detected and a very small percentage (<1%) showed a terminally differentiated phenotype. Looking at the type of memory, there was a significant polarization toward the effector compared to the central phenotype (Figure 3.4).

Whether this bias toward the effector memory phenotype affects outcomes of cancer therapy using the adoptive transfer of Vγ2Vδ2 T cells is not known.

6. Vγ2Vδ2 T cells produce the inflammatory cytokines IFN-γ and TNF-α specifically in response to phosphoantigen restimulation.

Since our main goal behind ex vivo expanding Vγ2Vδ2 T cells is to eventually infuse them back into TB infected macaques and inspect their potential anti MTB protective role, we sought to investigate their capability of secreting Th1 cytokines, important for controlling the infection.

IFN-γ activates macrophages to kill and eliminate the mycobacteria. It also enhances their expression of MHC class II molecules, which results in improved antigen presentation to T cells (44).

While TNF-α has many functions, it is considered central to the appropriate control of MTB infection. During the initial stages, TNF-α acts primarily as an alarm cytokine alerting surrounding cells to the presence of an infection. It is

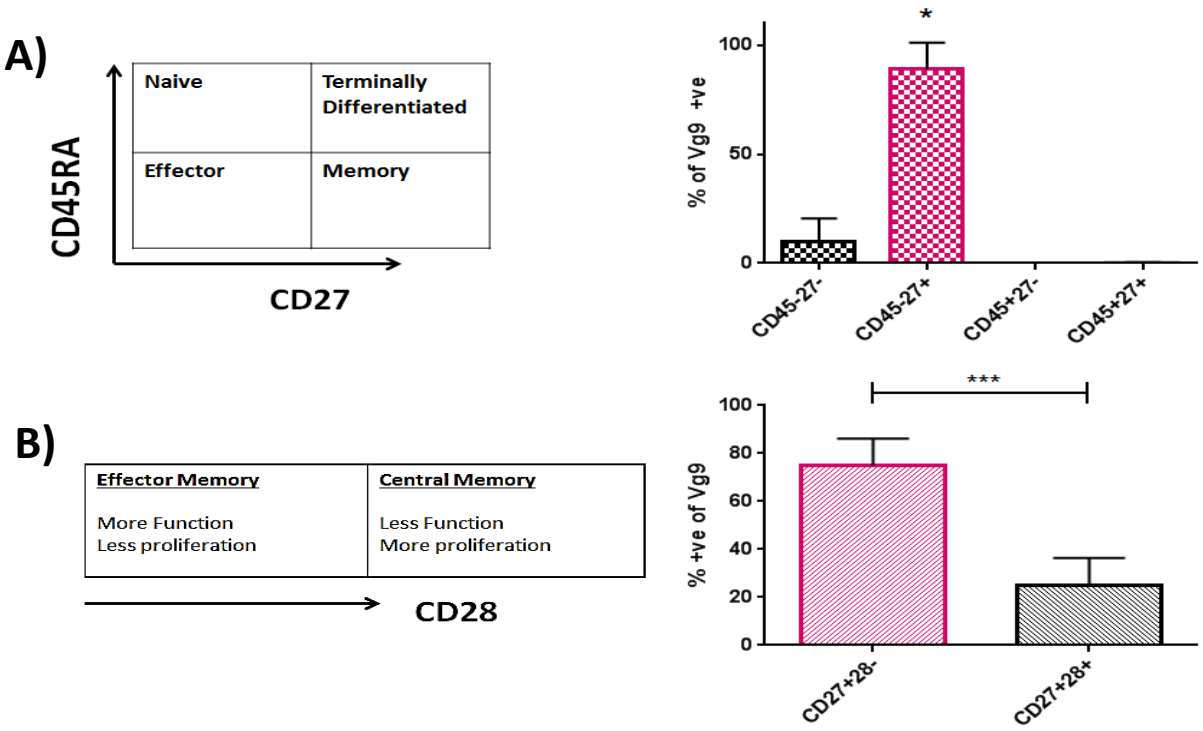


Figure 3.4: A) Ex vivo expanding Vɣ2Vδ2 T cells show both effector and central memory phenotypes with B) a polarization to the effector memory.

believed that macrophage derived TNF commences the recruitment of innate immune cells (46,47).

Ex vivo expanded Vɣ2Vδ2 T cells do produce IFN-ɣ and TNF-α specifically in response to restimulation with phosphoantigens (Figure 3.5); suggesting that these expanded cells have the potential of exerting protective functions against MTB.

7. Vɣ2Vδ2 T cells express the chemokine receptor CXCR3, a hallmark of activated T helper 1 cells.

Since the ultimate goal of expanding Vɣ2Vδ2 T cells is using them in immune intervention for the sake of therapy or for conducting basic research explorations related to the functions of these cells, it is very important to know their potential homing destinies after adoptive transfer. For that, we sought to determine whether these cells preferentially express certain homing receptors over others. We have explored the potential expression of the chemokine receptors CXCR2, CXCR3 and CXCR5 and the cell adhesion molecule CD62L.

CXCR2 is important for homing to sites of inflammation where its ligand, the inflammatory cytokine IL-8, is secreted by monocytes upon pathogen/danger sensing in the beginning of an immune response, and accumulating to recruit neutrophils and other innate cells (98). CXCR5 is a hallmark of T cells that reside in lymph nodes and are specialized in providing B cell help (99). CD62L, also known as L-selectin, acts as a homing receptor for lymphocytes to enter secondary lymphoid tissues via high endothelial venules.

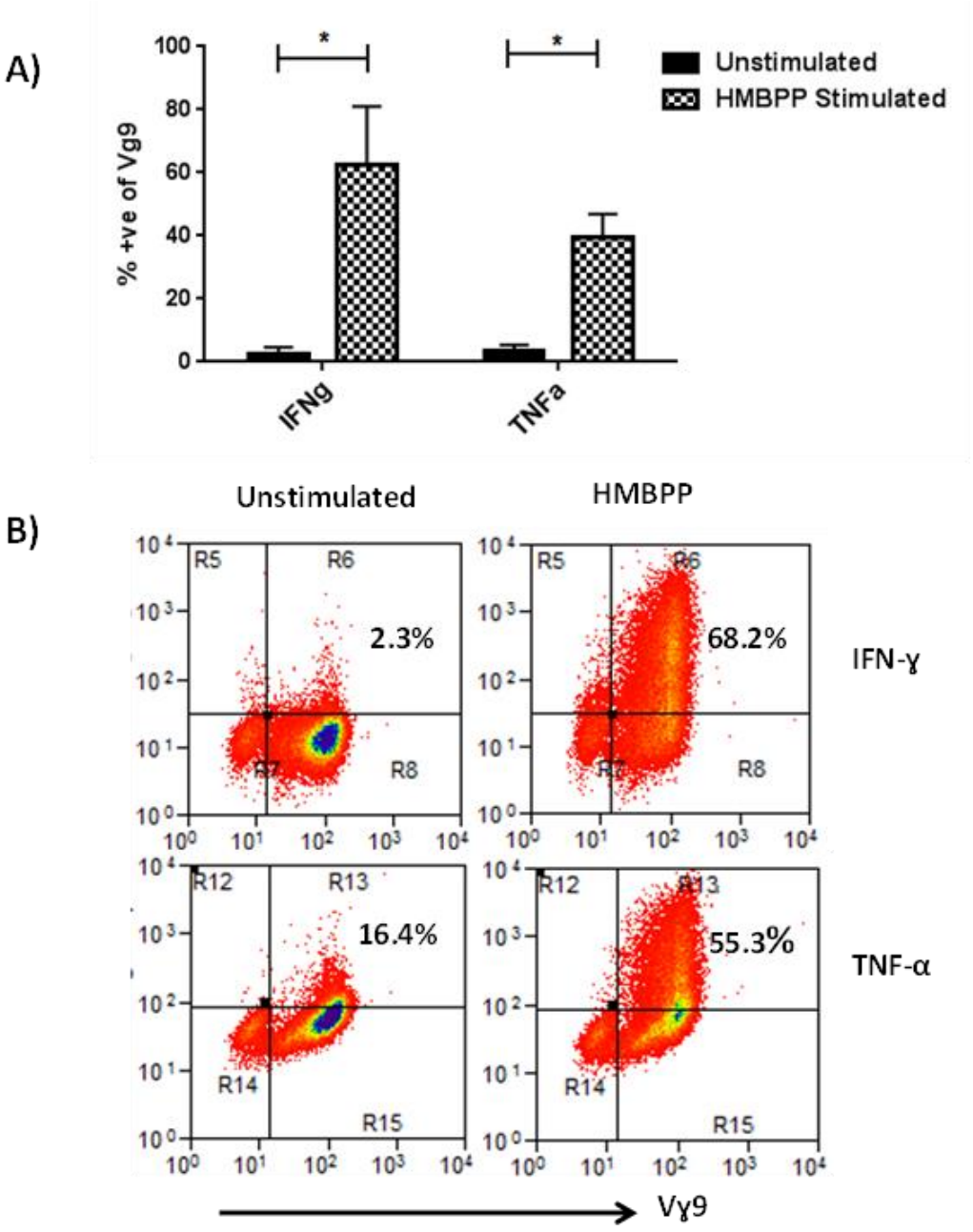


Figure 3.5: A) Vγ2Vδ2 T cells produce the inflammatory cytokines IFN-γ and TNF-α specifically in response to phosphoantigen restimulation. B) Representative histograms showing the specificity of IFN-γ and TNF-α secretion in response to HMBPP stimulation. IFN-γ: Interferon γ, TNF-α: Tumor Necrosis Factor α. HMBPP: E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate.

CD62L is usually expressed by naïve and central memory T cells (100).

CXCR3 is an inflammatory chemokine receptor whose expression is associated with CD4+ Th1 cells and CD8+ cytotoxic lymphocytes. CXCR3 and its ligands regulate the migration/recruitment of Th1 cells into sites of Th1 driven inflammation where IFN-ɣ is produced in abundance. Chemokine Ligands of CXCR3 (CXCL9, CXCL10 and CXCL11) are produced by monocytes, endothelial cells, fibroblasts and other cells upon IFN-ɣ induction. CXCR3 through binding of its chemokine ligands has been shown to coordinate inflammation in the periphery (101) .

Interestingly, we found that almost all of ex vivo expanded Vɣ2Vɣ2 T cells using zoledronate and IL-2 express CXCR3. In contrast very few expressed CXCR2, CXCR5 or CD62L (Figure 3.6).

More interestingly, a recent study has shown that TB specific CD4 T cells expressing CXCR3 have a higher potential of controlling MTB infection in mice, mainly due to their special ability of populating lung parenchyma tissue compared to cell that do not express CXCR3 (102-104).

The fact that our ex vivo expanded Vɣ2Vδ2 T cells exclusively express CXCR3 suggested a stronger potential of these cells to fight TB.

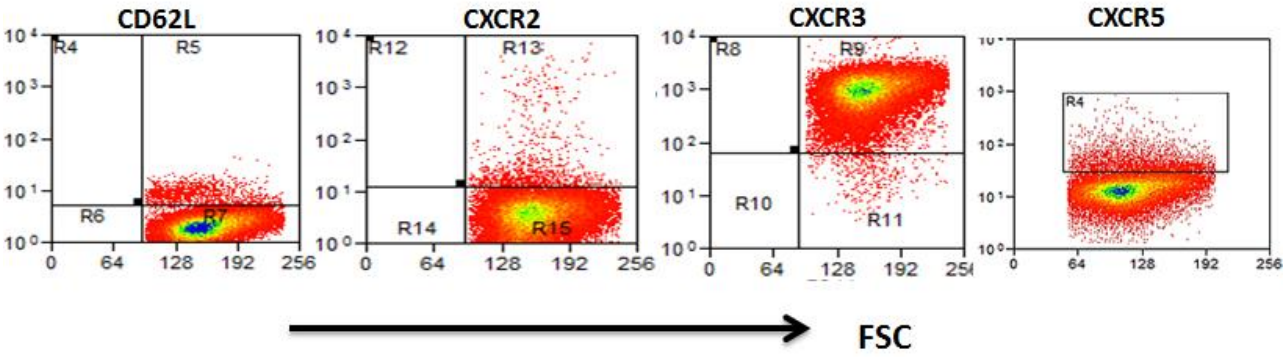


Figure 3.6: Vγ2Vδ2 T cells highly express the chemokine receptor CXCR3, a hallmark of activated Th1 T cells. Very few cells express CD62L (L-selectin), CXCR2 or CXCR5. FSC: forward scatter.

8. Vγ2Vδ2 T cells produce cytotoxic cytokines without the need for specific phosphoantigen restimulation.

We next sought to determine whether ex vivo expanded Vγ2Vδ2 T cells produce killing cytokines upon restimulation with PAgs. We have screened for the potential production of perforin, granulysin, granzyme A and granzyme B; known to play different and complementary roles in the killing action of cytotoxic/killer cells.

Perforin is a pore forming protein that can directly damage the cytoplasmic membrane of target cells. Upon secretion by cytotxic T cells or natural killer cells, perforin binds and inserts itself into the phospholipid bilayer of the target cell plasma membrane and polymerizes to form pores spanning target cell membrane. In addition, perforin is essential for the delivery of other components of the cytotoxic granules, such as granzymes, to the cytosol of target cells (105).

Granzymes are members of a family of serine proteases that are ultimately responsible for target cell death. Granzyme A and Granzyme B are the most abundant and thoroughly characterized in humans. While granzyme B is clearly cytotoxic by the induction of apoptosis in target cells, granzyme A has been shown to take a different role in killing. Mechanisms of granzyme A killing include the degradation of extracellular matrix, and the induction of adherent cells to manufacture proinflammatory cytokines (106).

Granulysin is a cytolytic protein which belongs to the saposin (lipid degrading) like protein family. Interestingly, granulysin have been shown to directly reduce the viability of a broad spectrum of pathogenic bacteria, fungi, and parasites. The underlying mechanism of granulysin's cytotoxicity may involve the insertion of its positively charged domain into the negatively charged surface of target microbes or cells, resulting in alteration of membrane permeability (105).

As demonstrated in Figure 3.7, ex vivo expanded Vɣ2Vδ2 T cells highly express both granzymes A and B and to a lower extent granulysin and perforin. To our surprise, this robust production of cytotoxic cytokines is not HMBPP specific. More strikingly, a reduction in cytokine production was noticed upon HMBPP stimulation compared to no stimulation in the case of granzyme A.

Although hard to explain, we believe that some speculations are worth further investigation. First, in our protocol, the "unstimulated" condition means lack of antigen of interest but still includes co-stimulatory signaling by the addition of anti CD28 and anti CD49d antibodies to the cells of interest. Whether co-stimulation alone is enough to induce the production of killing cytokines is unknown.

Second, in a previous experience of ours in expanding T regulatory cell using artificial beads coated with anti CD3 and anti CD28 antibodies in the beginning of the culture followed with a continuous supply of high dose IL-2, a similar pattern of robust granzymes and perforin production was noticed without the need of cell reactivation or the addition of costimulatory signaling.

Whether high production of killing cytokines is a manifestation of active cell division induced by IL-2 is unknown and worth investigation.

Third, it has been previously reported that cells become highly cytotoxic in advanced stages of effector memory when terminal differentiation is close to being reached (76). Although very few of our cells expressed the TEMRA terminally differentiated (TEMRA) phenotype, we believe that after such a robust expansion, they are in advanced stages of Effector memory.

A fourth possibility that needs exploring is the ability of these cells to degranulate as a function of antigen restimulation. Killing/cytotoxic cytokines are usually produced and packed inside exocytic granules in the cytoplasm of killing cells and degranulation is required for the accomplishment of killing upon antigen encounter. It would be interesting to know whether these highly produced killing cytokines are being secreted and whether this secretion is antigen specific or not. From our data, the reduction of cytokines produced upon HMBPP stimulation of Vγ2Vδ2 T cells at the end of expansion suggests such an antigen specific degranulation action. Looking at the literature, it has been reported that effector memory CD8+ T cells contained stored granzyme B that could be released upon antigen encounter, leading to some levels of immediate cytotoxicity. In contrast, only very low numbers of central memory cells had intracellularly stored granzyme B, and as a result, were incapable of immediate cytolytic activity (107,108). Inspecting CD107a, a marker of degranulation, can be useful for further investigations.

Another interesting finding of ours; expanded Vδ2Vδ2 T cells upregulated the expression of CD56, a marker associated with killing (109), upon restimulation with HMBPP (Figure 3.7C). This further supports the idea that degranulation of killing cytokines might be phosphoantigen specific, as opposed to the non-specific production of these cytokines.

9. Vɣ2Vδ2 T cells do not secrete pro-inflammatory or anti-inflammatory (suppressive) cytokines.

For a more complete screening, we have inspected the ability of expanded Vɣ2Vδ2 T cells to secrete the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and IL-17; all known to recruit innate cells to the site of inflammation; mainly neutrophils, monocytes and DCs (42,110). Production of these cytokines by our Vɣ2Vδ2 T cells was very minimal or even diminished (Figure 3.8A).

Similarly, we sought to determine whether expanded Vɣ2Vδ2 T cells produce anti-inflammatory (inhibitory) cytokines of IL-10 and TGF-β, normally produced by T regulatory cells (95). No production of suppressive cytokines was detected (Figure 3.8B).

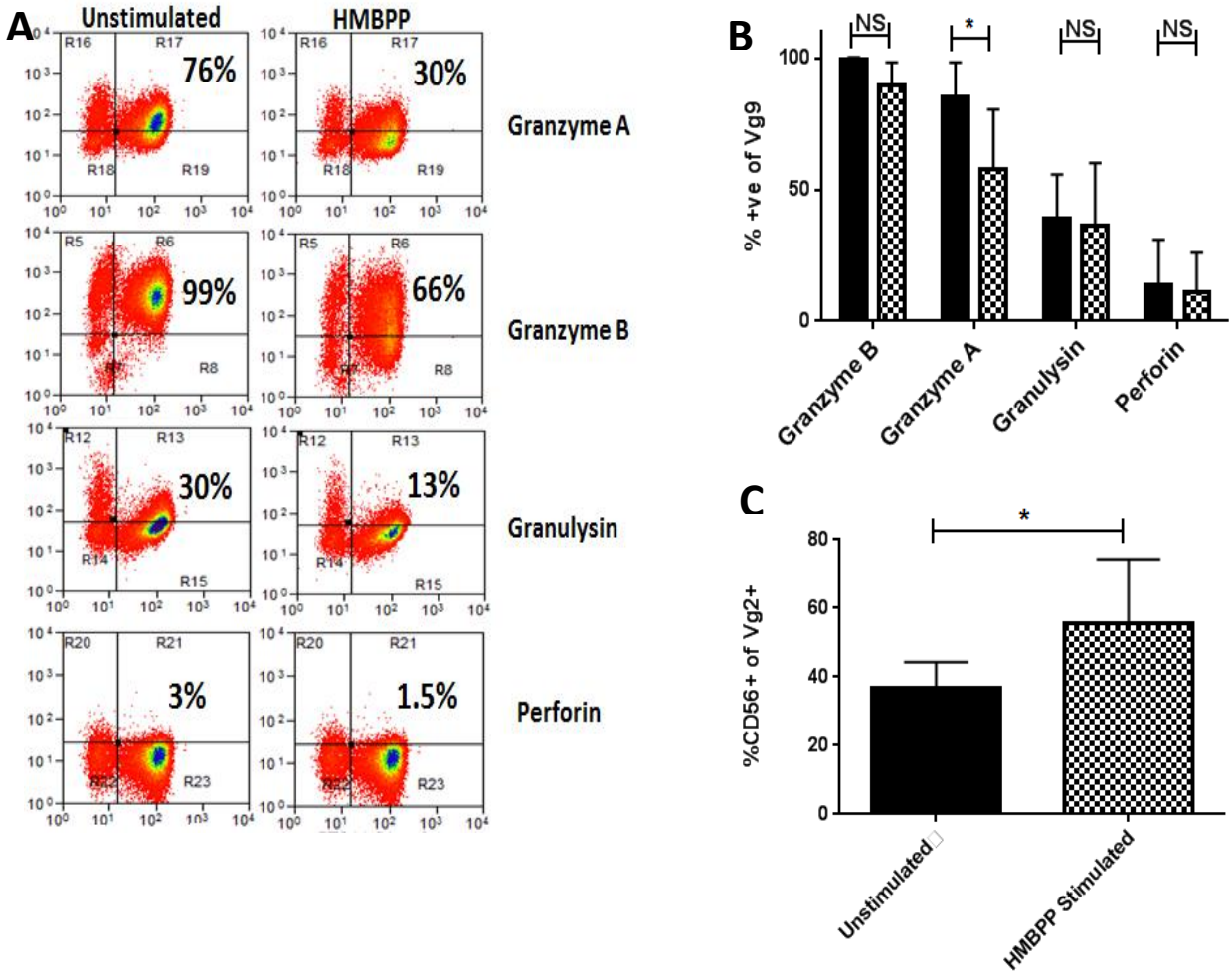


Figure 3.7: Vγ2Vδ2 T cells produce cytotoxic cytokines without the need for specific phosphoantigen restimulation. A) Representative flow histograms showing the % of Vγ2Vδ2 T cells producing killing cytokines with or without HMBPP stimualtion B) Statistical significance of HMBPP stimulation C) Vγ2Vδ2 T cells upregulate CD56 (marker of killer cells) upon HMBPP stimulation. HMBPP: E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate.

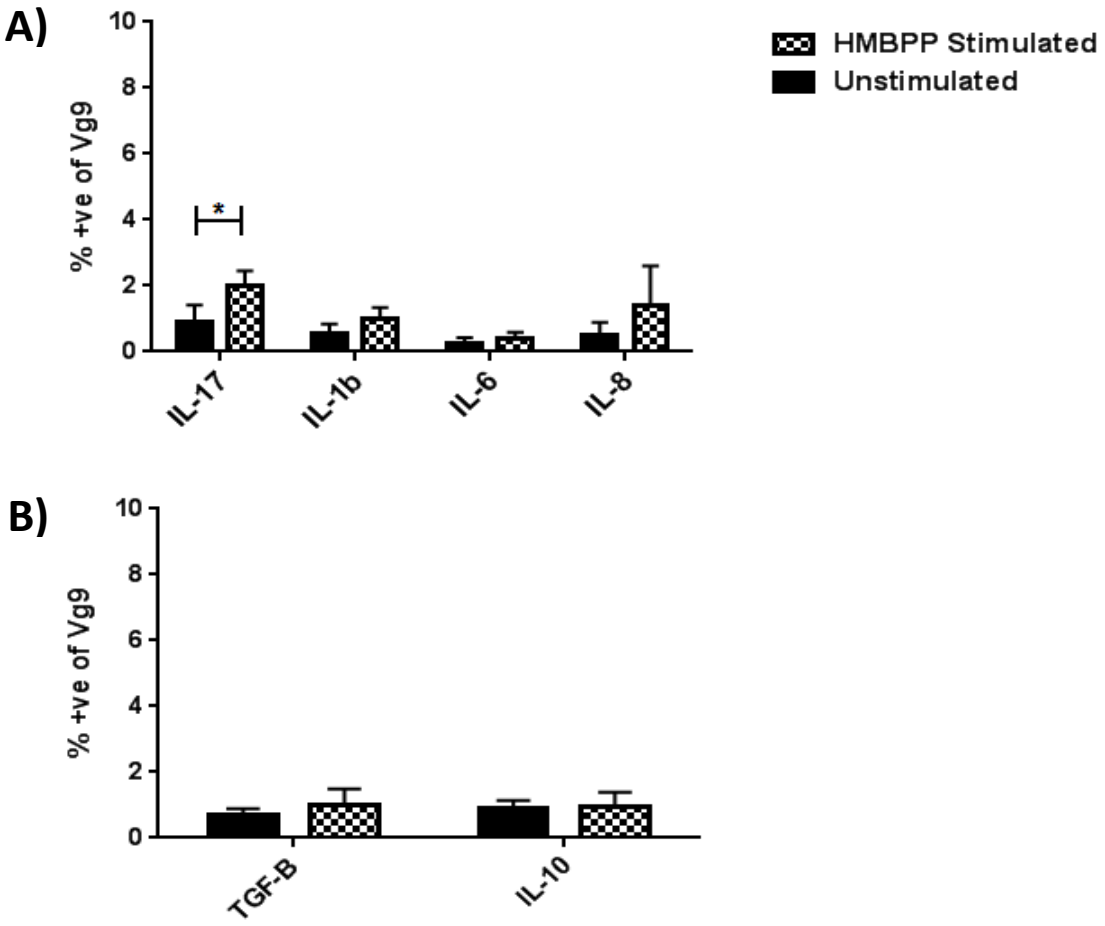


Figure 3.8: Vγ2Vδ2 T cells do not secrete A) pro-inflammatory or B) anti-inflammatory (suppressive) cytokines. IL-17: Interleukin 17, IL-1β: Interleukin 1 β, IL-6: Interleukin 6: IL-8: Interleukin 8, TGF-B: Tumor Growth Factor beta, IL-10: Interleukin 10. HMBPP: E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate.

10. Vγ2Vδ2 T cells are capable of inhibiting the intracellular growth of *Mycobacterium bovis* (BCG)

Since expanded Vγ2Vδ2 T cells have shown to robustly produce killing cytokines regardless of antigen stimulation, we needed to investigate their functional capability of cytotoxicity in vitro. For that, we performed an inhibition of growth assay where THP1 monocytes have been infected with BCG and co-cultured with expanded Vγ2Vδ2. In addition we have investigated the role of different cytokines in inhibiting the growth of mycobacteria using blocking antibodies against TNF-α, IFN-γ and granzyme A. Both IFN-γ and TNF-α are known to activate the phagocytic activity of macrophages (44,46,47), whereas granzyme A is known to induce the production of pro-inflammatory cytokines, such as TNF-α, and eventually activate phagocytosis (106).

As shown in Figure 3.9, at an effector:target ratio of 10:1, Vγ2Vδ2 T cells were able to kill/inhibit intracellular MTB at a rate of ~70%. The addition of blocking antibodies against different cytokines significantly affected this inhibition in all cases; with TNF-α being the most significant. In fact, in a recent study, granzyme A secreted by Vγ2Vδ2 T cells was shown to induce mycobacterium infected macrophages to produce TNF-α and consequently, significantly inhibited bacterial growth (111). This might explain the higher effect of TNF-α blocking shown here; as it might involve blocking of granzyme A activity as well.

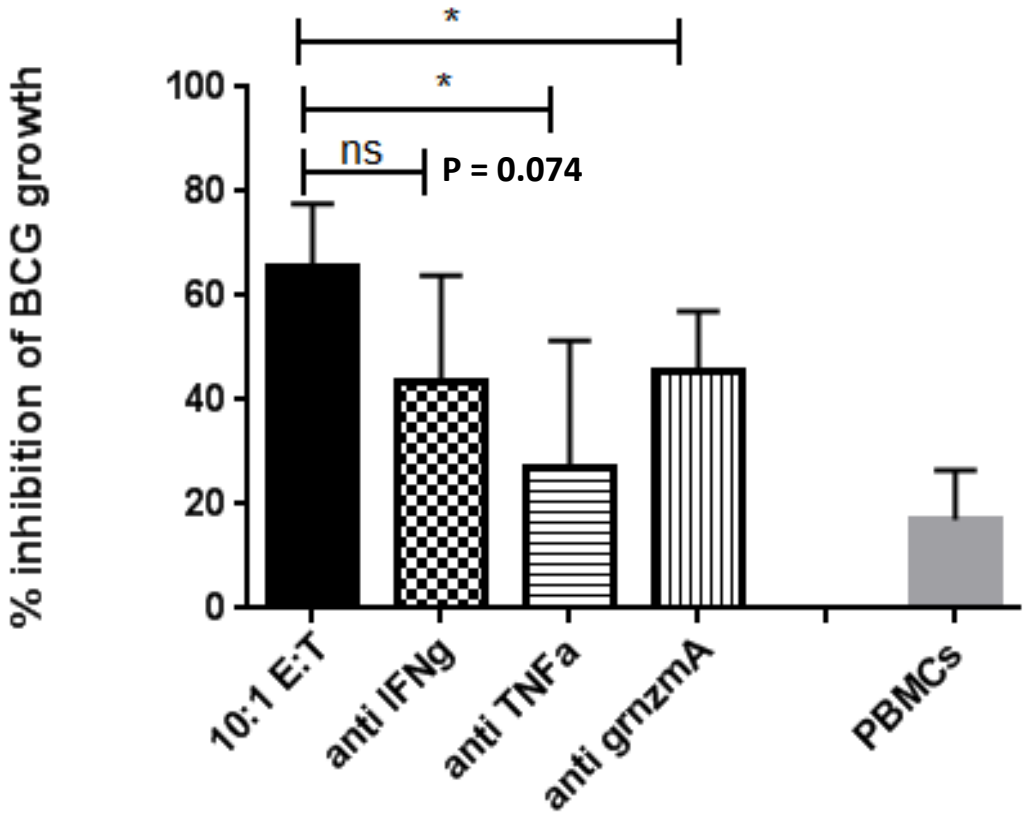


Figure 3.9: Vγ2Vδ2 T cells are capable of inhibiting the intracellular growth of M. bovis (BCG). BCG: Bacillus Calmette Guerin, E:T: Effector:Target, IFNg: Interferon ɣ, TNFa: Tumor Necrosis Factor α, grnzmA: Granzyme A, PBMCs: Peripheral Blood Mononuclear Cells.

Results of the inhibition of growth assay described here emphasizes that expanded Vɣ2Vδ2 T cells can recognize PAgs in the context of an infected cells and, as a result, respond by producing inflammatory and killing cytokines that significantly reduce the survival/growth of intracellular mycobacteria.

A more elaborative experiment using blocking antibodies against other cytokines; such as granzyme B, granulysin and perforin is worth following. In addition, the use of MTB instead of BCG and autologous monocytes instead of THP1 cells can make more accurate/specific conclusions in terms of describing the role Vɣ2Vδ2 T cells play in immunity against TB.

C. Concluding Remarks and Future Directions.

This study represents the first attempt to expand Vɣ2Vδ2 T cells from macaques. Ex vivo expansion of these cells was achievable using zoledronate and IL-2 as applied in protocols of human Vɣ2Vδ2 T cell expansion with some modifications.

At the end of expansion, most Vɣ2Vδ2 T cells express the effector memory phenotype (CD45RA-CD27+CD28-) and very few were terminally differentiated.

Ex vivo expanded Vɣ2Vδ2 T cells express the chemokine receptor CXCR3; important for homing to sites of Th1/IFN-ɣ induced inflammation. In addition, the cells specifically produce IFN-ɣ and TNF-α, associated with Th1 functions, upon restimulation with HMBPP. All indicating a suggestive protective role against MTB.

Ex vivo expanded Vγ2Vδ2 produce killing cytokines regardless of PAg restimulation. Looking for the potential of antigen specific degranulation is worth investigation.

The cells are capable of killing/inhibiting the growth of intracellular BCG. This action can be mediated by IFN-γ, granzyme A and most significantly TNF-α. Investigating the role of other killing cytokines can very informative of the potential multifunctionality of these cells.

As for future directions, we are looking forward to improve the expansion protocol by including inhibitors of cell exhaustion. We are hoping for better yields in terms of numbers and quality.

Chapter IV

Tracking of Fluorescently Labelled Vγ2Vδ2 T Cells in Blood and Broncho-Alveolar Lavage Fluids of Naïve Cynomolgus Macaques

A. Introduction and Rational:

As mentioned earlier, many problems are associated with in vivo expansion of Vγ2Vδ2 T cells. Continuous activation of Vγ2Vδ2 T cells using aminobisphosphonates (ABPs) and IL-2 leads to cell exhaustion, anergy and activation induced cell death (68,112). All that could eventually result in a systemic defect of Vγ2Vδ2 T cells in the body and, potentially, defective immune responses to come.

Similarly, immunotherapy using the adoptive cell transfer of ex vivo-expanded Vγ2Vδ2 T cells could have potential problems. Like αβ T cells, γδ T cells appear to have a homeostatic set point (113). Thus, additional γδ T cells infused intravenously may rapidly be lost, as there is insufficient 'space'. This loss has been a major problem in adoptive immunotherapy with tumor specific αβ T cells. In such cases, preconditioning the recipient by lymphodepletion using non-myeloablating chemotherapeutic agents resulted in better outcomes in terms of cancer treatment (114-116). Whether similar lymphodepletion approaches could help the persistence of Vγ2Vδ2 T cells after adoptive transfer has not yet been investigated. However, it is believed that supporting the

infused Vγ2Vδ2 T cell population with injections of low dose IL-2 can help the survival and, probably, the proliferation of these cells in the body. Interestingly, in a very recent study, Izumi et al (117) reported that Vγ2Vδ2 T cells persisted and increased in blood during and up to 12 weeks after the course of 8 weekly infusions without prior lymphodepletion or ongoing cytokine treatment. Importantly, infused cells maintained effector functions of IFN-γ production upon restimulation with PAgs, suggesting the possible presence of "some" endogenous factors that could support the persistence and functionality of these cells in vivo.

Another potential problem related to the adoptive transfer of Vγ2Vδ2 T cells is the homing/trafficking of these cells. Where these cells go after intravenous infusion could play a critical role in the success of treatment using the adoptive transfer strategy.

In a mouse model of human breast cancer, adoptively transferred γδ T cells labeled with radioisotope have shown to specifically localize to breast tumors. On the other hand, these cells were found to mostly localize to the spleen of healthy mice (118). Similarly, in humans, Vγ2Vδ2T cells were found to traffic to the lungs within the first hours of infusion and gradually homed to the spleen, liver and tumor site afterwards (89).

In terms of tumor progress, adoptively transferred human γδ T cells were able to control the growth of tumors generated by human breast cancer cells xenografted into athymic (nude) mice (118). In humans, treatment outcomes

have ranged from total failure to partial remissions to stable disease and rarely to cure. In general, Vγ2Vδ2 T cell based immunotherapy has shown to improve overall survival of cancer patients. Interestingly, outcomes correlated very well with the ability of Vγ2Vδ2 T cells to expand in vitro and with the number of cells infused back into the patient; suggesting that infused cells can persist to periods that allows them to reach tumors and exert effector functions against them (68,87-89,119).

To our knowledge, there is only one study that used the adoptive transfer of ex vivo expanded Vγ2Vδ2 T cells in the context of an infection. To investigate their anti-viral activity, highly purified expanded Vγ2Vδ2 T cells were transferred intravenously into influenza infected humanized mice after 2, 4 and 6 days of infection (120). Vγ2Vδ2 T cells peaked in the blood after the second infusion and then gradually decreased back to basal levels 10 days later. Mice treated with Vγ2Vδ2 T cells showed less viral replication in the lungs and reduced weight loss compared to control mice and, interestingly, more survived. This further suggested that ex vivo expanded Vγ2Vδ2 T cells are functional in vivo and play a role in controlling influenza infection.

As shown in Chapter 3 of this thesis, we have successfully expanded Vγ2Vδ2 T cells from macaques ex vivo. Expanded cells showed phenotypic and functional aspects similar to Th1 CD4+ cells and were able to inhibit the growth of mycobacteria when cultured with BCG infected monocytes. Our main goal for expanding these cells is to use them for studying their potential role in immune

protection against TB in a clean model of adoptive transfer without the interference of exogenous cytokines that potentially support/enhance function of other TB specific immune cells. It is of extreme importance to know whether these cells are able to persist and function in vivo after infusion. For that, we sought to infuse naïve monkeys with fluorescently labeled ex vivo expanded autologous Vγ2Vδ2 T cells and use flow cytometry to track cells in blood and BALF at several time points post infusion.

B. Results and Discussion:

1. Study Design

We have designed a tracking experiment where Vγ2Vδ2 T cells were fluorescently labelled and infused back into naïve monkeys and tracked them in blood and BALF at several time points after infusion. We have looked in the literature searching for a suitable fluorescent tracking dye that does not interfere with FITC, the dye marking our anti Vγ9 (Vγ2) antibody, and found PKH26. In order to secure enough cell numbers to be detected by flow cytometry after infusion, we collected and froze PBMCs from 4-5 blood draws; each forming 10% of the monkeys' total blood volume. All cells were thawed simultaneously and used for one large scale ex vivo expansion and one infusion. In order to reduce cell manipulation and because of the high toxicity of the PKH26 dye, only half of the total cells infused back into the monkeys were fluorescently labeled. PKH26 is a fluorescent lipophilic dye that mainly stains cell membranes and the working solution provided with the staining kit

is ethanol based. From our experience, PKH26 was very toxic to our cells causing considerable cell death and loss of function (data not shown). Labeled and unlabeled cells were mixed right before infusion.

As shown in Table 1, three monkeys were used in this experiment; each received different number of cells/Kg of body weight. For tracking, blood samples were collected 5 minutes, 30 minutes and 1, 3, 6, 12 and 24 hours after infusion and then every other day until day 7. BALF samples were collected 1, 6 and 24 hours after infusion, and then at days 3 and 7.

2. Vγ2Vδ2 showed a tendency to accumulate and persist in the lungs for at least 7 days post infusion

Interestingly, cells were detected in both blood and BALF samples after infusion (Figures 4.1, 4.2 and 4.3). Cells peaked in blood 5 minutes post infusion and started to disappear 30 minutes later in all 3 monkeys. In the case of 3 X 10^8 cells/Kg infusion, percent of Vγ2Vδ2 T cells in blood tripled at their peak compared to baseline and PKH26 +ve cells constituted around 50% of the total Vγ2Vδ2 population. Total percent of Vγ2Vδ2 T cells was still higher

Monkey ID	Number of Vγ2Vδ2 cells Infused
CN 8110	2×10^8 cells/Kg
CN 8365	3×10^8 cells/Kg
CN 8366	1.8×10^8 cells/Kg

Table 2: Number of Vγ2Vδ2 T cells infused by designated monkey. CN: Cynomolgus

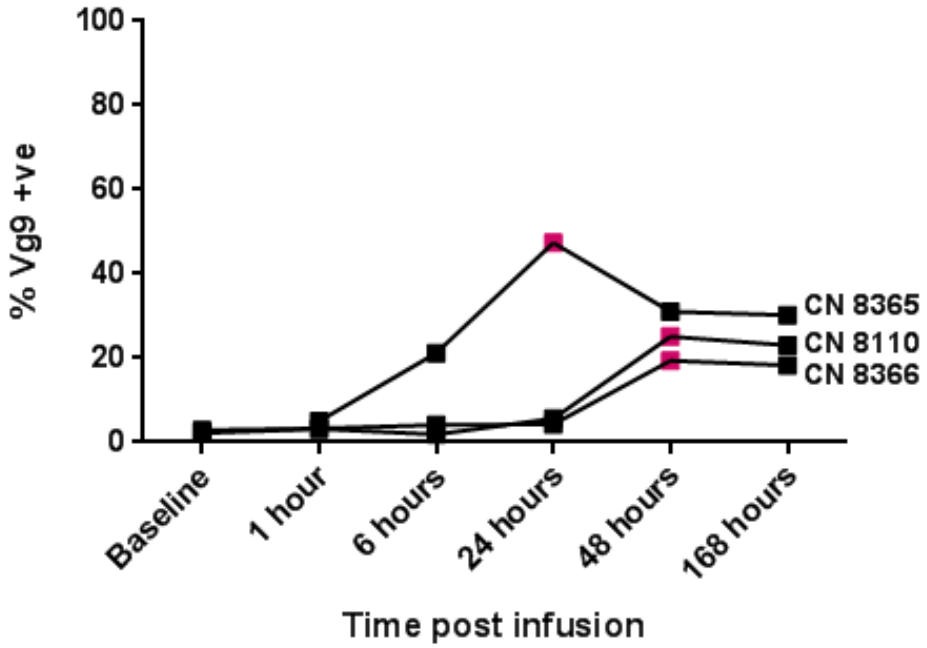


Figure 4.1: Vγ2Vδ2 cells peak in bronchoalveolar lavages of monkeys at 24-48 hours and still persist at day 7 post infusion.

than baseline up to 7 days post infusion suggesting that these cells were still circulating in the body.

In BALF, the appearance and peak of infused cells differed between monkeys. In the case of 3 X 10^8 cells/Kg infusion, cells showed up at 6 hours, peaked at 24 hours and were still highly detected at 7 days post infusion. Compared to baseline (~5% of total T cells), percent of Vγ2Vδ2 T cells was four times higher at 6 hours (~20% of total T cells) and around 10 times (50% of total T cells) at 24 hours post infusion. Monkeys that took 2X10^8 cells/Kg and <2X10^8 cells/Kg infusions showed some delay in trafficking to the lungs. An increase in percentage of Vγ2Vδ2 T cells started at 24 hours and peaked at 48 hours post infusion. Such apparent delay can be explained by possible low sensitivity of flow cytometry detection to small increases in cell numbers/percentages and might be over-represented due to the long gaps between the BALF collection time points between 6 and 24 hours post infusion. However, magnitudes of increase in percentages of Vγ2Vδ2 T cells correlated very well with number of cells infused per kilogram body weight. And still, cells were highly persistent at day 7 post infusion in all cases.

Interestingly, looking at the PKH26 +ve cells in BALF, generally, at all time points, these constituted around 10-15% of the infused Vγ2Vδ2 population compared to the original 50% before infusion. This indicates that infused PKH26 stained cells might have died in vivo due to toxicity of the dye. Since a portion of the PKH26 +ve cells was able to make it to the lungs, it is less likely

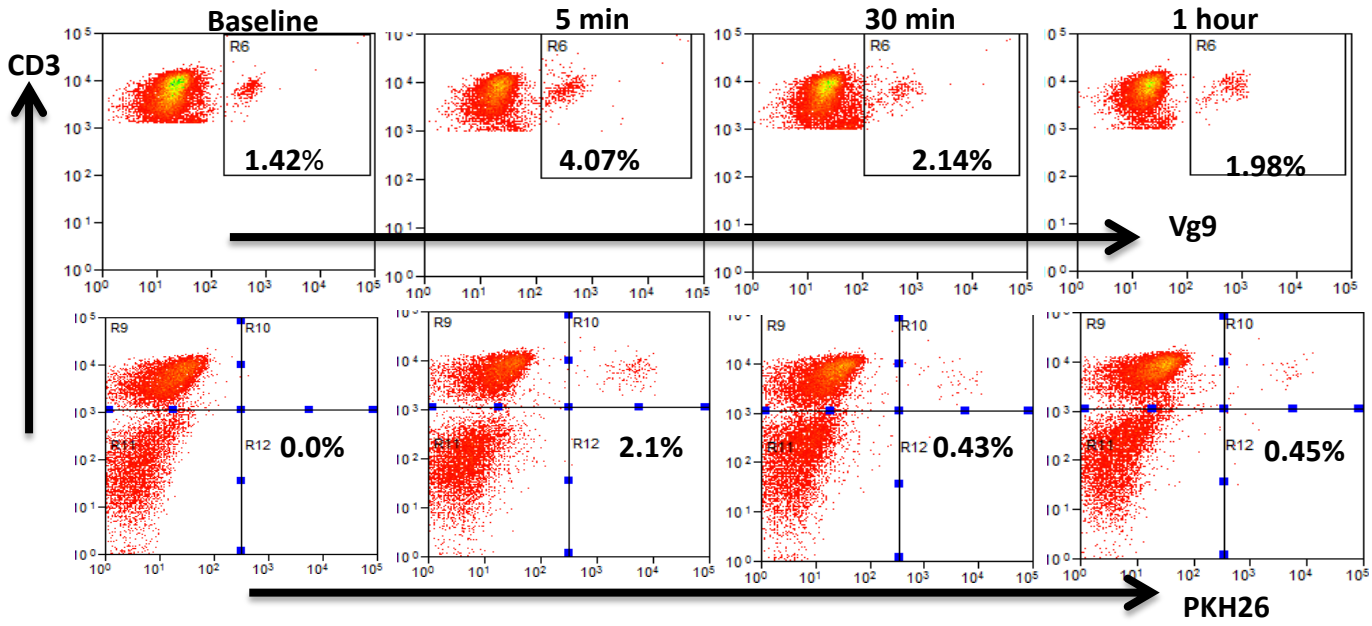


Figure 4.2: Vγ2Vδ2 cells peak in blood at 5 minutes post infusion. These are representative flow plots of samples collected from CN 8365 who received 3 X 10^8 cells/Kg. min: minutes.

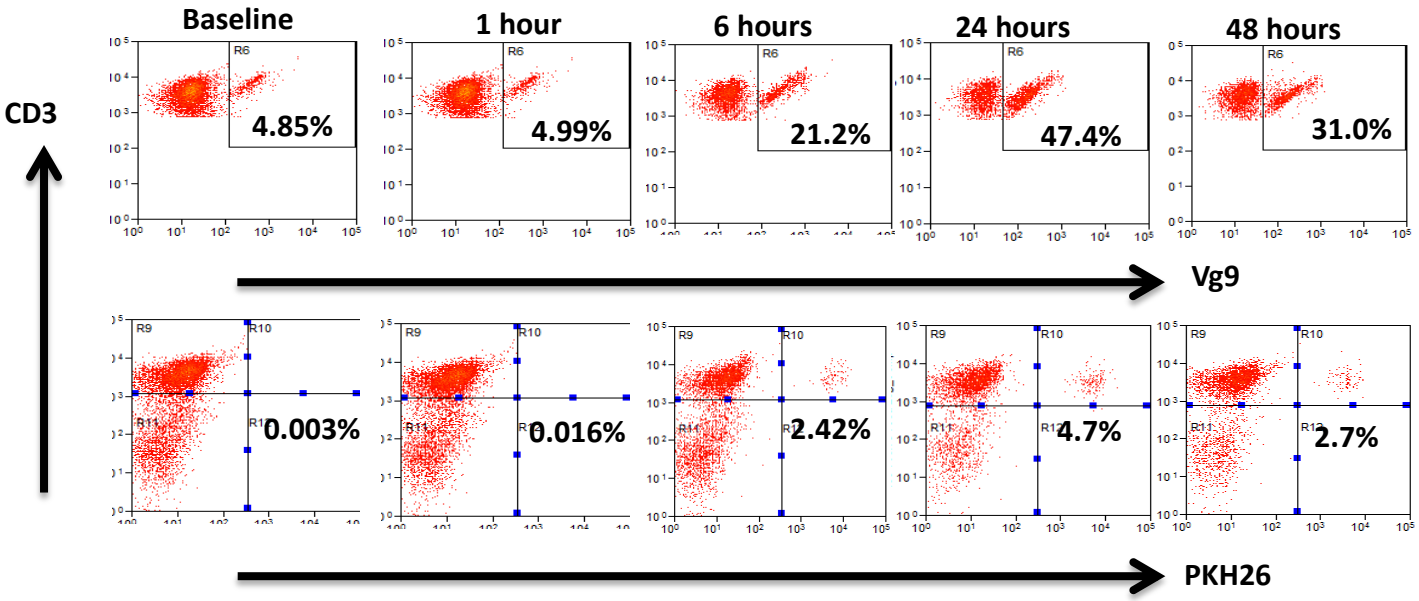


Figure 4.3: Vγ2Vδ2 cells peak in the bronchoalveolar lavage of monkeys at 24 to 48 hours post infusion. These are representative flow plots of samples collected from CN 8365 who received 3 X 10^8 cells/Kg.

that PKH26 could have affected the cell surface molecules responsible for adhesion and homing. In addition, PKH26 have been extensively used in stem cell research and has been shown to home to many different organs in vivo. However, consistent with our observations, PKH26 labelled cells proved to live shortly after labelling, up to 7 days (121).

From the above, it is very clear that infused Vɣ2Vδ2 T cells have a tendency to home or accumulate in the lung compartment and can highly persist there for a week after infusion with a very limited tendency to recirculate in the blood. How long these cells can stay in the lungs post transfer is worth exploring. In addition, is the lung compartment the only place where infused cells would accumulate? What about lymph nodes, spleen, liver, intestinal mucosa…etc. Such question can be answered by further tracking infused cells using radioactive labelling and whole body radio-scanning.

Another very important question is, why the lungs? Since these animals are naïve, there should not be any kind of specific inflammatory recruitment to the lungs. Previous adoptive cell transfer investigations of stem cells and SHIV specific CD8 T cells have reported the same lung accumulation phenomenon noticed here.in both mice and monkeys (122-126). In fact, monkey studies have shown that transferred cells persisted in BALFs for more than 8 weeks post infusion. Even in cases where cytotoxic T cells where transfected to express homing receptors to the intestinal mucosa, these cells still accumulated in the lungs and “apparently” never reached or never survived in

the intestines (123,124). The limited persistence of transferred cells in blood and long term persistence in the lungs (BALF) might be due to the route of cell infusion. Cells where infused via the cephalic or saphenous veins; known to convey initial passage through the pulmonary vasculature before gaining access to the systemic arterial circulation. It is possible that a substantial proportion of the infused cells were trapped in this tissue in a nonspecific manner and thus did not enter the arterial circulation and consequently was not able to go back to the circulation or home anywhere else in the body. Another possibility, homing to the lungs may be due to expression of adhesion molecules, such as lymphocyte function-associated antigen-1 (LFA-1), which mediate T cell entry into the airways (127,128). There is also evidence that the morphological rigidity or polarization of effector cells contributes to trapping within pulmonary capillaries (129,130). Interestingly, the adoptive transfer of bulk, unstimulated PBMCs does not result in accumulation of the transferred cells in the lungs in cynomolgus macaques (131), suggesting that this phenomenon may be dependent on the activated effector phenotype of the transferred cells.

However, it is important to emphasize that the long-term persistence of transferred T cells in the lungs (as detected in BALs), together with their preserved capacity to respond to Ag stimulation ex vivo, point to an intrinsic capacity of the infused cells to survive and maintain function in vivo.

To our knowledge this study represents the first documentation of Vɣ2Vδ2 T cell tracking after adoptive transfer in monkeys.

C. Conclusions and Future Directions:

In conclusion, this is the first effort to adoptively transfer and track ex vivo expanded Vɣ2Vδ2 T cells in macaques. Infusion of large numbers of these cells was safe with no adverse reactions detected. The infused cells persisted and remained functional in lungs for at least 7 days, but showed limited recirculation in the blood. The infused cells remained functional and produced IFN-ɣ upon restimulation with HMBPP when tested ex vivo at 24 hours post infusion (preliminary data not shown).

Further experiments will inspect the extent of persistence of infused Vɣ2Vδ2 T cells in BALFs beyond 7 days post infusion.

Knowing if the cells have the potential to home to organs other than the lungs can be done using radioactive tracking of infused cells and radioactive imaging.

Chapter V

The Adoptive Transfer of ex vivo Expanded Autologous Vγ2Vδ2 T Cells Confers Protection against the Dissemination of Mycobacterium Tuberculosis in Cynomolgus Macaques

A. Introduction and Rationale:

Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), is one of the oldest, most expanded and most lethal diseases in human history. Once thought to be controlled by antibiotic treatments and vaccination, TB re-emerged as a result of increasing poverty, the development of drug resistant strains and the spread of HIV/AIDS (132,133). The BCG vaccine, a live attenuated form of Mycobacteria, cannot be used in immunocompromised patients of HIV and others, and has proved to be non-protective for adults (134-138). There is a pressing need to develop a new protective vaccine and/or immunotherapeutics, and this cannot be done without a deep understanding of the immune response against MTB.

We have been studying immune responses against TB using macaque non-human primate TB models for over a decade now. We have investigated the role different subsets of T cells play in fighting MTB and made various investigations to study the potential therapeutic/protective capacities certain cytokines and bacterially derived compounds might have against the disease.

A main focus of our lab is the Vγ2Vδ2 T cells, a major human γδ T cell subset that exists only in primates and not in other animals. These cells constitute 65–90% of total circulating γδ T cells in humans and are known to contribute to both innate and adaptive-like immune responses in various infections (90,139-141). Vγ2Vδ2 T cells recognize phosphate rich compounds, such as HMBPP, essentially generated by a variety of microbes including MTB and, host IPP, accumulating in cells undergoing stress; such as cancer cells and certain virally infected cells. (56,67,93,142,143)

Our decades-long studies in non-human primate models highly contributed to illustrating biology and immune responses of human Vγ2Vδ2 T cells in MTB and other infections. We showed that administration of phosphoantigen compound plus IL-2 induced remarkable expansion of Vγ2Vδ2 T cells, antagonized cytokine-induced Foxp3+ T regulatory cells, reversed T regulatory cell mediated immune suppression, and attenuated pneumonic plague lesions in non-human primates (83,144-146).

Early studies have shown that infections with HMBPP producing bacteria including BCG and MTB could remarkably activate, expand and recall expand Vγ2Vδ2 T cells that could readily traffic to and accumulate in the lung compartment of macaques (68,86). Interestingly these cells were found to accumulate as part of the T cell population within TB granulomas. Pathogen induced Vγ2Vδ2 T cell activation/expansion in these studies produced various

cytokines that correlate well with immune protection against TB, such as IFN-ɣ and cytotoxic cytokines.

In our latest attempt to understand the function of Vγ2Vδ2 T cells in immunity against TB, we have used HMBPP and IL-2 to expand these cells in vivo in the context of a non-human primate TB infection model (84). Results from this study suggested that Vγ2Vδ2 T cells confer high protection against the disease. However, this protection could not be attributed solely to Vγ2Vδ2 cells, as IL-2 also expands other immune cells. In fact IL-2 alone treatment did confer immune protection against TB in macaques. Interestingly, treating MTB infected macaques with high dose IL-2 before and after infection did result in similar outcomes in terms of pathology reduction and to a lower extent in terms of bacterial burdens (91). However, this was accompanied with a huge expansion of T regulatory cells and not Vγ2Vδ2 T cells; re-emphasizing a role of IL-2 driven expansion of non- Vγ2Vδ2 T cells in immune protection against TB.

Consequently, studying the exact role that Vγ2Vδ2 play in fighting TB needs a cleaner strategy. Such strategy can be achieved by either depletion of Vγ2Vδ2 T cells before and during TB infection or by the adoptive transfer of these autologous Vγ2Vδ2 T cells into MTB infected macaques. Since there is no depleting antibody for these cells in the market yet, the only option left is that of autologous adoptive transfer. The percentage of Vγ2Vδ2 T cells in the circulation is very low (1-2% of the total T cell population), there is no way of collecting cells for adoptive transfer without expanding them ex vivo before

infusing them back into monkeys. As described in Chapters 3 and 4, for the first time, we have successfully expanded Vɣ2Vδ2 T cells from macaques using zoledronate and IL-2 to numbers high enough for adoptive transfer and without losing functional characteristics of the cells. In addition, these cells were found to accumulate in the lungs and persist for at least 7 days.

This study represents the first of its kind where Vɣ2Vδ2 T cells are adoptively transferred in macaques in the context of MTB infection and aims to reveal the role they might play in immune protection against the disease.

B. Results and Discussion:

1. Study Design:

For this study, we have chosen to use long tailed crab eating "cynomolgus" macaques (*Macaca fasicularis*) for two major reasons. First, "the spectrum of disease observed in these monkeys is similar to humans, and this model can be very useful for studying pathogenesis and immunology of tuberculosis, as well as testing vaccines, diagnostic reagents, and drugs prior to use in human populations" (147,148). Second, these monkeys are small in size compared to other species, average weight for adult males ranges between 4.7 and 8.3 Kg and for adult females between 2.5 and 5.7 kg (149). Monkey weight matters in our case, because that would reduce the number of cells needed for infusion. As mentioned in chapter 3, large scale ex vivo expansion of Vɣ2Vδ2 T cells can be very challenging in terms of required care, labor and incubator space needed.

The use of 500 CFU MTB for infection is considered a high dose infection that leads to severe TB. Previous studies in the lab (150) have shown that 500 CFU will not lead to rapid death in adult monkeys including cynomolgus macaques. Two to three months after infection, monkeys are not expected to develop clinical signs of TB but will have developed apparent inflammation and lesions; allowing us to evaluate the effect of infused Vγ2Vδ2 T cells on disease progression in terms of gross pathology and bacterial burdens.

To determine the number of autologous Vγ2Vδ2 T cells to be infused back into monkeys after TB infection, we have looked at similar adoptive transfer studies performed in mice and humans. We have carefully inspected studies where the adoptive transfer showed beneficial outcomes in terms of tumor progress and/or protection against viral infection. Generally, number of cells given per infusion is $\geq 10^6$ cells for mice (118,120) and $\geq 10^9$ for humans (87-89). The average weight of a laboratory mouse is 20-30 g and that of an adult human is 50-80 Kg. Cynomolgus macaques weigh 3-6 Kg in average. Based on that, we thought that 10^8 cells per monkey could be equally effective.

In addition, we looked back into the results of our recent study that showed that HMBPP/IL-2 treatment of TB infected monkeys expands Vγ2Vδ2 T cells to up to 60% of the CD3 T cell population. As mentioned previously, these monkeys showed a significant reduction in granulomatous and necrotic tissues typical of the disease (84). Monkeys had an average of 1×10^6 Vγ2Vδ2 cells/mL of blood. Since, the average total blood volume of these cynomolgus macaques

is 300 mL, the total number of Vγ2Vδ2 cells is 300 X 10^6 cells/entire blood. Based on that, we aimed at using the same number of cells and infuse ≥100 X 10^6 (1X10^8) Vγ2Vδ2 cells shortly after the establishment of infection (on day 4) and ≥1X10^8 cells 2 and 4 weeks after the initial infusion, respectively (Table 2 and Table 3). The reason why the total number of cells was given on three separate infusions with 2 week period laps is to replenish the body with more Vγ2Vδ2 cells since we believe that most previously infused cells will have accumulated in infected lungs and that some might have eventually died after 2 weeks.

2. Monkeys that received Vγ2Vδ2 T cells showed less bacterial burdens in broncho-alveolar lavages throughout the course of tuberculosis infection.

One measurement of active TB is the presence of bacteria in the BALFs. As Figure 5.1 shows, bacterial CFUs recovered from BALFs of monkeys over time were significantly less in the monkeys from the Vγ2Vδ2 T cell group compared to the PBL group, suggesting that infused Vγ2Vδ2 T cells might have contributed to the killing/inhibition of MTB in the lungs of infected macaques.

3. Monkeys that received Vγ2Vδ2 T cells showed less weight loss throughout the course of tuberculosis infection.

Another measurement of active TB is weight loss. MTB has been frequently referred to as the "queen of consumption". Interestingly, weight loss is significantly more prominent in patients with disseminated extra-pulmonary TB (151). As shown in Figure 5.2, monkeys that received Vγ2Vδ2 T cells

	Day 1	Day 4	Day 18	Day 32	Day 60
Group 1	*M. tuberculosis* (500 CFU)	Vγ2Vδ2 T-cell (≥10^8cells)	As day 4	As day 4	Euthanazia Termination
Group 2	*M. tuberculosis* (500 CFU)	Peripheral blood lymphocytes (PBL) (≥10^8cells)	As day 4	As day 4	Euthanazia Termination
Group 3	*M. tuberculosis* (500 CFU)	1 mL saline	As day 4	As day 4	Euthanazia Termination

Table 2: Animal Groups and Treatments. *M. tuberculosis*: *Mycobacterium tuberculosis*, CFU: Colony Forming Unit.

Monkey #	D 3	D 17	D 28	Total cells infused/Kg (purity)
Vγ2Vδ2				
CN 7826	125 X 10^6	210 X 10^6	75 X 10^6	102 X 10^6 (91%)
CN 8343	125 X 10^6	210 X 10^6	17 X 10^6	83 X 10^6 (82%)
CN 8345	125 X 10^6	230 X 10^6	161 X 10^6	112 X 10^6 (86%)
CN 8346	125 X 10^6	260 X 10^6	225 X 10^6	55 X 10^6 (88%)
PBL				
CN 8334	125 X 10^6	120 X 10^6	85 X 10^6	72 X 10^6
CN 7828	125 X 10^6	125 X 10^6	125 X 10^6	90 X 10^6
CN 7834	125 X 10^6	125 X 10^6	125 X 10^6	110 X 10^6
CN 7837	125 X 10^6	125 X 10^6	125 X 10^6	150 X 10^6
CN 7840	125 X 10^6	125 X 10^6	125 X 10^6	95 X 10^6
Saline				
CN 7833				Saline

Table 3: Animals and Treatments. D: Day, CN: Cynomolgus, PBL: Peripheral Blood Leukocytes.

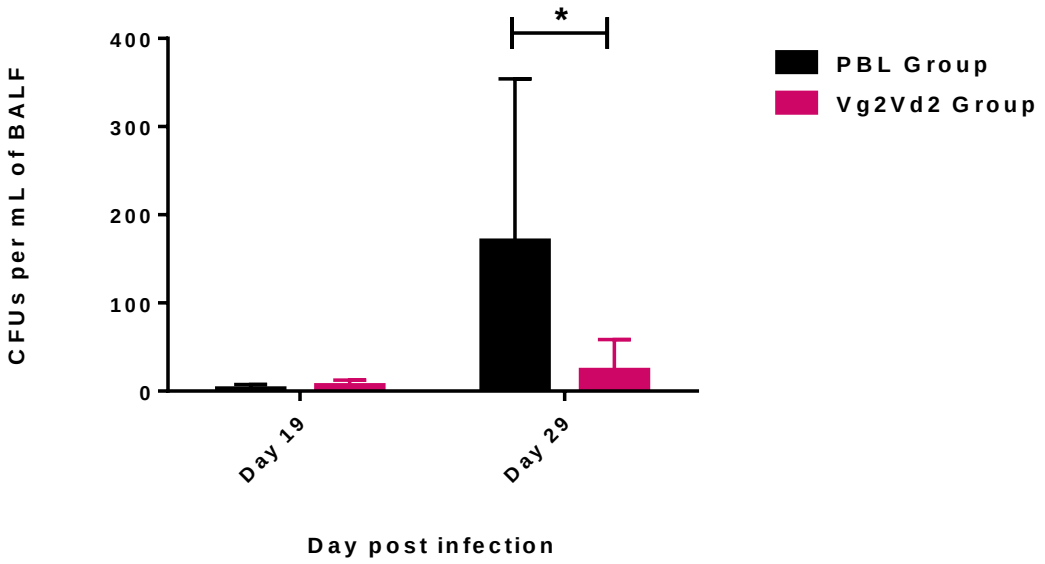


Figure 5.1: Monkeys that received Vγ2Vδ2 T cells showed less bacterial burdens in BALFs throughout the course of TB infection. PBL: Peripheral Blood Leukocytes, CFUs: Colony Forming Units, BALF: Bronchoalveolar Lavage.

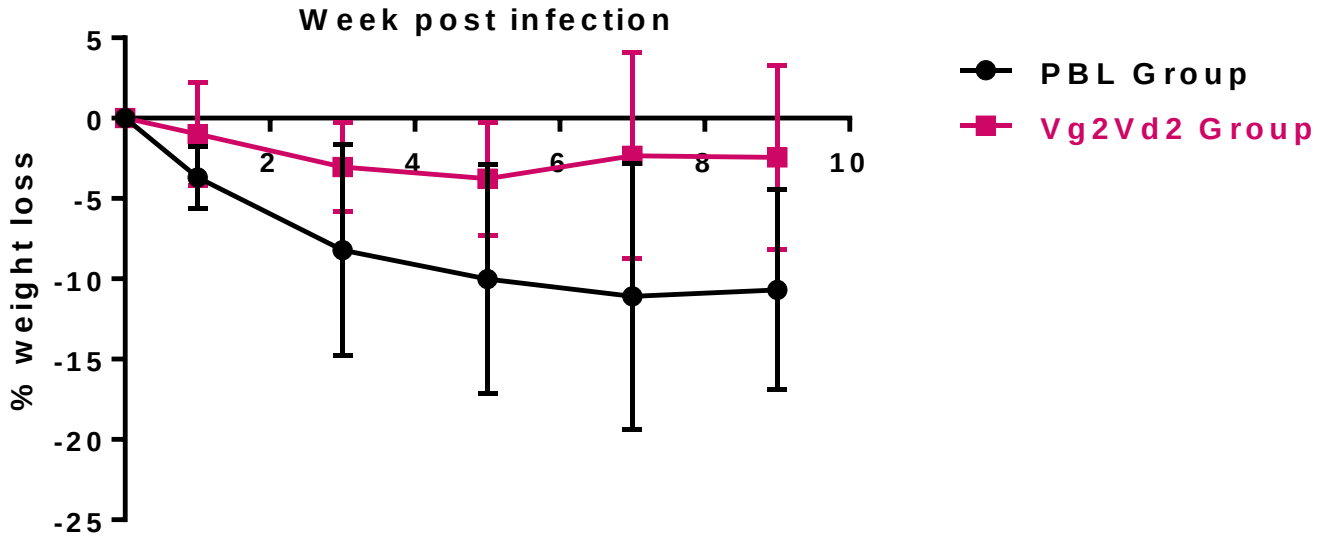


Figure 5.2 : Monkeys that received Vγ2Vδ2 T cells showed less weight loss throughout the course of TB infection compared to monkeys that received PBLs. PBLs: Peripheral Blood leukocytes..

experienced reduced weight loss (~5%) compared to those that received PBLs over time (~15%). This suggests that infused Vγ2Vδ2 T cells might have controlled the dissemination of MTB to extrapulmonary compartments.

4. Monkeys that received Vγ2Vδ2 T cells showed less lymphocytopenia throughout the course of tuberculosis infection.

Lymphocytopenia, is another criterion associated with active TB. For unclear reasons, disease severity is associated with greater depression of the total lymphocyte numbers in blood, especially CD4 T cell counts, that would return back to normal after therapy. Several studies suggested that MTB itself can impact CD4 T cell homeostasis. Another reason could be the continuous recruitment of lymphocytes to the site(s) of infection where ongoing inflammation is taking place. Interestingly, it has been repeatedly reported that TB patients with T cell lymphocytopenia usually have a more severe disease which involves extra pulmonary compartments (152-155).

Looking at results from our monkeys, the group that received Vγ2Vδ2 T cells showed reduced lymphocytopenia compared to the group that received PBLs (Figure 5.3). This might be an indication that Vγ2Vδ2 T cells participate in infection containment and limiting extrapulmonary dissemination.

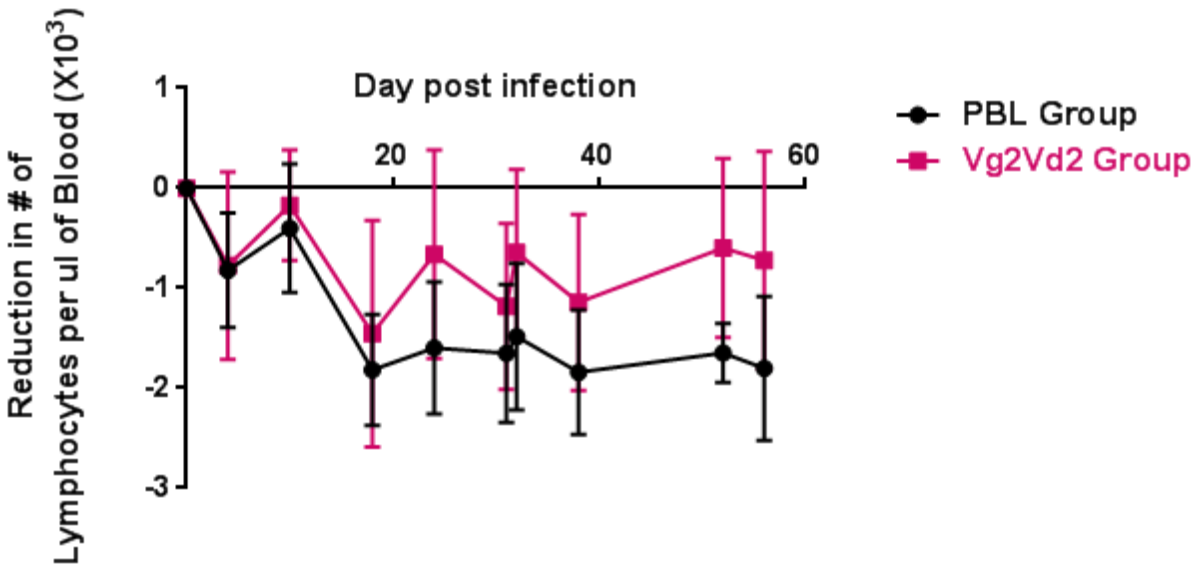


Figure 5.3: Monkeys that received Vγ2Vδ2 T cells showed less lymphocytopenia throughout the course of TB infection. PBL: Peripheral Blood Leukocytes.

5. Adoptively transferred Vγ2Vδ2 T cells led to reduction in pulmonary and extra-pulmonary *M. tuberculosis* bacterial burdens.

We next sought to examine whether the adoptive transfer of Vγ2Vδ2 T cells could lead to changes in bacterial burdens after pulmonary MTB infection. We measured bacterial CFU counts in lung tissue homogenates from the right caudal lung lobe (infection site), right middle lobe and left caudal lobe at day 56, the end point after MTB infection. The Vγ2Vδ2 treated group exhibited significantly lower numbers of bacilli in lung tissue homogenates from the lung lobe of infection and all other lung lobes compared to the PBL control group (Figure 5.4 A). Same applied to extra-pulmonary tissues including liver, spleen and kidneys (Figure 5.4 B). These findings suggest that ex vivo expanded Vγ2Vδ2 T cells were able to recognize MTB infected cells and exerted anti TB functions to help reduce bacterial burdens.

6. Adoptively transferred Vγ2Vδ2 T cells confer immune resistance to tuberculosis dissemination after pulmonary *M. tuberculosis* infection

We then sought to determine whether the adoptive transfer of Vγ2Vδ2 T cells would affect the extent of TB pathology/lesions in lungs after MTB infection. We conducted complete necropsy and gross pathology examination at day 56 after infection to examine TB associated pathology and lesions in different groups. Interestingly, Vγ2Vδ2 T cells treated macaques exhibited reduced TB lesions in lungs compared to the PBL treated group. One of the four animals that received Vγ2Vδ2 T cells displayed localized TB lesions confined to the right

caudal lobe (infection site) of the lungs whereas the other 3 monkeys showed limited dissemination to right middle and/or right cranial lobes only but not to the left side of the lungs. In contrast, all control macaques treated with PBLs or saline (only 1 monkey) exhibited severe/extensive caseating TB lesions and displayed extensive coalescing granulomas in right caudal, middle, cranial lobes and left side of the lungs. TB lesions in these control macaques were readily disseminated to the thoracic cavity and to extrathoracic organs including liver, spleen and kidneys. In contrast, macaques treated with Vγ2Vδ2 T cells showed minor extrapulmonary dissemination limited to only one of the four animals in the group.

When the gross TB pathology was evaluated in details and compared between groups using the scoring system as we and others previously described, a significant difference was found between groups. Results obtained in this study are relatively similar (to a lower extent) to those obtained previously by us when we treated TB infected macaques with HMBPP and IL-2 to expand their Vγ2Vδ2 T cells in vivo. Gross pathology, bacterial dissemination and bacterial burdens were significantly reduced. It is noteworthy that macaques treated with IL-2 alone, although showed relatively reduced TB lesions, could not effectively reduce MTB bacterial burdens compared to saline controls (84,91).

All the described above provide accumulative evidence that Vγ2Vδ2 T cells play an important role in limiting bacterial dissemination when present in abundance at early stages of infection.

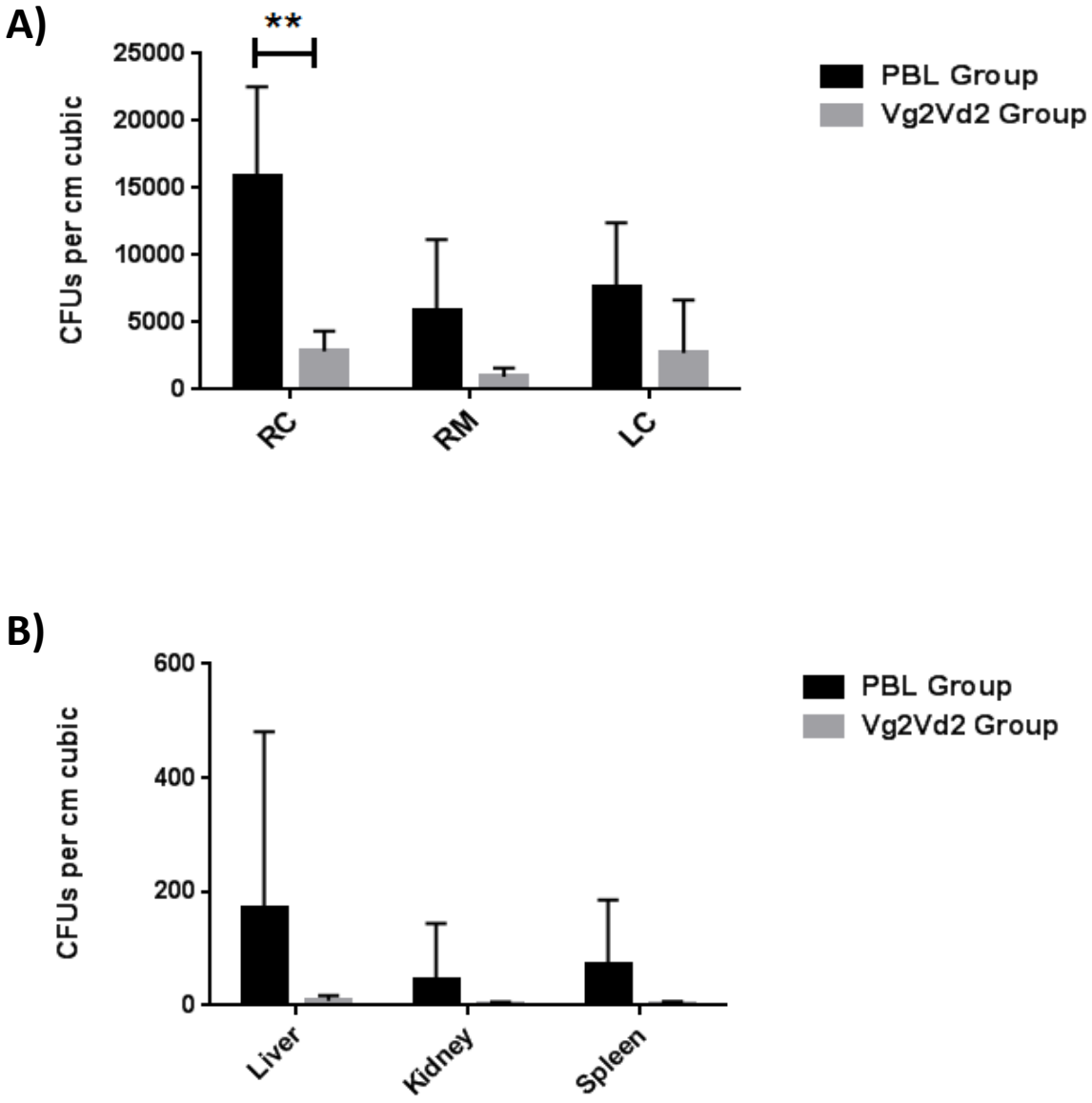


Figure 5.4: Adoptively transferred Vγ2Vδ2 T cells led to reduction in A) pulmonary and B) extra-pulmonary MTB bacterial burdens. RC: Right Caudal, RM: Right Middle, LC: Left Caudal, CFUs: Colony Forming Units, PBL: Peripheral Blood Leukocytes.

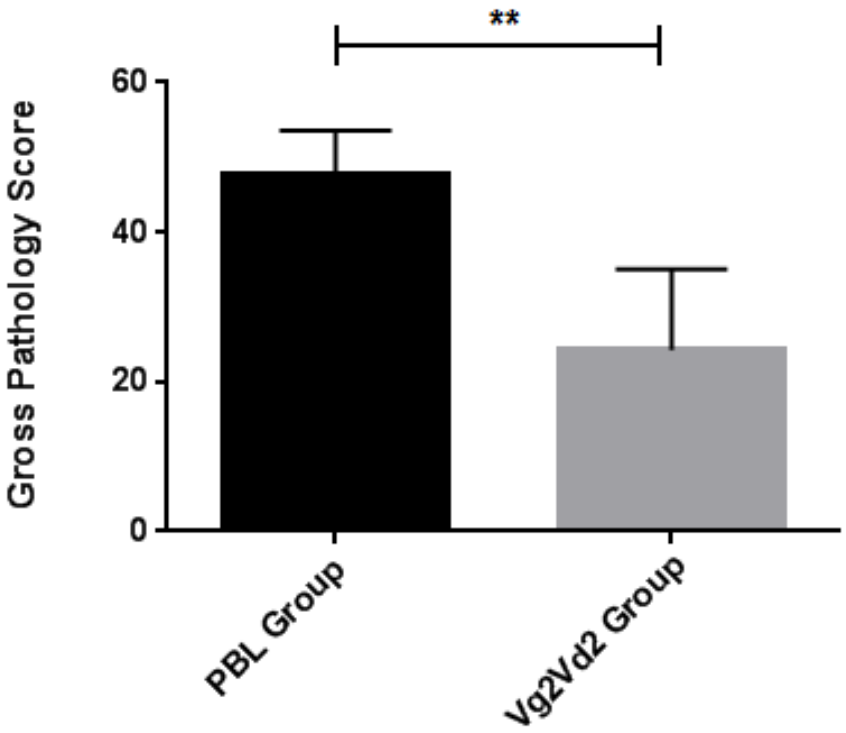
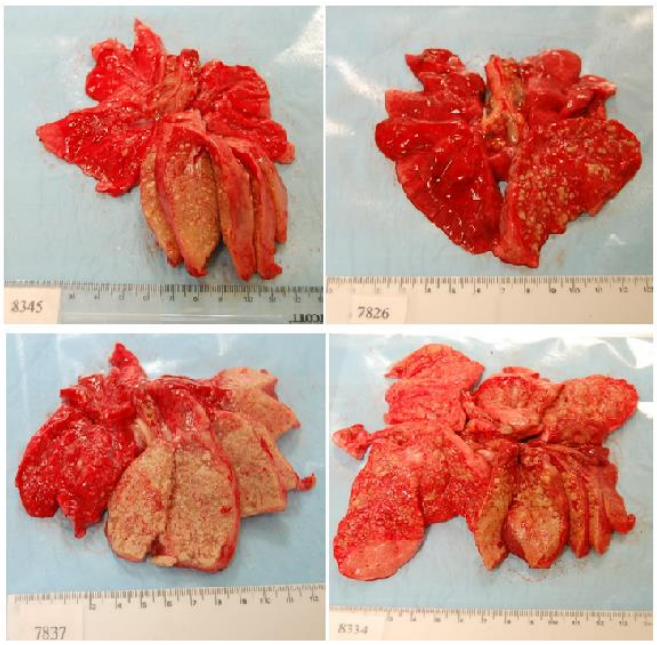


Figure 5.5: Monkeys that received Vγ2Vδ T cells showed reduced gross pathology compared to the PBL (peripheral blood lymphocytes) group.

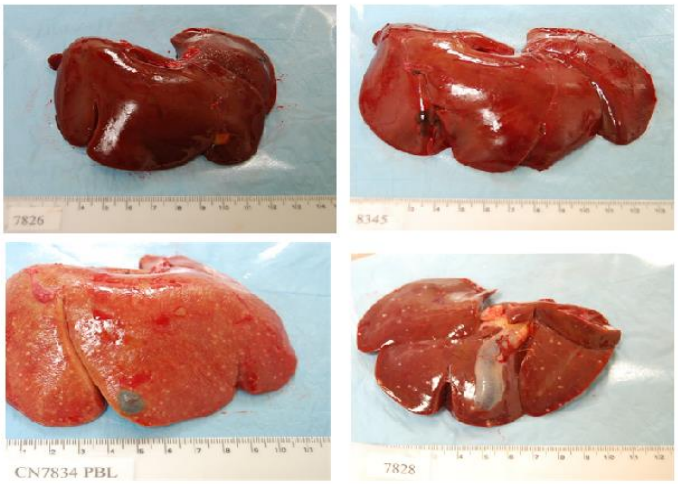
A)



Vγ2Vδ2 group

PBL group

B)



Vγ2Vδ2 Group

PBL Group

Figure 5.6: Adoptively transferred Vɣ2Vδ2 T cells led to reduction in TB induced pathology and dissemination in A) pulmonary and B) extra-pulmonary (liver) compartments of infected monkeys. PBL: Peripheral Blood Leukocytes.

C. Final discussion, Conclusions and Future Directions:

We are the first to expand Vγ2Vδ2 T cells from macaques and adoptively transfer them into a TB infection model. This study generated the first direct evidence that Vγ2Vδ2 T cells confer protection against MTB and can give new insights for the design of new vaccines against the disease.

Monkeys treated with ex vivo expanded Vγ2Vδ2 T cells showed reduced manifestations of MTB infection compared to the those treated with PBLs. Throughout the infection, Vγ2Vδ2 treated monkeys showed reduced weight loss, reduced lymphocytopenia and reduced bacterial burdens in their BALFs. In addition, at the end of the study, 2 months after infection, Vγ2Vδ2 T cells treated monkeys showed reduced TB associated pathology/lesions both in pulmonary and extra-pulmonary compartments. All mentioned is consistent with reduced dissemination of MTB and is indicative of the protective role these cells play in fighting the infection.

As detailed in Chapter 3, zoledronic acid/IL-2 driven expansion of macaque Vγ2Vδ2 T cells resulted in a polarization of function towards the Th1 signature; known to be essentially protective against MTB. The Majority of cells was of the "effector" memory type; associated with high functional potentials. Cells specifically produced IFN-γ and TNF-α in response to ex vivo stimulation with HMBPP. They showed a robust production of cytotoxic cytokines that variably participate in killing target cells using different mechanisms. Importantly, expanded cells were able to inhibit/kill the intracellular growth of

mycobacteria. Furthermore, Vɣ2Vδ2 T cells exclusively expressed CXCR3, known to drive cells to sites of IFN-ɣ (Th1) induced inflammation.

After expansion, cells used for this experiment were all of high purity (>80% Vɣ2Vδ2). Cells were infused back into monkeys at days 3, 18 and 32 post infection. Generally, in the course of MTB infection, the first 5 days show a minor measurable recruitment of innate cells; especially DCs. Even later when DCs start accumulating at the site of infection, their maturation is delayed by the induction of a suppressive environment. Seven to nine days post infection, mature DCs traffic out of the lung to the closest draining lymph nodes in order to prime antigen specific T cell responses. Upon activation, T cells expand clonally and upregulate the expression of various adhesion molecules and chemokine receptors to drive their homing to the site of infection. Of these chemokine receptors, CXCR3 is the most associated with protection against MTB. T cells start homing to the lungs 18-21 days post infection where they start their fight to control and contain the infection by completing granuloma formation.

As explained above, expanded Vɣ2Vδ2 T cells were infused back to the monkeys in critical time points when a protective T cell response is delayed by MTB. As shown in Chapter 4, expanded Vɣ2Vδ2 T cells showed a tendency to traffic, accumulate and highly persist in the lungs for 7 days at least in monkeys; without the need for an inflammation driven recruitment. Whether infused cells would specifically home to the site of infection (right caudal lobe of

the lungs) is something that we don't know about. However, it is worth mentioning that researchers in the field of cancer therapy have reported that expanded Vɣ2Vδ2 T cells show an antigen driven recruitment to the tumor site, where the phosphoantigen IPP is highly presented. Since our cells express CXCR3, we expect them to specifically move according to the chemokine gradient of its ligands; CXCL9, 10 and 11. How early these are secreted after MTB infection is unknown. Still since the production of these chemokines is induced by IFN-ɣ, we assume them to be most concentrated at the site of infection at the time IFN-ɣ producing Th1 cells are trafficking and functioning in the lungs (18-21 days post infection). Based on that, we expect that Vɣ2Vδ2 T cells infused at 18 and 32 days post infection, at least, will specifically home to the site of infection driven by their CXCR3 expression. Interestingly, as proved recently by others, expression of CXCR3 could help cells specifically locate to the lung parenchyma tissue where granulomas are forming.

Our assumption is that; cells of the first infusion (day 3 post infection) would form an early supply of Th1-like functioning cells when TB specicifc T cells would not have arrived yet. On the other hand, those infused at days 18 and 32 would be an additive supply of protective T cells.

By their specific production of IFN-ɣ and TNF-α, infused Vɣ2Vδ2 T cells could have caused earlier recruitment of innate and adaptive T cells to the site of infection, as both cytokines induce the production of recruiting chemokines and adhesion molecules. In addition, infused Vɣ2Vδ2 T cells could have

enhanced phagocytosis and antigen presentation and helped killing MTB and MTB infected cells.

Since infused cells were able to produce cytolytic/toxic granzymes, perforin and granulysin and inhibit/kill MTB infected cells in vitro, we assume that same happened in vivo, further contributing to the control of infection.

All in all, these early additive sources of Th1 and killing cytokines can help better control of MTB growth, earlier containment of infection by T cell surrounded granulomas and consequently, reduced MTB dissemination.

Still, many questions are left unanswered. Very little is known about the natural history of Vɣ2Vδ2 T cells in the context of MTB infection of the lungs. Although abundantly present in the lung mucosa and peripheral blood, when exactly these cells start interacting with MTB infected macrophages is unknown. Whether MTB can evade or manipulate the function of these cells early on infection, as the case with most innate cells, is not known. In addition, since the function of Vɣ2Vδ2 T cells is very plastic depending on the cytokine environment in their surrounding (detailed in the Chapter 1). Is the cytokine milieu present very early after infection supportive of a Th1 polarization, like the case of IL-2? The answer to this question can help determine the role these cells play in early stages the immune response against MTB.

If these cells function secrete Th1 and killing, as CD4+ and CD8+ MTB specific T cells do, what is their unique contribution to the immune protection against

MTB. It would be interesting to know whether infusing CD4+ TB specific T cells at early time points post infection, as we did here, would give similar results.

The relative importance of cell infusion timing can be revealed by more infusion experiments. Can these cells help controlling TB reactivation from latency for example? Answers to such questions can help design therapeutic strategies for cases of severe TB.

Results from this thesis strongly suggest a protective role of Vɣ2Vδ2 T cells in protecting against MTB dissemination. Still, a better and supportive proof would be achieved by depleting these cells from the body before infection. For that, a depleting antibody needs to be generated.

Finally, how the suggestive protective role of Vɣ2Vδ2 T cells against TB dissemination concluded from this study can be translated into better vaccine or immunotherapeutic candidates should be carefully studied.

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Appendix A

UIC 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

December 6, 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 **8/20/2013**. *The protocol was not initiated until final clarifications were reviewed and approved on* **11/21/2013**. *The protocol is approved for a period of 3* years with annual continuation.

Title of Application: The Role of Gamma/Delta (Vgamma2Vdelta2) T Cells in Immunity

ACC Number: 13-128

Condition of Initiation: Radiation portion of the study cannot be initiated until all protocol staff that will be involved with husbandry during this portion of the study have completed both standard husbandry training and radiation hazard husbandry training.

Initial Approval Period: 11/21/2013 to 8/20/2014

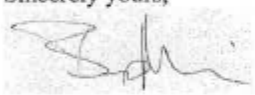
Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

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, 13-122, and 13-129*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding 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 (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, Arwa Qaqish

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VITA

Arwa Mahmoud Qaqish

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Publications and Conferences:

Qaqish, AM, Nasrieh, MA, Al-Qaoud, KM, Craig, PS, Abdel-Hafez, SK. The seroprevalences of cystic echinococcosis, and the associated risk factors, in rural-agricultural, bedouin and semi-bedouin communities in Jordan. Ann Trop Med Parasitol (2003); 97(5):511-20.

The Third Conference in Medical & Biological Sciences. April 2004. Faculty of Applied Sciences-Zarka Private University-Zarka-Jordan. Oral Presentation: Immunoglobulin G Subclass Analysis for the Serodiagnosis of Cystic Echinococcosis.

Boutennoune H, **Qaqish A**, Al-Aghbar M, Abdel-Hafez S, Al-Qaoud K. Induction of T helper 1 response by immunization of BALB/c mice with the gene encoding the second subunit of *Echinococcus granulosus* antigen B (EgAgB8/2). Parasite (2012); 19(2):183-8.

6th International Gamma Delta T cell Conference. May 2014. University of Illinois at Chicago. Oral Presentation: Adoptively transferred Vγ2Vδ2 T cells confer immune protection against tuberculosis (TB) in *Mycobacterium tuberculosis*-infected macaques.

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