Analysis of mycobacteria-specific T cell functions in HIV-infected individuals and healthy controls

ABSTRACT

Tuberculosis caused by *Mycobacterium tuberculosis* (MTb) continues to represent a major public health problem worldwide and it is the main cause of death among people living with HIV/AIDS. Mycobacteria-specific CD4 T cells play an important role in the control of mycobacterial infection and seem to be impaired in HIV patients, including those receiving highly active antiretroviral therapy. The aim of this project was to establish and use *in vitro* assays to characterize mycobacteria-specific effector T cell functions in HIV-1 infected patients in comparison with healthy controls.

For establishing the methods, blood samples from healthy donors were used and peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation. To analyze frequencies of mycobacteria-specific T cells in peripheral blood, PBMCs were stimulated overnight with different mycobacterial antigens. After intracellular staining, effector cytokine production (IFN-γ, TNF and IL-2) from T cells was analyzed by multicolor flow-cytometry.

Viability and mycobacteria-specific T cell effector cytokine production was assessed in fresh cells as well as after cryopreservation. Mycobacteria-specific T cells were found to occur at very low frequencies in peripheral blood. Therefore a protocol was established to expand mycobacteria-specific T cells *in vitro* and to analyze mycobacteria-specific effector T cell functions in a human antigen-specific setting. These enriched mycobacteria-specific T cells were used to analyze kinetics of T cell effector cytokine production in response to stimulation with infected macrophages. In addition, antigen-expanded T cells were used to analyze the inhibitory effect of T cells on growth of mycobacteria in macrophages.

Another aim of this project was to analyze frequencies and functionality of mycobacteria-specific T cells in blood samples from HIV-1 patients and healthy controls. Therefore the protocol to quantify CD4 effector T cell subsets was optimized to give low background and allow sensitive and reproducible T cell analysis directly from isolated blood cells. Stimulation was tested with live *Mycobacterium avium* (*M. avium*), causing opportunistic infections in HIV-patients, as well as purified antigens from MTb and *M. avium*. At the end of the project, samples from two HIV patients were analyzed. Results pointed towards an impaired mycobacteria-specific immune response in the HIV patients with differences in polyfunctionality of the T cell

responses. These preliminary findings encourage the use of the technique in further studies comparing T cell responses towards MTb and *M. avium* antigens as well as live *M. avium* in healthy donors and HIV patients at different stages of infection.

The tools established in this study are open for a variety of different analyses such as studies with different antigens, modified macrophages or modified mycobacteria and might contribute to a better understanding of still incompletely understood mechanisms involved in anti-mycobacterial immunity.

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ACRONYM

AIDS	Acquired Immunodeficiency Virus
APC	Allophycocyanin
APCs	Antigen presenting cells
BCG	Bacille Calmette-Guérin
BD	Becton Dickinson
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
FBS	Fetal bovine serum
FC	Fragment of crystallization
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
L major	*Leshmania major*
MDM	Monocyte derived macrophages
MHC	Major histocompatibility
MOI	Multiplicity of infection
MOP	Mycobacterial optimal promoter
MTb	*M. tuberculosis*
NKT	Natural killer T cells
NOD	Nucleotide Oligomerization Domain receptor
OADC	Oleic Acid Albumin Dextrose Complex
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PE-CY7	Phycoerythrin & cyanine tandem conjugate
PFA	Paraformaldehyde
PMA	Phorbol 12-Myristate 13-Acetate

PPD	Purified protein derivative
PRR	Pattern recognition receptor
RBC	Red blood cells
RLU	Relative light unit
SOP	Standard operating procedures
SSC	Side ward scatter
Tb	Tuberculosis
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
WBC	White blood cells
κ	Kappa

1. INTRODUCTION

1.1 Background

Tuberculosis (Tb), a disease caused by *M. tuberculosis* (MTb) represents a major public health problem worldwide and it is the leading cause of death among people living with HIV/AIDS [1]. Despite a lot of efforts, there is currently no effective vaccine developed against infection. In addition the drugs available today are losing their efficacy mainly due to the prolonged nature of treatment as well as the occurrence of multidrug-resistant strains. To discover new therapeutic targets we need to learn more about host-mycobacterium interactions; for rational vaccine design we need to learn what constitutes protective immunity against mycobacteria. And, ultimately, we need to understand the impact of concomitant HIV-infection [2].

1.2 Pathogenesis of mycobacterial infections

M. tuberculosis is an acid-fast rod shaped slow growing intracellular pathogen with strict oxygen requirements for its growth. The Tb bacillus is transmitted from person to person when an individual inhales droplets containing bacilli from the air, which is expelled by patients with active pulmonary tuberculosis. Tuberculosis primarily affects the lung, which is the main port of entry for the Tb bacilli and site of disease manifestation. Only less than 10% of all cases will develop extrapulmonary tuberculosis [3].

Subsequent to inhalation of the droplet containing bacilli, the bacilli are phagocytosed by resident alveolar macrophages in the lung. In contrast to many other pathogens, which are destroyed by these professional phagocytic cells, *M. tuberculosis* is able to survive the antimicrobial defense mechanism and makes these cells its host. Mycobacteria that resist destruction start to multiply and disrupt the macrophages, which then release chemokines that attract blood monocytes and other inflammatory cells to the lung. Inflammatory monocytes will differentiate into macrophages, which readily ingest but do not destroy the mycobacteria. In this stage of the infection, the mycobacteria grow logarithmically [4].

About two to three weeks after infection, MTb-specific T cells are stimulated in the draining lymph nodes, which then activate infected macrophages to kill the Tb bacilli. However, the

immune response to MTb does not result in complete eradication of the bacilli; rather it leads to the containment of the bacilli in small solid granulomatous lesions in the lung consisting of a central area of infected macrophages surrounded by central memory T cells and B cells. Failure of the immune response to clear the bacteria results in a dynamic balance between bacterial persistence and host defense. In fact the majority of infected individuals maintain this balance for their entire lifetime without developing clinical disease, also called latent tuberculosis. Small proportions of infected individuals about 10% will develop active tuberculosis during their life time [3, 5]. In immunocompetent adults active tuberculosis usually occurs as a result of reactivation of latent TB, whereas in immunologically incompetent individuals such as newborns, elderly, and HIV infected patients active disease can develop following primary infection. Subsequent to failure of the immune system the granuloma loses its solidity and a cavitary lesion develops. At this stage the bacilli start to grow uncontrollably and can spread aerogenically (Figure 1).

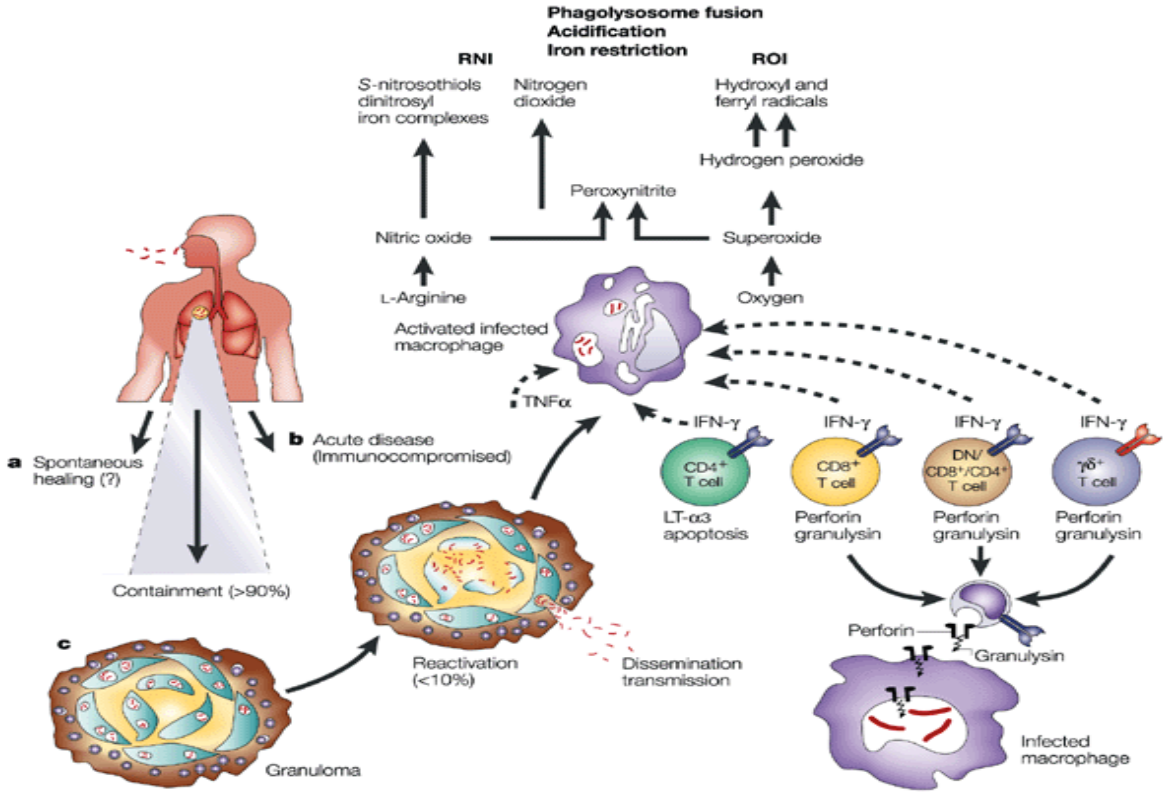


Figure 1: Overview of the course of MTb infection and immune response against tuberculosis(adapted from reference[3]) .

1.3 Innate immunity to mycobacteria

Successful control of mycobacterial infection requires the combined action of cells of the innate and adaptive immune system. Even though other cells of the innate immunity such as neutrophils are involved, macrophages play a central role in defense against mycobacterial infection. They are involved in phagocytosis, intracellular killing and initiation of adaptive immune response.

In the lung the Tb bacilli is ingested by resident alveolar macrophages by phagocytosis [3], a process that involves membrane invagination and formation of phagosome. The Tb bacilli use various receptors for entering into alveolar macrophages, which includes complement, mannose, Fc, CD-14 and scavenger receptors on macrophages [6].

Following internalization of microbes in to the phagosome, the phagosome undergoes a progressive maturational process that finally results in the formation of a less hospitable hybrid organelle, the phagolysosome. The phagolysosome possesses antimicrobial properties such as very low pH, hydrolytic enzymes, defensins and the ability to generate toxic oxidative compounds, that leads to the degradation of the microbes. However, MTb interferes with phagosome maturation and resist destruction by macrophages and survive within these cells.

Acquisition of antimicrobial properties of the phagosome as it matures depends on its interaction with the endocytic pathway, which comprises organelles ranging from early endosome to lysosome [7]. The phagosome fuses within 3 to 10, 10 to 30 and 15 to 60 minutes after ingestion by macrophages with early endosome, late endosome and lysosome, respectively. During the fusion event there would be an exchange of internal content as well as membrane constituents. Early sorting endosomes, which are recognized by the presence of Rab5 and early endosomal antigen 1 (EEA1), are relatively poor in proteases and their pH is 6-6.5. In contrast late endosomes are relatively more acidic (pH5-6) and contain more hydrolytic enzymes. They are recognized by the presence of Rab7, Rab9, lysobisphosphatidic acid, mannose-6-phosphate receptor and lysosomal-associated membrane proteins (LAMPs). The last vesicle in endocytic pathway is the lysosome. It contains bulk of active proteases and lipases and has a much lower pH (4-5.5). MTb arrests the phagosome maturation at the early endosomal stage and avoids fusion of the phagosome with the lysosome. MTb also interferes with phagosome acidification, which is a key event in the maturation process. In addition to its role in the activation of

cathepsins a family of acidic proteases that degrades phagocytosed particle and antigen presentation; it also has a role in membrane trafficking [7-9]. MTb is able to arrest phagosome acidification at pH 6.4, which is significantly higher than the pH in the phagolysosome, as well as the downstream events thus avoiding destruction by macrophages and survive within the macrophages. Activation of macrophages by proinflamatory cytokine IFN-γ, promotes the delivery of MTb in to mature phagolysosome [10].

In addition to the recognition of mycobacteria by phagocytic receptors, the recognition of mycobacterial products by evolutionarily conserved pattern recognition receptors (PRR) is important for an effective host protective immune response. Different classes of PRR such as Toll like receptors (TLRs), C-type lectin receptors and NOD-like receptors are required for the recognition of pathogen associated molecular patterns (PAMPs) of M. tuberculosis. Sensing of mycobacteria-specific PAMPs by PRR induces intracellular signaling that leads to the production of cytokines, chemokines, and initiation of adaptive immune response. Several proinflamatory cytokines are produced by activated macrophages including TNF-α, IL-1 β, IL-6, IL-12, IL-18 and IL-15. TNF-α is a prototype of proinflamatory cytokine, which plays an important role in granuloma formation, induction of macrophage activation and immunoregulation. IL-1 β and IL-6 also have protective roles in mycobacterial infection, however, IL-6 is also reported to promote mycobacterial infection by inhibiting production of TNF-α, and IL-1 β. IL-12 plays a central role in defense against *M. tuberculosis*. It links the innate and adaptive immune response to mycobacteria and its protective effect is mainly through the induction of IFN-γ. Similarly IL-18 and IL-15 are important in the induction of IFN-γ [4, 11].

Even though the correlate of protective immune response to *M. tuberculosis* is not completely understood, the role of IFN-γ is well established, primarily in the context of antigen specific T cell immunity. Initiation of mycobacteria-specific adaptive T cell response requires presentation mycobacterial antigens, co-stimulatory signal and cytokine production by professional antigen-presenting cells (APCs) such as macrophages and dendritic cells [11].

Studies in mice show that several types of antigen presenting cells including alveolar macrophages, lung macrophages and dendritic cells are infected with *M. tuberculosis* or *M. bovis* in the lung following aerosol infection. Infected antigen presenting cells migrate to regional lymphoid tissues and present antigen to T cells. *M. tuberculosis* antigens are processed by APCs

through intravaceolar proteolysis, loaded to major histocompatibility complex class II molecule and translocated to the cell surface for presentation of *M. tuberculosis* peptides to CD4 T cells. MTb peptides are also presented to CD8 cells through major histocompatibility class I molecule. There are functional differences among different APCs. While dendritic cells are primarily responsible for priming naïve T cells to become effector and memory T cells, macrophages play a role in activation of effector and memory T cells at the site of infection [12].

In addition to presentation of antigen to T cells APCs also provide costimulatory signals for stimulation of T cells. The costimulatory molecules expressed on macrophages and dendritic cells B-7.1 (CD80) and B-7.2 (CD 86) bind to CD28 and CTLA-4 on T cells for proper stimulation of T cells during antigen presentation. Antigen presentation in the absence of costimulation may lead to increased apoptosis of T cells.

Furthermore, a number of cytokines produced by macrophages and dendritic cells are essential at early stages of infection to initiate the immune system as well as at later stages to sustain and regulate it. Among these, type one cytokines including IL-12, IL-27 and IL-23 are required for the development of optimal T helper type1 (Th1) response and play an important role in promoting T cell mediated immunity against intracellular pathogens such as MTb [11, 13].

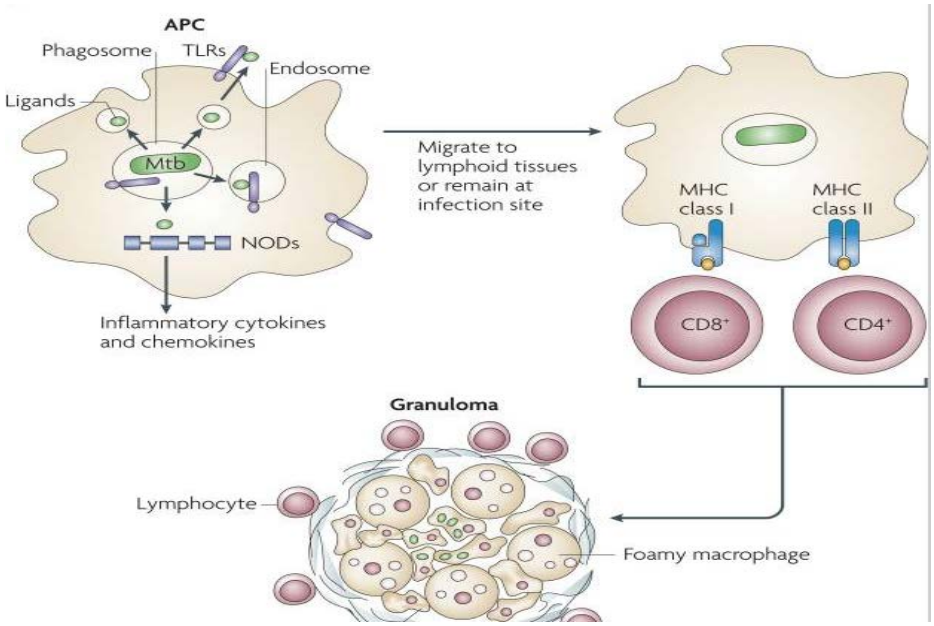


Figure 2: Antigen presenting cells (APCs) link innate and adaptive immune response to mycobacteria. Following internalization by APCs such as macrophages and dendritic cells, MTb resists destruction and survives in the phagosome. Recognition of MTb ligands by PRR such as TLRs and NOD proteins of APCs leads to secretion of inflammatory cytokines and chemokines that are required for induction of adaptive immune response. Antigen presentation(priming) by APCs in the regional lymphnodes, leads to activation & expansion of mycobacteria specific CD4 & CD8 T cells, which then translocate to the site of infection to control the growth of MTb. (Adapted & modified from reference [12]).

1.4 T cell immunity to mycobacteria

Besides the innate immune defense mechanism the adaptive immune response particularly T cell mediated immunity plays a central role in defense against mycobacterial infection. T cells originate from hematopoietic stem cells in the bone marrow but their precursors migrate through the blood to the thymus to complete their maturation process. In the thymus the developing T cells also called thymocytes, rearrange their T cell receptor genes and undergo a selection process that depends on their interaction with the thymic cells. The developing thymocytes give rise to three distinct T cell lineages, which include γ:δ T cells, α:β T cells, and NKT cells. The α:β T cells represent the major population of the mature repertoire of T cells. They recognize linear peptide antigens bound to major histocompatibility complex. Immature T cells with α:β T cell receptor that recognize self-MHC molecule and those that do not interact strongly with self-antigen are allowed to mature, while the T cells that do not match this positive and negative selection criteria will be eliminated by apoptosis. Upon completion of the maturation process the T cells leave the thymus. These T cells express cell surface markers that defines mature T cells including CD3: T cell receptor complex, and the co-receptor protein either CD4 or CD8 [14, 15].

Mature T cells that have never encountered an antigen also called naïve T cells continuously recirculate between blood and secondary lymphoid tissues such as lymph nodes, peyer's patch tonsils and spleen, until they die or encounter an antigen without entering other tissues [16]. This preferential homing of naïve T cells to secondary lymphoid tissue is mediated by adhesion molecules and signaling receptors expressed on lymphocytes and endothelial walls. Activation of naïve T cells in response to invading pathogens occurs in these tissues. Dendritic cells collect antigens in the peripheral tissues and migrate to secondary lymphoid tissues and present pathogen-derived antigens to naïve T cells bearing TCR specific for that antigen. Priming of T cells by dendritic cells induces expression of activation markers, cytokine secretion, massive proliferation and differentiation of naïve T cells to effector and memory T cells [17, 18].

In contrast to naïve T cells, effector T cells have acquired new attributes that enable them to respond quickly and efficiently when they encounter specific antigens on target cells. Effector T cells can synthesize all molecules required for their specialized effector or cytotoxic function and they do not need a co-stimulatory signal to perform their effector function. In addition the

changes that occurred on their cell surface allow them to enter sites of infection and carry out their specific effector function [15].

Naïve CD8 T cells differentiate into cytotoxic T cells after they recognize peptide-MHC I complexes on dendritic cells. Effector CD8 T cells produce cytotoxins, which kills infected target cell, as well as cytokines such as IFN-γ and TNF-α that activates microbicidal activity of infected cells [19]. Unlike CD8 T cells, which differentiate mainly in to cytotoxic T cells, naïve CD4 T cells differentiate into diverse functional subtypes after they recognize peptide-MHC II complexes on dendritic cells. CD4 T cells play an important role to defend our body from invading pathogens while avoiding immunopathology from excessive immune response. They are involved in activation of the cells of innate immune system, B cells, and cytotoxic T cells and play a role in the suppression of the immune system. The differentiation of naïve CD4 T cells in to different functional subtypes depends on the cytokine milieu at the time of priming, lineage specific transcription factors and epigenetic modification. Besides the classical T helper 1 (Th1) T helper 2 (Th2) cells, additional subtypes have been identified. This includes T helper 17 (Th17), regulatory T cell (Treg), follicular helper T cell and T helper 9 cells, each with its characteristic cytokine profile and distinct role[20]. Of these, Th1 cells play an important role in the control of intracellular pathogens such as *M. tuberculosis* or *M. avium*.

As described before *M. tuberculosis* resist killing by macrophages and survives within the phagosome of these cells [10]. Recognition of infected macrophages by antigen specific Th1 cells promotes elimination of these pathogens through the production of membrane-associated proteins as well as soluble cytokines by Th1 cells that activate macrophages. Th1 cells produce mainly IFN-γ, lymphotoxin α, TNF α and IL-2 and express the CD40 ligand. IFN-γ is important for activation of macrophages, whereas the CD40 ligand expressed on Th1 cells sensitizes macrophages to respond to IFN-γ. Activation of macrophages by IFN-γ promotes fusion of the phagosome with lysosome; induce the production of nitric oxide and superoxide radicals that enables the macrophages to destroy pathogens [15, 20]. In addition, MTb specific T cells promote the destruction MTb bacteria in infected macrophages through autophagy in a contact dependent mechanism [21].

Subsequent to clearance of the pathogen the majority of effector T cells (90%-95%) die by apoptosis, while leaving behind small fraction of long-lived resting memory T cells that respond

rapidly and effectively to future encounters with the same pathogen. The enhanced ability of memory T cells to control secondary exposure to the same pathogen is attributed to increased frequency antigen specific memory T cells, rapid acquisition of effector function and localization to peripheral sites of infection [22, 23].

Memory T cells are categorized in to central memory and effector memory based on surface phenotype and function. Central memory T cells are CD45RO+; and constitutively express CCR7 and CD62L, which are required for homing to secondary lymphoid tissue. In contrast to naïve T cells which are CD45RA+ and also home to secondary lymphoid tissue, central memory T cells have higher sensitivity to antigenic stimulation, are less dependent on costimulation and up regulate CD40L to a greater extent, thus providing effective stimulatory feedback to dendritic cells and B cells. Upon antigenic stimulation central memory T cells produce mainly IL-2, proliferate and differentiate to effector T cells. On the other hand, effector memory T cells which are also CD45RO+ lack homing receptor to secondary lymphoid tissue, but express characteristic set of chemokine receptors and adhesion molecules required for homing to inflamed tissue. Compared to central memory T cells, effector memory cells are characterized by a rapid effector function, responding within hours of antigenic stimulation [24].

1.5 Importance of mycobacteria specific T cells in the control of mycobacterial infections

Although different subsets of T cells are assumed to play a role in controlling mycobacterial infection, the relative importance of CD4 T cells in protection against *M. tuberculosis* infection is well established in animal model as well as in humans. Transgenic mice deficient in CD4 molecules show increased susceptibility to *M. tuberculosis* compared with wild type mice [25, 26]. Similarly individuals infected with human Immunodeficiency virus, which is characterized by progressive depletion and dysfunction of CD4 T cells, are at high risk of primary or reactivated tuberculosis indicating the importance of these cells in the control of mycobacterial infection in humans[27]. T helper type 1 CD4 T cells, which mainly produce IFN-γ, are particularly important in this respect [20]. Mice with disrupted IFN- γ gene were unable to contain or control normally sublethal doses of *M. tuberculosis* delivered intravenously or aerogenically [28]. IFN-γ is also important in humans since individuals with IFN-γ or IL-12 receptor deficiency are highly susceptible to mycobacterial infections [29, 30]. Even though,

IFN-γ is important in the control of mycobacterial infections, it is not sufficient on its own. For instance, individuals with certain autoimmune diseases receiving anti-TNF-α treatment have increased susceptibility to tuberculosis, suggesting an important role of this cytokine in control of *M. tuberculosis* infection [31].

Recent studies in chronic viral, parasitic and bacterial infections have indicated that the quality (the degree of multifunctionality) of T cell immune response rather than the quantity(frequency of antigen specific CD4/CD8 T cell or bulk measurements, such as total cytokine secretion) represents an important correlate of protection against pathogens or better clinical outcome [32]. Hence, analysis of antigen-specific T cells simultaneously producing multiple cytokines: IFN-γ, TNF-α, and IL-2, termed polyfunctional T cells has been suggested to evaluate vaccine elicited immune response against-specific infections that requires T cells for protection, instead of analyzing single cytokine producing T cells such as IFN-γ or TNF-α only, which does not always correlate with disease protection [32].

Polyfunctional T cells are associated with superior functional capacity than single cytokine producing cells and represented a higher percentage of total cytokine response in viral infections that are completely or well controlled such as cytomegalovirus, influenza and smallpox viruses [33]. Similarly polyfunctional T cells are correlated with better clinical outcome or control of HIV-1 and HIV-2 infections [34-36]. These studies show that single cytokine producing cells are predominant in uncontrolled or in acute infections when antigen load is higher, whereas multiple cytokine producing cells dominate the overall cytokine response in individuals that control or clear pathogens such as following antiretroviral treatment or naturally as in case of HIV long term non-progressors. However, it is not known whether the maintenance of polyfunctional T cells is the cause or consequence of viral control.

More direct evidence suggesting the protective role of polyfunctional Th1 cells comes from study of *Leshmania major* infection in the mouse model [37]. Challenging mice with live *L major* parasite, following immunization with different types of vaccines that elicited similar magnitude of Th1 response but different quality has different levels of protection. While mice with high frequency of polyfunctional T cells were protected those mice that produce single cytokine (IFN-γ only) were not. Taken together, these studies show the importance of

polyfunctional T cells that produce multiple cytokine are better correlates of protection than single cytokine producing cells.

Nevertheless studies of polyfunctional T cells producing IFN-γ, TNF-α and IL-2 in patients with pulmonary tuberculosis, latent *M. tuberculosis* and healthy controls end up with different conclusions regarding the distribution of cytokine producing CD4 T cells in different groups. While some report that polyfunctional T cells are associated with active tuberculosis [38, 39], others report that polyfunctional T cells are better correlates of protection [40, 41]. These differences in cytokine production profile of MTb specific CD4 T cells may be due to the use of different antigens or may be due to methodological differences in analyzing antigen specific T cells response and shows the difficulty of studying correlates of protective immunity to tuberculosis.

On the other hand studying mycobacteria-specific T cell mediated immune response in HIV infected patients before and after onset of anti-retroviral treatment in comparison with healthy individuals, in addition to providing information on how HIV infection impairs mycobacteria specific immune response it may lead to better understanding of correlates of protective immune response to mycobacteria as highly susceptible individuals become less susceptible following treatment.

1.6 Mycobacterial infections in HIV patients

Human immunodeficiency virus type 1 (HIV1) is one of the leading global health problem caused by an infectious agent. According to a recent world health organization (WHO) report there were approximately 34 million people worldwide living with HIV in 2010 [42]. There were also 2.7 million new HIV cases and 1.8 million AIDS deaths in this year. HIV patients experience increasing immunodeficiency due to loss of CD4+ T helper cells and are prone to opportunistic infections by a range of pathogens. The majority of complications are caused by co-infections with mycobacteria, namely MTb and MAC-disease (*M. avium* complex), caused by MAC organisms like *M. avium* and *M. intracellulare*. More than 10 million HIV infected people are estimated to be co-infected with *M. tuberculosis*, the causative agent of tuberculosis. Co-infected individuals are 21 to 34 times more likely to develop active tuberculosis disease than people without HIV. About 25% of death among people living with HIV is attributed to Tb [43].

After infection with HIV, CD4+ T cell numbers slowly decline over time. During this period CD4+ T cell counts of patients are regularly monitored and in case CD4+ T cell counts fall below a certain threshold, highly active antiretroviral therapy (HAART) is initiated. In Norway today, HAART is usually started in HIV patients at CD4+ T cell counts around 350 / mm^3. Close to 100 % of the Norwegian HIV-patients receive HAART if necessary, thus avoiding patient conditions with extremely low CD4+ counts.

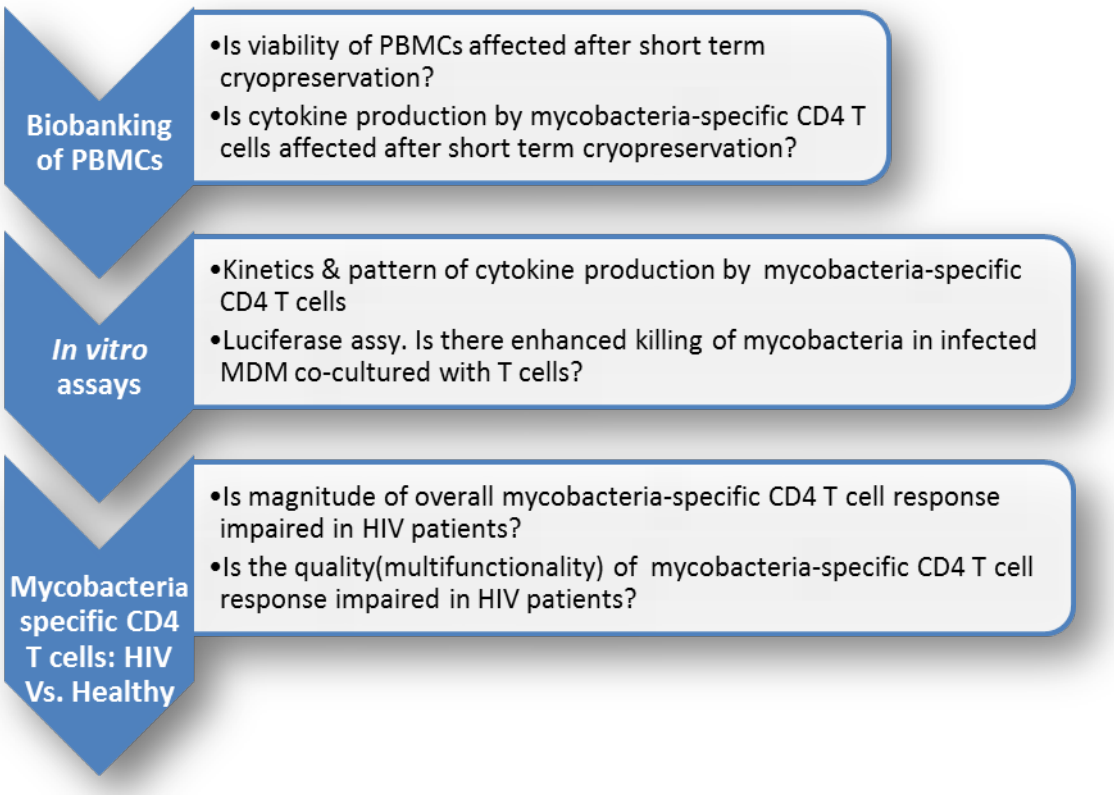
Even though antiretroviral treatment is associated with significant reduction on the incidence of *M. tuberculosis* [44], the risk still remains higher in treated patients compared to HIV uninfected individuals [45]. Moreover, unlike other opportunistic pathogens MTb causes disease early in the course of HIV infection in patients with relatively normal CD4 count [46]. This suggests that there is a qualitative defect in immune response to MTb, independent of the total CD4 count. This defect could be on cells of innate or adaptive immunity. As CD4 T cells are crucial to control of mycobacterial infections analyzing the effect of underlying HIV-1 infection on function of CD4 T cells may lead to a better understanding of the mechanism of increased susceptibility of HIV patients to MTb. At the same time this information would give us indirect evidence of the correlates of immune protection to MTb which is required for optimal vaccine design against tuberculosis.

2. AIM OF THE STUDY

The overall aim of the project was to characterize mycobacteria-specific polyfunctional CD4 T cell response in HIV-1 infected patients in comparison with healthy control subjects.

Specific Objectives

- Establishing methods for biobanking of PBMCs from healthy donors and HIV patients.
- Establishing *in vitro* procedures for cultivation and expansion of mycobacteria-specific T cells and macrophages from PBMC samples as well as assays for analysis of different anti-mycobacterial CD4 effector T cell functions.
- Analyze polyfunctional CD4 T cell responses to mycobacterial antigens in HIV patients in comparison with healthy control subjects.



3. MATERIALS AND METHODS

Detailed procedures for isolation and biobanking of PBMCs as well as for analysis of mycobacteria specific T cell function can be found in appendix I. HIV patient samples were handled under BL3 condition.

3.1 Study participants

3.1.1 Healthy controls

Five healthy controls were recruited from working group at the department of cancer research and molecular medicine (IKM) of Norwegian University of Science and Technology. One individual was recruited among the blood donors at St. Olav's hospital, who consented his blood to be used for research purpose. All of them were vaccinated with BCG during child hood. While five of them were Caucasians, who had no past history of tuberculosis and not lived in tuberculosis endemic areas for more than one month, one of the donors was African, who lived in tuberculosis endemic area for long periods of time, though without history of active tuberculosis. Gender distribution was equal. Even though the healthy donors were not tested for HIV, they represent low risk group for HIV infection.

3.1.2 HIV patients

The patients in our study were recruited from the pool of outdoor patients regularly visiting St. Olav's hospital. This pool consists of 120 well characterized HIV-infected patients at various stages of disease with and without ongoing HAART. Unfortunately due to late ethical approval we were able to acquire sample from only two patients, who consented to participate in the study. Both of them were male. One of the patients is relatively younger and was taking Truvada and Reyataz, while the other one who was relatively older and had severely impaired reconstitution of CD4 T cells was taking a combination of Truvada, Prezista, Norvir, and Isentress. Sample acquisition from patients was facilitated by Professor Jan K. Damås Department of Infectious Diseases, St Olav's Hospital. Study participant characteristics are summarized in table 1.

	Number of participant	Age(years)	Baseline CD4 T cell count(cells /µl of blood)	Current CD4 T cell count(cells /µl of blood)	Duration of disease(years)	Duration HAART(months)	Baseline viral load(copies/ml)	Current viral load(copies/ml)
HIV patients	2							
Median		49.5	190	400	13.5	11	56,500	35
Range		45-54	170-210	350-450	12-15	10-12	35-78x10^3	20-50
Healthy control	6							
Median		32	NA	NA	NA	NA	NA	NA
Range		24-40	NA	NA	NA	NA	NA	NA

Table 1 Socio-demographic and clinical characteristics of study participants. NA: not applicable or not available.

3.2 Cell culture, media and Reagents

3.2.1 Cell culture

Primary cells obtained from healthy donors and HIV patients were used in all experiments in this study. Cell culture was set up in 24 or 96 well microtiter plate depending on the type of experiment. For cytokine analysis from short term stimulation or after expansion of mycobacteria specific T cells as well as for growth inhibition assay, 10% A+ heat inactivated human serum in RPMI supplemented with 10mM HEPES and 2mM glutamine were used. For preparing of monocyte derived macrophages 30% A+ human serum used instead of 10%. Hereafter, these media will be mentioned as 10% and 30% media.

3.2.2 Reagents used for intracellular cytokine assay

For identifying T cell lineage fluorescent conjugated anti-human CD3 (eFluor 450, clone OKT3) and anti-human CD4 (FITC, clone OKT4) were used. To further delineate the functional profile anti-human IFN-γ (PE, clone 4S.B3), anti-human TNF-α (APC, clone MAb11) and anti-IL-2 (PE-CY7, clone MQ1-17H2) were used. Costimulation was provided by purified anti-human CD28 (clone CD28.2) and anti-human CD49d (clone 9F10) antibodies. Protein secretion was inhibited by Protein Transport Inhibitor Cocktail (500X) and Cell Stimulation Cocktail (500X) were used as a positive control. Dead cells were excluded from analysis with Fixable Viability Dye eFluor® 780. All of them except antiIL-2 antibody were obtained from eBioscience. AntiIL-2 antibody was obtained from biolegend. In addition 4% Para-formaldehyde and 0.5% saponin in 2% FCS/DPBS were used for fixation and permeablization, respectively.

3.2.3 Antigens

Different mycobacteria derived purified proteins as well as live *M. avium* bacteria were used to assess mycobacteria-specific memory CD4 T cell response in healthy controls and HIV patients. The preparation of live *M. avium* bacteria will be explained later (section 3.6). *M. tuberculosis* and *M. avium* derived purified proteins (PPD) were kind gift from Ingrid Olsen, Norwegian Veterinary Institute, Oslo.

M. tuberculosis derived purified protein (MTb PPD) is prepared from cultured filtrates from virulent strains of *M. tuberculosis* [47]. It is a soluble protein that contains more than 200 different mycobacterial antigens. Most of these proteins are also expressed by other mycobacteria such as *M. bovis* BCG [48]. Similarly *M. avium* derived purified protein (*M. avium* PPD) is also prepared from cultured filtrates of *M. avium* [47].

3.3 Peripheral blood mononuclear cell (PBMC) isolation and biobanking

3.3.1 PBMC isolation principle

The isolation of PBMCs requires separation of whole blood by centrifugation through a density gradient. Whole blood is layered onto a sterile aqueous medium containing polysaccharide and sodium diatrizoate at a predetermined density of 1.077 g/ml at 25°C. Gentle centrifugation at room temperature results in the separation of PBMCs at the blood/ ficoll interface, with the other white blood cells (WBCs) and red blood cells (RBCs) passing through the interface and collecting at the bottom of the tube.

The PBMC interface was collected and washed with sterile Phosphate Buffered Saline (PBS) to remove any contaminating separation medium. The PBMCs were then resuspended in 2% FCS in PBS and the cell count and viability of the cells was determined (detailed procedures described in appendix I).

3.3.2 Cell count and viability

After resuspension in the required volume of 2% FCS in PBS, PBMCs were counted with Countess™ Automated Cell Counter, which uses an image analysis system for cell counting and

for distinguishing live cells from dead cells. The instrument identifies objects in the field as cells based on their similarity to the average size of all the objects in the image, and their degree of circularity.

The method is based on the dye exclusion principle, i.e. live cells possess intact cell membrane that excludes certain dyes (in this experiment 0.4% trypan blue), whereas dead cells do not. An aliquot of cells is mixed with an equal volume of 0.4% trypan blue, and 10 µL of the mixture is transferred to a non-gridded disposable Countess™ chamber slide. The slide is inserted into the instrument, and the image that appears on the screen represents the same area as four 1 x 1 mm squares on a hemocytometer. Using the zoom function and fine focusing knob, image adjustments are made to bring the objects into proper alignment. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a uniform blue cytoplasm. Then, a single touch of a button on the touchscreen starts the automatic process of acquiring and analyzing the image, resulting in readout of total cells/mL, live cells/mL, dead cells/mL, and percent viability. The live cell number was used for the different experiments. Cell count was verified with coulter counter.

3.3.3 Cryopreservation of PBMCs

To avoid inter-assay variability while running experiments separately at different time points, we planned to freeze and analyze patient samples in batch. Cryopreservation of PBMCs is used to maintain structural and functional integrity of cells. Following isolation the PBMCs were resuspended in the cryopreservation media (10% DMSO in FCS), transferred to controlled rate freezing container and stored in -80^0C freezer for short period of time (procedure described in detail in annex I).

3.4 Preparation of Monocyte derived Macrophages (MDM)

PBMCs were isolated as described in 3.3.1. Following washing and cell counting, the PBMCs were resuspended in 10% media at a concentration of 4 million PBMCs per ml and 100µl of cell suspension was added to each well in a 96 well flat-bottomed plate for growth inhibition assay and cytokine assay from antigen-expanded T cells. After one hour incubation at 37 ^{0}C 5% CO_2

non-adherent cells were removed and the plate was washed twice with warm sterile PBS. Then 100µl of 30% media was added and the cells were further incubated at 37 ^{0}C 5% CO_2 on average for 6 days (5-7 days) to allow the monocytes to differentiate in to macrophages (detailed procedure in appendix I).

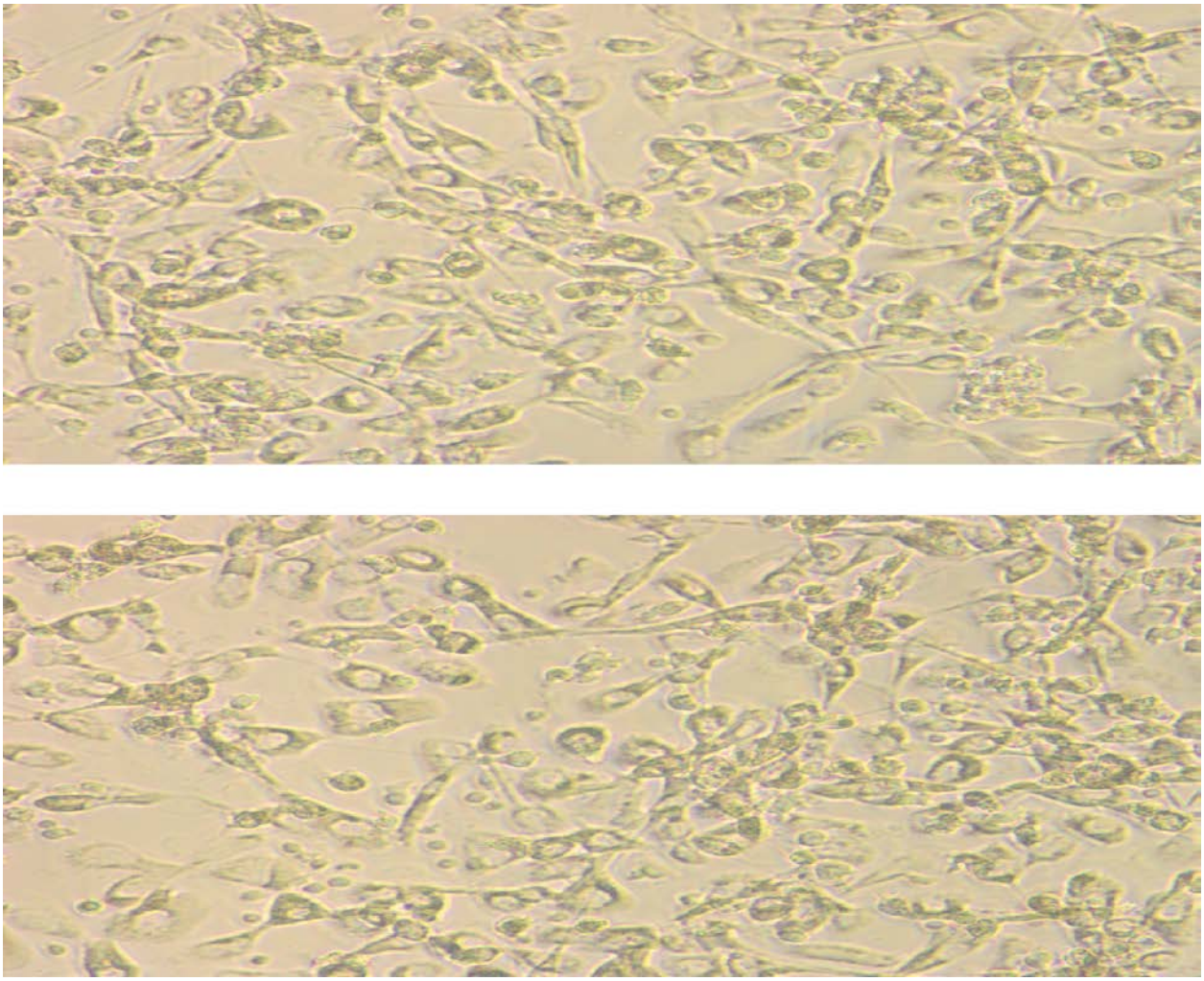


Figure 3: Monocyte derived macrophages from two different wells after 5 day incubation at 37 ^{0}C 5% CO_2 in a 96 well plate.

3.5 Mycobacteria specific T cell expansion

PBMCs were prepared as described in 3.3.1 and resuspended at concentration of 2 million per ml in 10% media. 1ml of cell suspension was added to each well in a 24 well plate. The PBMCs in 4 wells were stimulated with heat-killed *M. avium* bacteria (MOI 5x) or MTb &/or *M. avium* PPD, while one well used as unstimulated control containing only PBMCs and media. Subsequently the cells were incubated at 37 ^{0}C 5% CO_2 on average for 6 days (5-7days) before they were used for growth inhibition and cytokine assay (Detailed procedure described in appendix I).

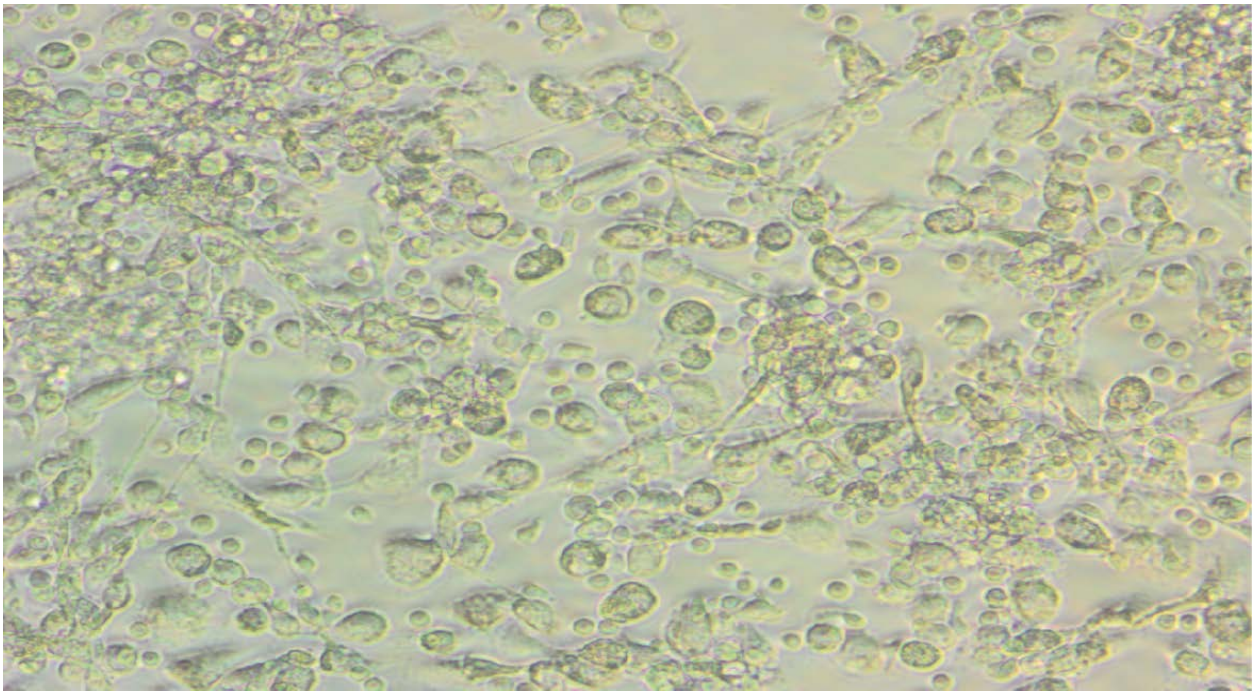


Figure 4: Heat-killed *M. avium* stimulated PBMCs after 5 day incubation at 37 ^{0}C 5% CO_2 in a 24 well plate.

3.6 Cultivation and preparation of *M. avium*

For growth inhibition as well as for T cell cytokine assay slightly modified *M. avium* bacteria were used in addition to other mycobacterial antigens that were used for T cell cytokine assay. All procedures involving the use of live *M. avium* were carried out according to the standard

operating procedure (SOP) for work with *M. avium* with special precautions applicable in mycobacteria-lab.

M. avium is an acid-fast rod shaped slow growing, non-motile, non-spore forming bacteria that requires oxygen for its growth. In this experiment *M. avium* strain 104 pMH109 C were used. Strain 104 (fully sequenced), is stably transfected with pMH109 containing MOP (mycobacterial optimal promoter) driving firefly luciferase and acr (16-kDa α-crystallin homolog) driving lacZ. It confers resistance to hygromycin. It has an *E coli* origin of replication and integrates in mycobacterial chromosome at a tRNA locus. Clone C is from Gerald cangelosi's lab at SBRI (Seattle Biomedical Research Institute) (Kirsten Hauge). The strain is smooth, transparent and virulent. T1/2 is ~20 hours.

A stock culture of *M. avium* strain 104 pMH109 C was maintained on Middlebrook 7H10 plates supplemented with Oleic Acid Albumin Dextrose Complex (OADC) (BD/Difco). Plates were kept in the in polyethylene plastic bag and incubated at 37^0C for a week and then re-plated on to fresh 7H9 plates. Every 2-3 month stock culture was replaced by fresh stock from freezer.

At least one day before starting the experiment smooth and transparent colonies was picked from the plate and grown at 37^0C on an incubator shaker in 10ml of Middlebrook 7H 9 media supplemented with Albumin Dextrose Complex (ADC) in 50 ml conical tube. Immediately before use of the bacteria, the broth culture was vortexed and sonicated for 30 seconds (3 times). Then the optical density (OD) of the broth culture was determined by spectrophotometer at 600nm wavelength, with OD=1 at 600nm representing $4.5x10^8$ bacteria per ml. hence, the number of bacteria in the broth was calculated in reference to this number after determining the OD of the broth. The bacteria in broth culture were used for the experiments when the OD was between 0.3 and 0.6, at which log phase of bacterial growth is assumed.

After determining the OD of the broth the bacteria was washed twice with sterile PBS and resuspend in sterile PBS corresponding to the original volume of broth. Then the bacteria were used for the various experiments at different multiplicity of infection (MOI).

3.7 Growth inhibition assay

For determining the ability of heat-killed *M. avium* expanded effector T cells to inhibit the growth of live *M. avium* bacteria in infected MDM, luciferase assay was used.

Luciferase assay principle

Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase, a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP•Mg2+ as a co-substrate. The intensity of emitted light measured by a luminometer is directly proportional to luciferase concentration, hence to the number of live bacteria in the lysate[49].

$$\text{Bettle Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{\text{Firefly Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

For growth inhibition assay autologous monocyte derived macrophages prepared as described above were used (section 3.4). On day five the media from MDM removed and the MDM washed with warm PBS twice before antigen expanded effector T cells and live *M. avium* bacteria were added. Expanded effector T cells(PBMCs) from same donor transferred from 24 well plate in to 15ml conical tube after 5 day stimulation with heat-killed *M. avium* at MOI 5X, washed twice with 2% FCS in PBS and the cell count and viability was determined. The PBMCs are then resuspended in 10% media at concentration of 3.6 million per ml and 100µl of cell suspension added to MDM in a 96 well plate as described in the table below. At the same time *M. avium* bacteria prepared as described above added to 96 well plate at low dose(MOI 5X) and high dose(MOI 10X) as shown in the table below. Then the plate incubated at 37^0C 5%CO_2 for 72 hours.

UNSTIMULATED 1	HIGH DOSE + T CELL 1
UNSTIMULATED 2	HIGH DOSE + T CELL 2
LOW DOSE + T CELL 1	HIGH DOSE – T CELL 1
LOW DOSE + T CELL 2	HIGH DOSE – T CELL 2
LOW DOSE – T CELL 1	
LOW DOSE – T CELL 2	

Table 2: Growth inhibition assay experiment layout. MDM were infected with live *M. avium* in low (MOI5x) or high (MOI 10x) dose. Plus & minus sign indicates incubation of MDM with & without T cell co-culture, respectively.

After 72 hour incubation non-adherent cells and excess bacteria were removed and the adherent infected MDM washed twice with pure PBS. Then MDM were lysed with 120µl of 1x cell culture lysis buffer (promega) for 1 minute and pipetted 5 times up and down and 50µl of lysate transferred to luciferase assay plate as follows (OptiPlex 96 well plate).

UNSTIMULATED 1	LOW DOSE - T CELL 1	HIGH DOSE - T CELL 1
UNSTIMULATED 1	LOW DOSE - T CELL 1	HIGH DOSE - T CELL 1
UNSTIMULATED 2	LOW DOSE - T CELL 2	HIGH DOSE - T CELL 2
UNSTIMULATED 2	LOW DOSE - T CELL 2	HIGH DOSE - T CELL 2
LOW DOSE + T CELL 1	HIGH DOSE + T CELL 1	
LOW DOSE + T CELL 1	HIGH DOSE + T CELL 1	
LOW DOSE + T CELL 2	HIGH DOSE + T CELL 2	
LOW DOSE + T CELL 2	HIGH DOSE + T CELL 2	

Table 3: Luciferase assay lay out. Plus & minus sign indicates incubation of MDM with & without T cell co-culture, respectively.

Then 50µl of firefly luciferase assay substrate (promega) added to each well and luciferase activity was measured within 10 minutes using luminometer (victor 1420 multilabel counter).

3.8 Measurement of effector cytokine production by CD4 T cells in response to mycobacterial antigens

Measurement of qualitative and quantitative antigen specific T cell response is commonly used for monitoring of immune status during disease and for assessment of vaccine efficacy. To assess the impact of HIV infection on mycobacteria specific T cell response, we established in this project methods to analyze mycobacteria specific effector cytokine production by activated T cells. This method will be used to analyze cytokine response from healthy individual and HIV infected patients. Even though there are different methods for the analysis of cytokine production by antigen stimulated T cells the use intracellular cytokine staining allows analysis of multiple

parameters per cell, which is in line with our objective to analyze multifunctional T cell response.

3.8.1 Intracellular cytokine staining (ICS)

This method is based on the use of high affinity anti cytokine antibody that binds cytokine accumulated inside the cell following inhibition of protein secretion, cell fixation and permeablization procedures. The technique allows analysis of functional memory T cell response to specific antigens in short term stimulation. Cytokine from both fresh (unexpanded) and antigen expanded T cells (PBMCs) were analyzed. Antigen expanded T cells was prepared as described before (section 3.5) washed, counted and resuspended in appropriate amount of 10% media to make the final concentration 3.6 million PBMCs per ml. Autologous MDM prepared as described before (section 3.4) were used as antigen presenting cells for antigen expanded T cells. 10% of PBMCs are estimated to be monocytes and be adherent i.e.0.04 million MDM in 96 well plate, so 0.36million of expanded T (PBMCs) cells were added to make the final concentration 0.4 million.

Both fresh and antigen expanded PBMCs were stimulated in a 96 well flat-bottomed plate at a concentration of 0.4 million PBMCs per well with different mycobacterial antigens, positive control. Anti-CD28 and anti-CD49 antibodies were added as costimulatory molecules. After two hour incubation at 37^0C 5%CO_2, the secretion of newly synthesized proteins were inhibited by protein transport inhibitor cocktail and the cells were further incubated on average for 14 hour at 37^0C 5% CO_2. Subsequently the cells were transferred to tubes washed, stained for surface markers (CD3, CD4), fixed, permeablized and stained for intracellular cytokines (IFNγ, TNF-α, and IL-2). Live dead stain was also used to exclude dead cell from analysis. Finally, the cells were analyzed by flow-cytometry (detailed procedure described in annex I).

3.9 Flow-cytometry acquisition and analysis

3.9.1 Principle of Flowcytometry

Flow-cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or

internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

There are four main components of flow cytometer. The first component is the fluidics system of the instrument. The heart of the fluidics system on all flow-cytometers is the "nozzle". This is the component where cells are injected into the sheath-fluid stream, are hydrodynamically focused, and in cuvette-nozzle designs where the cells are illuminated by the laser beams. Requirements for steering and focusing the laser beams and for collecting and detecting the fluorescence and scatter emissions require sophisticated optics. The collected light energy must be converted to electrical energy and this in turn converted to digital information. This is the function of the electronics of the cytometer. Finally in order for the operator/investigator to interpret what happened within the cytometer the data must be collected by a computer and interpreted by software into appropriate figures[50].

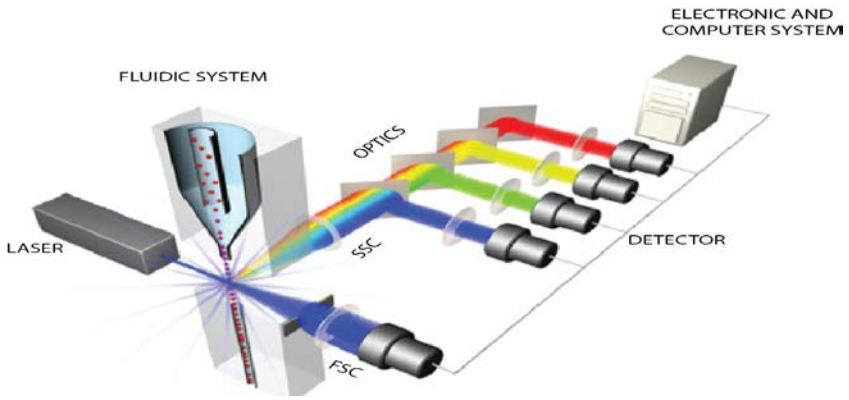


Figure 5: Main components of a flow-cytometer(adapted and modified from [51]).

3.9.2 Compensation

Simultaneous measurement of multiple characteristics of cells using polychromatic flow-cytometry is often subjected to intrinsic spectral overlap of the different fluorochrome used. Compensation is the process which corrects the detected "spillover" of the emission of one fluorochrome into the detector designed to collect the emission from another fluorochrome. The primary purpose is to allow the measurement of the true fluorescence in the fluorescence channel contaminated by the spillover.

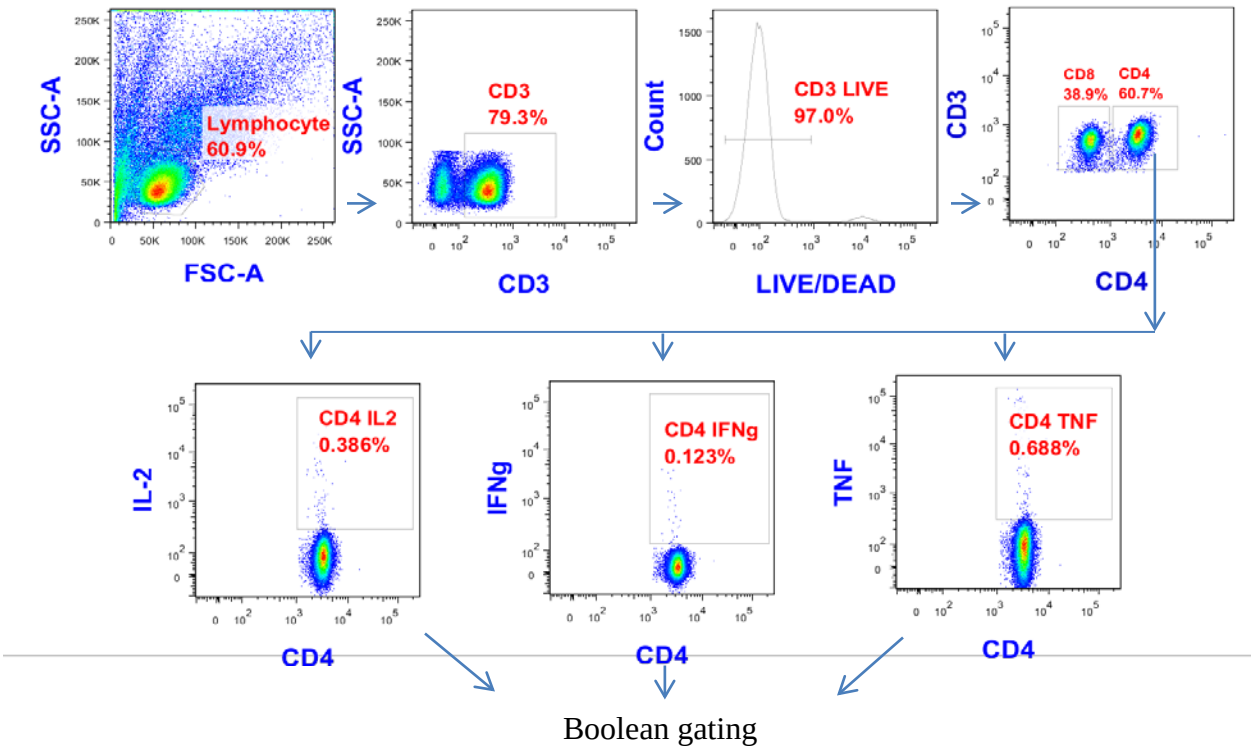
BD Polymeric microparticles were used to set up compensation for all fluorochromes used in our experiment, except for live/dead fixable dye for which an equal mixture of live and heat-killed cells (PBMCs) was used. Two kinds of particles were used. The first one have been coupled to an antibody specific for the IgG, κ light chain of mouse, which binds to the light chain of each fluorochrome labeled antibody used in our experiment. The second one is a negative control which is labeled with FBS that have no binding capacity. The compensation was calculated and was applied to subsequent experiments automatically by the software installed in the computer (procedure described at the end in appendix I).

3.9.3 Analysis of mycobacteria-specific polyfunctional CD4 T cell function by multicolor flow-cytometry

Six-color flow-cytometry was used to characterize polyfunctional CD4 T cell response to mycobacterial antigens (Figure 6 A). Two colors (CD3 and CD4) were used for identification of the T cell lineage and one color for excluding dead cells from analysis and the other three different colors were used to analyze effector T cell function(cytokine production). Gating was done as follows: Lymphocytes were identified based on FSC/SSC properties →T cells were identified based on CD3 surface marker → exclude dead cells → live CD3 →CD4+ → then analyze the CD4+ T cells for IFN-γ, TNF-α, and IL-2 expression(Figure 6 A). Similar gating strategy was used when only one or two cytokines were analyzed from fresh (unexpanded) or expanded T cells. Cytokine producing cells are shown as % of CD4 gated T cells. Live/dead stain was used for analysis of cytokines from fresh PBMCs in the last part of the project (section 4.4) for the two healthy donors and two HIV patients.

After analyzing the CD4 T cell response using flowjo software for individual cytokines, by applying Boolean gating it is possible to identify all possible combinations of cytokine producing CD4 T cells (Figure 5 B). Simultaneous measurement of three cytokines (IFN-γ, TNF-α, and IL-2) gives us seven possible functional states, one triple cytokine producer state (CD4 IFN-γ+ IL-2+ TNF-α+), three double cytokine producer states (CD4 IFN-γ+ IL-2+ TNF-α-, CD4 IFN-γ+ IL-2- TNF-α+, CD4 IFN-γ- IL-2+ TNF-α+) and three single cytokine producer state (CD4 IFN-γ- IL-2- TNF-α+, CD4 IFN-γ- IL-2+ TNF-α-, CD4 IFN-γ + IL-2- TNF-α-). The relative contribution of each functional subset to the overall response can be shown by using pie chart.

A)



B)

IFN-γ	+	-	+	+	-	-	+
IL-2	+	+	-	+	-	+	-
TNF-α	+	+	+	-	+	-	-
	Triple producers		Double producers			Single producers	

Figure 6: Flow-cytometric characterization of CD4 T cells for cytokine production A. representative dot plot from single individual and gating strategy. CD4 T cells were identified based on the surface markers CD3 and CD4. Dead cells were excluded from analysis. Subsequently the CD4 T cells were analyzed for each cytokine (here representative data from MTb stimulated CD4 T cells), followed by Boolean gating to identify each functional subset. B. Representation of the seven functional subsets of CD4 T cells gated as described above, which produce various combinations of cytokines when the three cytokines were analyzed.

3.10 Ethics

The project was approved by the Regional Ethics Committee (REK) at the Norwegian University of Science and Technology (NTNU). Oral and written consent was obtained from study participants before sample collection. All parts of the study were conducted according to the ethical guidelines at our hospital in conformity with the Helsinki declaration.

3.11 Data analysis

Flow-cytometric data was analyzed by using FlowJo software (version 7.6.5 Tree Star). Further data analysis and graphical display was done by GraphPad Prism software version 5 (Software MacKiev, GraphPad, San Diego, CA) and Microsoft Excel (2010).

4. RESULTS

4.1 Establishing protocols for biobanking of PBMCs from HIV patients and healthy controls

The response of isolated peripheral blood mononuclear cells (PBMCs) to different antigenic stimuli is often used as surrogate for *in vivo* immune responses to adaptive and innate immune stimuli. Cryopreservation of isolated PBMCs is commonly used to preserve the structural and functional integrity of mononuclear cells and to standardize immunological measurement. The use of cryopreserved PBMCs enables batch analysis of patient samples hence avoids inter and intralaboratory variability [52]. For cryopreservation of PBMCs the protocol described in appendix I was used. In the first part of this project to determine whether short term cryopreservation has an effect on the viability and functional response of CD4 T cells we have analyzed the viability and cytokine response of PBMCs to mycobacterial antigens initially at time of isolation of PBMCs and after short term cryopreservation at -80^{o}C.

4.1.1 Viability of PBMCs was not affected by short term cryopreservation

To test the effect of short term cryopreservation on viability, PBMCs from four healthy donors were isolated by ficoll-hypaque density gradient centrifugation technique. Their viability was determined immediately after isolation as well as after thawing one and two week later from -80^{o}C freezer. The mean percent viability for the three time points was 96%, 94%, and 93.4%, respectively (Figure 7). The viability does not seem to be affected by short term cryopreservation and it was always greater than 90 percent.

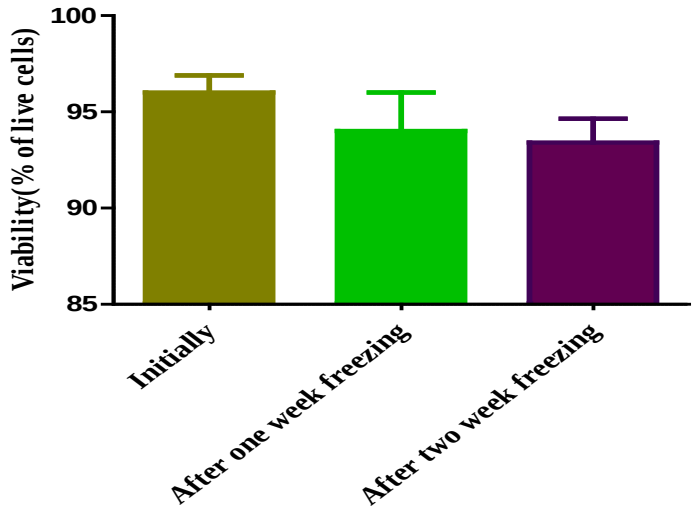


Figure 7: Viability of PBMCS before and after short term cryopreservation. The viability was determined with Countess™ Automated Cell Counter. Data displayed as mean percent viability and standard deviation of the four donors.

4.1.2 Effector cytokine production from fresh and frozen (biobanked) PBMCs

Effector cytokine production in response to stimulation with a specific antigen is central function CD4 T cell subsets [20]. In order to determine the effect of short term cryopreservation on the function of CD4 T cells, mycobacteria-specific effector cytokine response of CD4 T cells (IFN-γ production) was analyzed before and after cryopreservation of PBMCS for one week in -80^{0}C freezer. Fresh and frozen PBMCs from four healthy BCG-vaccinated donors were stimulated overnight using *M. tuberculosis* derived purified protein (MTb PPD). After overnight stimulation, IFN-γ production was analyzed by intracellular cytokine staining. Comparable results were obtained with fresh and frozen cells. The mean percent and standard deviation of IFN-γ producing CD4 T cells in response to MTb PPD was 0.04%+/- 0.011 and 0.041+/-0.007 for fresh and frozen PBMCs, respectively (Figure 8).

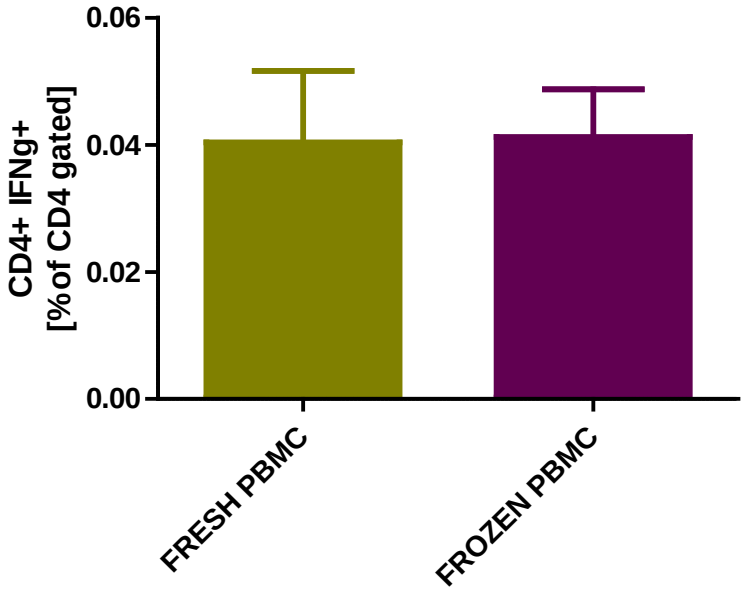


Figure 8: Cytokine production by CD4 T cells before and after short term cryopreservation. Both fresh and frozen PBMCs were stimulated overnight with MTb PPD and IFN -γ production from CD4 T cells was analyzed by ICS. Data is shown as mean percent +/- standard deviation of IFN-γ producing cells within CD3+CD4+ gated T cells. Background staining was low and subtracted from both fresh and frozen samples.

4.2 Establishing an in vitro assay for analysis of mycobacteria-specific CD4 effector T cell function

PBMCs isolated from whole blood and buffy coat samples from healthy BCG vaccinated individuals were used to establish method for analyzing T cell function such as cytokine production (Kinetics & Pattern) and the ability of antigen expanded T cells to inhibit the growth of intracellular bacteria in infected monocyte derived macrophages.

4.2.1 Expansion of mycobacteria-specific T cells for analysis of antigen-specific effector T cell functions

Mycobacteria-specific CD4 T cells were found at lower frequency (usually less than 0.1%) in the donors we tested in our study (Figure 8). Activated memory T cells recognizing their specific antigen enter the cell cycle and start proliferating[24]. Thus, mycobacteria specific T cells present at very low frequencies with in the PBMC population can be expanded and enriched by stimulation with mycobacterial antigen [53]. To increase the frequency of antigen specific CD4 T cells, PBMCs were stimulated and expanded on average for 6 days (5-7 days) with different mycobacterial antigens (as described in section 3.5 & appendix I) and expanded T cells were used for different assays.

4.2.1.1 Expanded mycobacteria-specific T cells show increased frequency cytokine producing mycobacteria-specific CD4 T cells

After 5-7 day expansion of mycobacteria specific T cells with MTb derived PPD, effector cytokine production in response to antigen presented from MDM was assessed as discussed in 3.8.1. In order to determine the frequency of antigen-specific CD4 T cells after expansion & optimal time for measuring cytokines by intracellular cytokine staining, MTb PPD expanded T cells were co-cultured with autologous monocyte derived macrophages, and re-stimulated with MTb and *M. avium* PPD. Then cytokine production by CD4 T cells was analyzed by flow-cytometer after six hour and overnight incubation.

Increased frequency of cytokine producing CD4 T cells was observed after expansion (Figure 9) compared to unexpanded T cells (Figure 8). In addition the cytokine production by CD4 T cells as measured by intracellular cytokine staining (ICS) for IFN-γ seemed to be higher when the expanded T cells were re-stimulated overnight compared to six hour re-stimulation. The

frequencies of responding CD4 T cells were 0.156 vs. 0.82 for MTb PPD and 0.08 vs. 0.71 for *M. avium* PPD, following six hour and overnight stimulation, respectively (Figure 9). Similar pattern were observed when cytokines were analyzed from unexpanded T cells (Data not shown). Therefore for subsequent experiments overnight stimulation was chosen (16 hours).

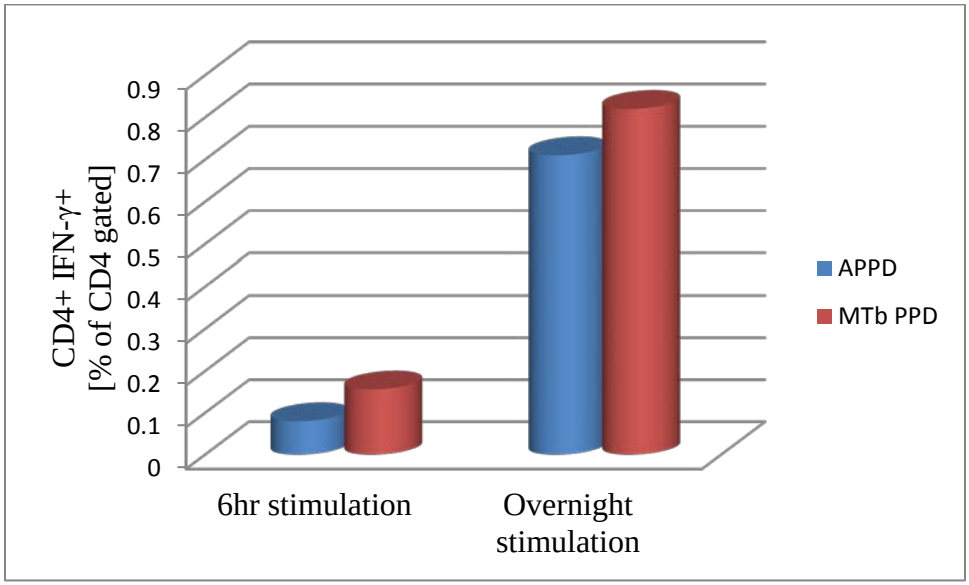


Figure 9: Frequency of IFN-γ producing CD4 T cells after 6 hour & overnight stimulation. PBMCs were expanded with MTb PPD for 6 days to increase the frequency of antigen-specific CD4 T cells. Following 6 hour and overnight re-stimulation with MTb & M avium PPD the frequency of antigen-specific CD4 T cells determined by staining for IFN-γ effector cytokine production in response to mycobacterial antigen by ICS as described in the materials and methods section. The data shown as mean frequency of IFNγ producing CD4 T cells from two healthy BCG vaccinated donors. APPD: *M. avium* PPD. MTb PPD: *M. tuberculosis* PPD. Background was subtracted from analysis.

4.2.1.2 Kinetics of effector cytokine production analyzed from expanded mycobacteria-specific CD4 T cells

Even though increased frequency cytokine producing CD4 T cells was observed after overnight stimulation (Figure 9), integrated single time point measurement of cytokine production by intracellular cytokine staining might obscure the dynamic evolution and persistence of individual cytokine production as well as life time of co-expression of multiple cytokines at a single cell

level. To explore the dynamics of cytokine production following stimulation of PBMCs with live *M. avium* bacteria at different doses, we examined cytokine production in two different conditions. In the first part of the experiment (1^{st}condition) the cytokine secretion was inhibited by protein transport inhibitor cocktail two hours after co-culturing antigen expanded T cells with infected MDM; in the second part of experiment (2^{nd}condition) the cytokine secretion was inhibited in the last four hour of the total incubation time. For both conditions the total incubation time was 20 hour. Finally, the cytokine production was determined by intracellular cytokine staining.

Although the percentage of cytokine producing cells rise as the dose of bacteria increased, the frequency of cytokine producing CD4 T cells decline over time as shown by decreased percentage single as well as double cytokine producing cells when the protein secretion was inhibited in the last four hour compared to after two hour inhibition(Figure 10 A, B, & C). While the decline was more pronounced for single TNF-α producing CD4 T cells (Figure 10 B), IFN-γ producing cells despite the decline persist at relatively a higher level over the entire incubation time (Figure 10 A). Concomitant expression IFN-γ and TNF-α was also relatively short lived (Figure 10 C).

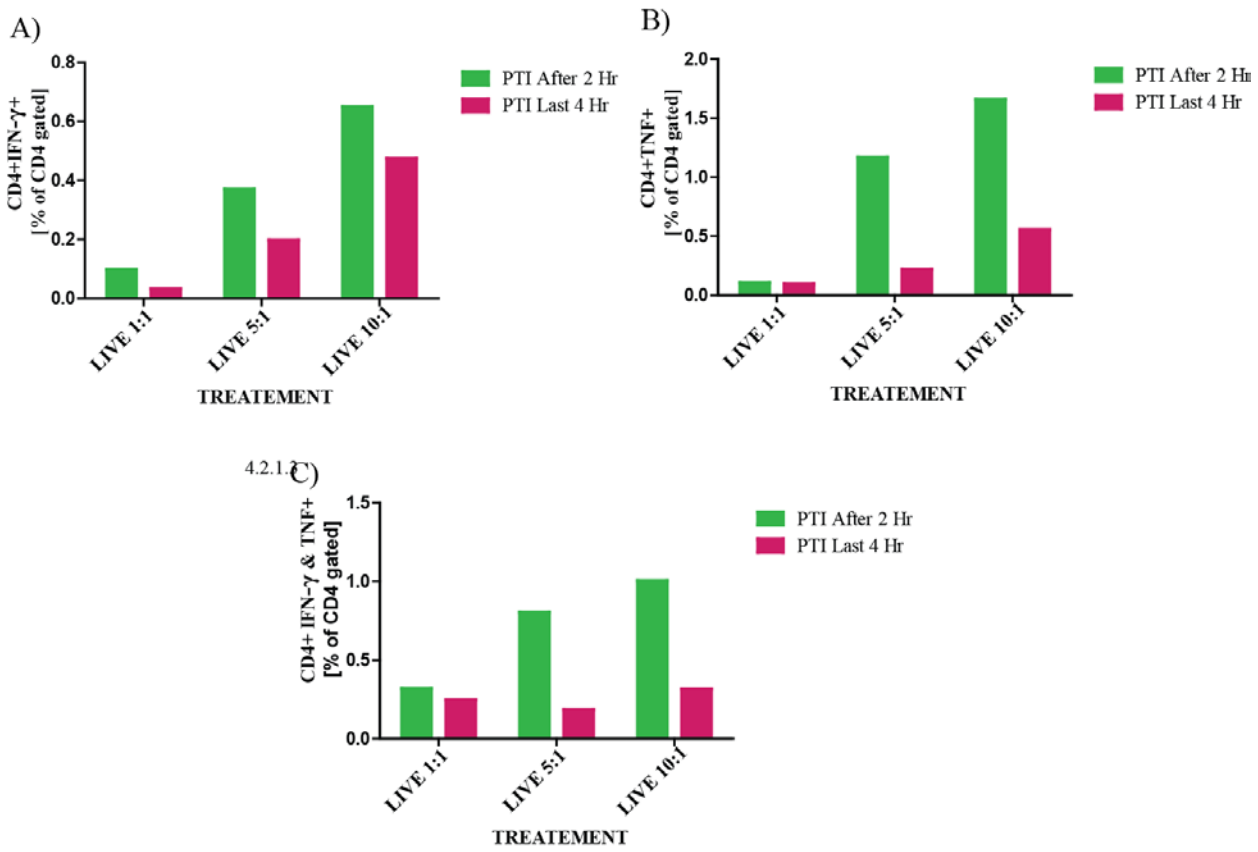


Figure 10: Kinetics of cytokine production by antigen expanded CD4 T cells. PBMCs were stimulated for 6 days with heat-killed *M. avium* bacteria to expand the antigen specific T cells and re-stimulated overnight with increasing doses of live *M. avium* bacteria. Protein secretion inhibited in first two hours and last four hour. Cytokine production by CD4 T cells was analyzed by ICS. The data is shown as frequency cytokine producing cells from a single donor. Background was subtracted from all conditions. Live 1:1, 5:1, 10:1 represents the ratio of live *M. avium* bacteria to MDM in a 96 well plate. PTI: protein transport inhibitor.

4.2.1.3 Cytokine production by antigen-expanded CD4 T cells was dependent on antigen dose.

Besides characterization of the kinetics of cytokine production by mycobacteria-specific CD4 T cells, antigen expanded T cells was also used to study donor to donor variation, antigen dose dependent response and to determine optimal dose of live *M. avium* needed for growth inhibition assay. To this end samples from two healthy BCG vaccinated donors were obtained and expanded with heat killed *M. avium* bacteria as described in section 3.5. To determine characteristic pattern of cytokine production by antigen expanded mycobacteria-specific CD4 T as well as to determine the optimal dose of bacteria for growth inhibition assay, heat-killed *M. avium* expanded T cells were re-stimulated overnight with increasing doses of live *M. avium* bacteria. *In vitro* generated autologous MDM were used as antigen presenting cells. Following overnight stimulation cytokine production was analyzed by intracellular cytokine staining.

The frequency of responding CD4 T cells after expansion as measured by IFNγ was higher (up to 11% Figure 11 A) compared to unexpanded CD4 T cell, which was less than 1% (data not shown) for the first donor (Figure 11 A, B, & C). Similarly, the frequencies of single TNF-α as well as double cytokine producing CD4 T cells after expansion were higher compared to unexpanded CD4 T cell response for the first donor. For the second donor (Figure 11 D, E, and F) only expanded T cells were analyzed. The frequency of CD4 T cells producing single cytokine (IFN-γ or TNF-α) as well as double positives (simultaneously producing IFN-γ and TNF-α) was increased proportionally as the dose of bacteria increased from low to high (Figure 11 A-F). The highest frequency of responding CD4 T cells was observed at multiplicity of infection 10X both for live and heat-killed *M. avium* stimulated PBMCs. However, the response to heat-killed bacteria was less than the response for live stimulated ones (first donor). The frequency of responding CD4 T cells was also different in the two donors. The first donor (Figure 11 A, B, & C) has higher frequency of responding CD4 T cells compared to the second one (Figure 11 D, E, and F).

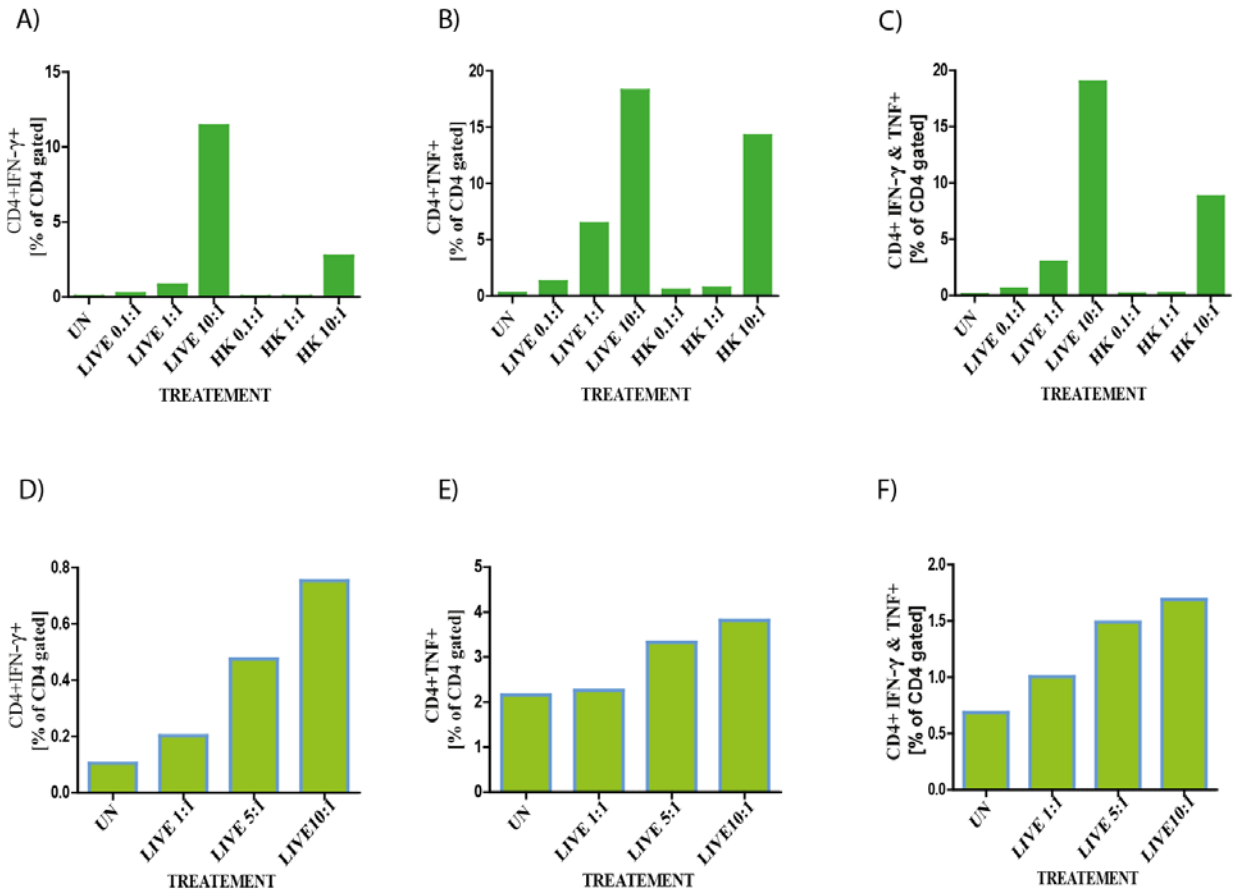


Figure 11: Cytokine production by heat-killed *M. avium* expanded CD4 T cells. Cytokine (IFN-γ, TNF-α or both cytokines) production by CD4 T cells analyzed by ICS after overnight re-stimulation of heat-killed *M. avium* expanded T cells with increasing doses of live *M. avium* bacteria. Autologous MDM were used as antigen presenting cells. The data is shown as frequency of cytokine producing CD4 T cells from two healthy donors. The data in the upper row (A, B, & C) is from donor one and the lower row (D, E, & F) from second donor. UN: unstimulated. HK: heat killed. Live/HK 0.1:1, 1:1, 5:1, 10:1 represents the ratio of live/HK *M. avium* bacteria to MDM in a 96 well plate.

4.3 Effect of antigen expanded T cells on mycobacterial growth in infected MDM

Macrophages are component of the innate immune system and they are capable of ingesting and destroying most invading microorganisms without help from cells of adaptive immune system. However, there are certain pathogens that pose a challenge on antimicrobial defense mechanism mounted by macrophages and survive in this cell. In these instances macrophages needs help from other cells including cells of the innate immune system such as natural killer cells, as well as antigen specific T cells to destroy the ingested pathogens. Mycobacterial infections caused by *M. tuberculosis* or *M. avium* are examples of such pathogens that resist destruction subsequent to ingestion by macrophages[10]. Even though different cell subsets are known to provide help to infected macrophages through the production of soluble molecules or membrane associated proteins, the role of T helper type I CD4 T cells [25, 26, 28] is paramount importance in activation macrophages. Activation of infected macrophages by antigen specific Th1 CD4 T cells promotes fusion of phagosome with lysosome; induce the production of nitric oxide and superoxide radicals that enables the macrophages to destroy pathogens.

4.3.1 Enhanced killing of *M. avium* in infected MDM co-cultured with T cells

In order to determine the ability of mycobacteria-specific memory CD4 T cells to inhibit the growth of live *M. avium* bacteria by activating infected monocyte derived macrophages, T cells were co-cultured with infected MDM for 72 hours in a 96 well plate. As mycobacteria specific T cells were found at lower frequency in the donors we tested, these cells were first expanded with heat-killed *M. avium* bacteria. From the previous experiment (section 4.2.1.3) for determining cytokine production from heat-killed *M. avium* expanded CD4 T cells we found higher frequency of cytokine producing CD4 T cells when MDM were infected with live *M. avium* at MOI 5X and MOI 10X, suggesting most of the MDM were infected and served as antigen presenting cells. Therefore, for determining the growth inhibition capacity of expanded T cells this multiplicities of infection were used. Infected MDM were cultured with and without heat-killed *M. avium* expanded T cells for both multiplicities of infection. Uninfected MDM with expanded T cells were used as a negative control.

Enhanced killing of live *M. avium* bacteria was observed when infected MDM were co-cultured with expanded T cells for both multiplicities of infection as shown by decreased intensity of light (RLU) in infected MDM co-cultured with expanded T cells in comparison with infected MDM not co-cultured with T cells (Figure 12). In co-cultured wells on average there was 1.5 times less bacterial load compared to infected MDM that was not co-cultured with expanded T cells. On the other hand there were less background reading (RLU) in unstimulated control indicting that the test was specific and the instrument reading were solely due to the presence of live bacteria.

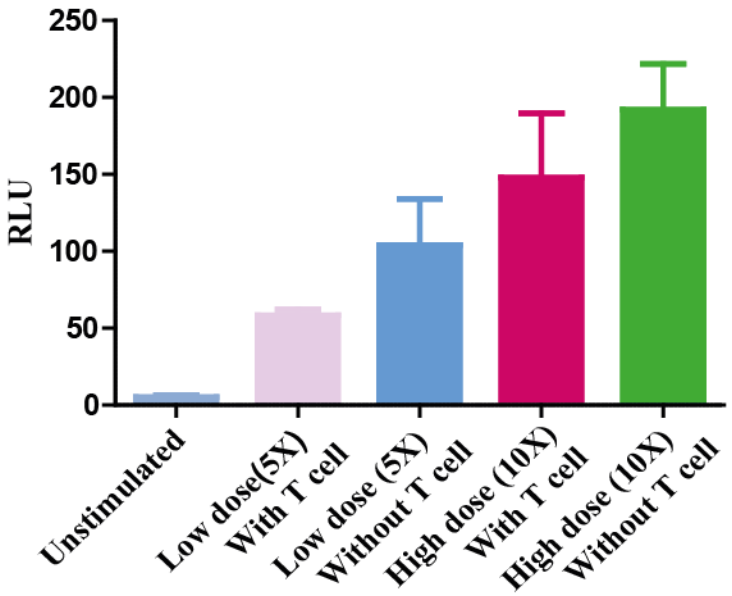


Figure 12: Growth inhibition by heat-killed *M. avium* expanded T cells. Infected MDM were cultured in a 96 well plate with & without expanded T cells for 72 hours. Inhibition of growth of bacteria in infected MDM was determined with luciferase assay as described in section 3.7. The amount of emitted light (RLU), which is directly proportional to the number of bacteria, was measured by luminometer. The data shown as mean RLU and standard deviation from four replicate wells for each condition from single donor.

4.4 Mycobacteria specific CD4 T cell function in HIV patients in comparison with healthy control

Having established the method for analyzing mycobacteria specific CD4 T cell response using PBMCs from healthy donors, we used this method to compare memory CD4 T cell response between BCG vaccinated HIV-1 infected patients and healthy controls. Whole blood sample were obtained from two healthy donors and two HIV infected patients. Following isolation of PBMCs, they were stimulated with MTb and *M. avium* derived purified protein (PPD) and analyzed by flow-cytometer after overnight incubation as described in section 3.9.3.

4.4.1 CD4 T cells are depleted in Blood of HIV-1 infected patients

Infection with HIV-1 is characterized by progressive depletion of CD4 T cells; however, following initiation antiretroviral treatment HIV plasma RNA level will be reduced and the CD4 T cell start to increase to normal level [54]. Before comparing mycobacteria specific CD4 T cell response in HIV-1 infected patients with healthy controls, to determine whether the two groups have comparable CD4 T cells we evaluated the proportion of CD3 T cells expressing CD4 molecule. Even though the two HIV patients have been on a combination of antiretroviral treatment for long period of time (mean 11 years) and had very low viral load, they have lower proportion of CD3 T cells expressing CD4 molecule. The mean proportion of CD3 positive T cells expressing CD4 was 61% and 30% in healthy controls and HIV infected patients respectively (Figure 13).

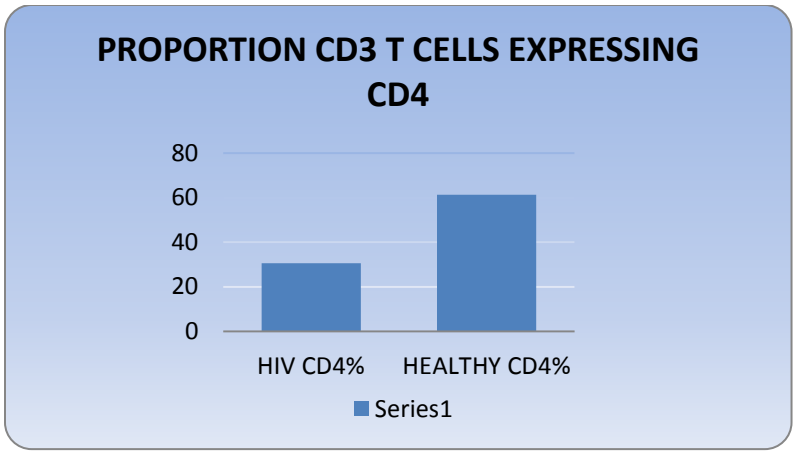


Figure 13: Proportion of CD4 T cells in HIV patients and healthy controls. The graph shows the mean percentage of CD3 T cells expressing CD4 for HIV patients and healthy controls.

4.4.2 HIV patients have lower overall frequency of mycobacteria specific CD4 T cells

Since HIV infected patients in our study had lower proportion of CD3 T cells expressing CD4 despite being on antiretroviral treatment for long period of time in comparison with healthy controls, for determining whether this was also reflected on mycobacteria specific CD4 T cell response, we compared the overall absolute frequency of cytokine producing CD4 T cells in HIV patients with healthy control. The absolute frequency of each of the seven functional state for the three cytokines analyzed (IFN-γ, IL-2, TNF-α) was summed up to obtain the overall frequency of responding CD4 T cells. Similar to the proportion of CD4 T cells, the magnitude of overall mycobacteria specific CD4 T cells response (cytokine production) to mycobacterial antigens was lower in HIV patients in comparison with healthy control (Figure 14).

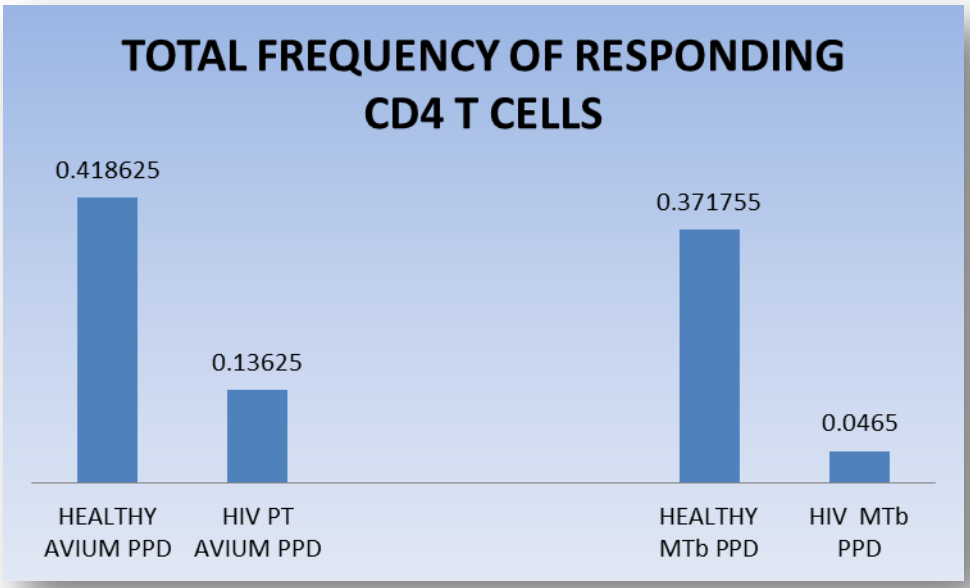


Figure 14: Mycobacteria specific memory CD4 T cell response in HIV patients and healthy control. The data represents the mean percentage of overall responding CD4 T cells to MTb and *M. avium* derived purified proteins (PPD).

4.4.3 HIV patients have lower frequency of mycobacteria specific polyfunctional CD4 T cells

In order to test the hypothesis that HIV increases the risk of tuberculosis by depletion of mycobacteria specific polyfunctional CD4 T cells that simultaneously produce IFN-γ, IL-2, and TNF-α, and to infer indirectly the role of polyfunctional CD4 T cells in protection against tuberculosis, we compared the polyfunctional CD4 T cell response to mycobacterial antigens in HIV patients and healthy control. HIV patients have lower frequency of mycobacteria specific polyfunctional CD4 T cells, as shown by decreased absolute frequency of CD4 T cells simultaneously producing IFN-γ, IL-2, and TNF-α (Figure 15 A & B), following stimulation with *M. tuberculosis* and *M. avium* derived purified proteins(PPD) compared to healthy controls.

However, since the HIV patients in our study have lower number of CD4 T cell than healthy controls; direct comparison of the difference in absolute frequency might not reflect the true

difference in the two groups, in other words the two groups are not directly comparable. Therefore, instead of direct comparison the data were normalized and the contribution of polyfunctional CD4 T cells that simultaneously produce all the three cytokines (IFN-γ, IL-2, and TNF-α) tested to the overall frequency of cytokine producing CD4 T cells were determined. Accordingly, HIV patients have lower proportion of responding polyfunctional CD4 T cells compared to healthy controls, for both antigens used (MTb PPD mean 13% vs.7%; *M. avium* PPD, 13% vs. 5% Figure 15 C, D, & E).

The relative proportion and composition of monofunctional (any one cytokine), bifunctional (any two cytokine) and polyfunctional (all three cytokines) were also analyzed. For both groups monofunctional response represented a larger proportion of the overall responding CD4 T cells, while the monofunctional response to MTb PPD in HIV patients is dominated by TNF-α and IFN-γ single cytokine producing cells, the response in healthy controls was predominantly TNF-α and IL-2. Single IL-2 producing CD4 T cells was absent in HIV patients following stimulation with MTb PPD. The bifunctional response to MTb PPD in HIV patients was exclusively due to CD4 T cells simultaneously producing TNF-α and IL-2 and comprised higher proportion of overall response compared to the proportion of bifunctional response in healthy controls. Even though the bifunctional response in healthy controls is also comprised of mainly CD4 T cells that simultaneously produce TNF-α and IL-2, unlike the HIV patients they do also have small proportion of CD4 T cells that simultaneously producing IFN-γ & IL-2, and IFN-γ & TNF-α. Although the polyfunctional CD4 T cells response doesn't represent highest proportion of the overall response for both groups, HIV patients have lower proportion of polyfunctional CD4 T cells compared to healthy controls following stimulation with MTb PPD. Similar pattern was observed for *M. avium* PPD stimulated CD4 T cells for both groups.

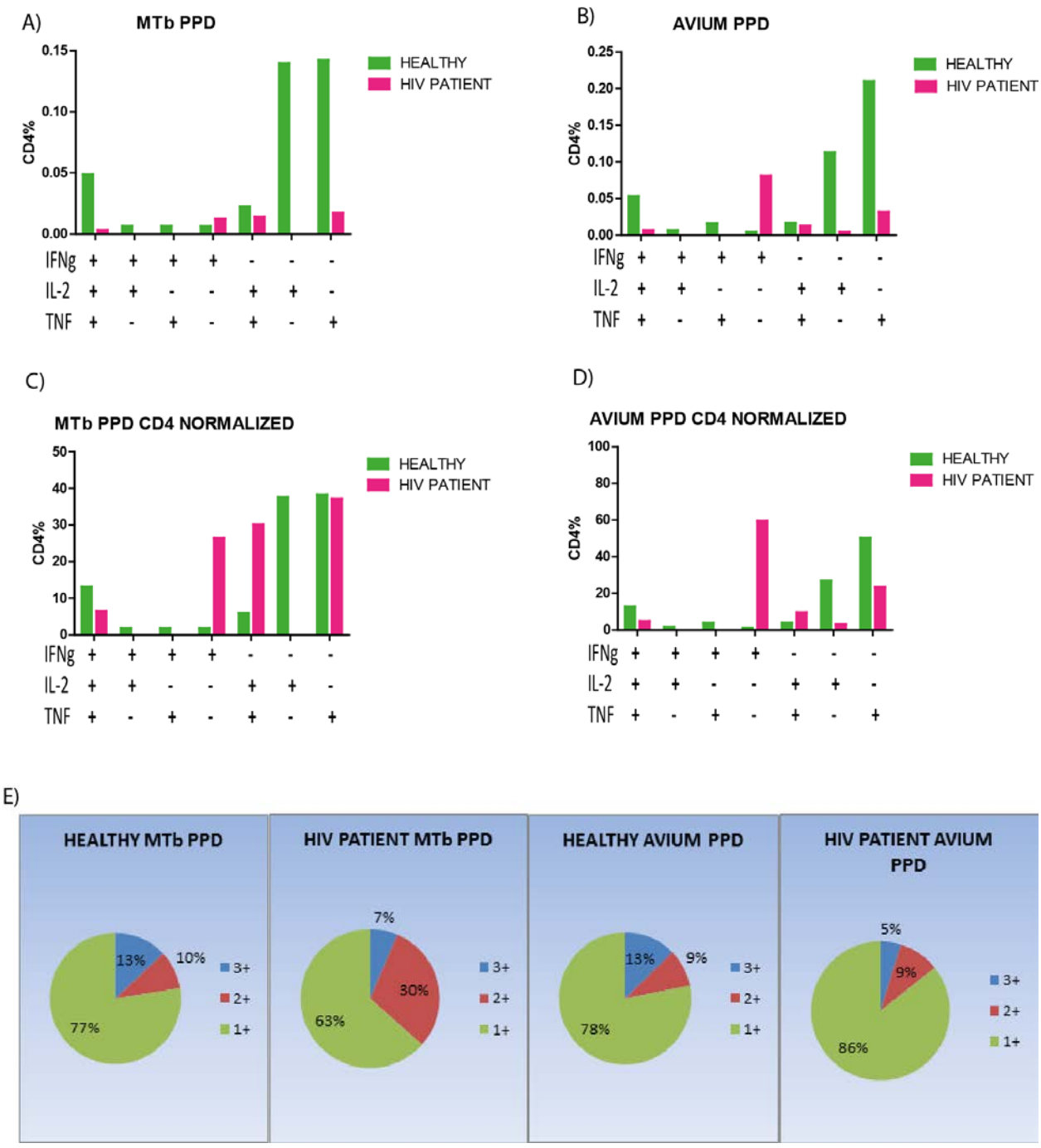


Figure 15: Functional subset of antigen specific memory CD4 T cell response in HIV patients and healthy controls. A & B) The mean frequency of the different functional subset of responding CD4 T cells following stimulation with MTb & *M. avium* derived purified proteins (PPD). C & D) represents the relative frequency of responding CD4 T cells shown in A, and B as absolute frequency. E) The pie chart shows the summary of triple (3+, blue), double (2+, red) and single (1+, green) cytokine producing CD4 T cells in HIV patients and healthy controls.

5 DISCUSSION

The overall aim of the project was to establish a method for cell isolation and biobanking of PBMCs as well as an *in vitro* assay to characterize mycobacteria specific CD4 T cell function in HIV patients and healthy controls. For establishing the method we used whole blood and buffy coat samples from healthy BCG vaccinated donors. Hence in the first part of the project we used a protocol (see appendix I) for isolation and cryopreservation PBMCs and determined the effect of short term cryopreservation on viability and function of CD4 T cells.

The use of cryopreserved PBMCs to characterize the phenotype and function of CD4 T cells is essential to avoid the inter assay variability since it allows batch analysis of samples from different donors collected at different times. However, the *in vitro* assay has to be adapted and should be validated for the use of cryopreserved PBMCs and the quality of cryopreserved cells has to be monitored to ensure reliable and accurate results. Previous studies show that function of cryopreserved PBMCs was associated with viability of PBMCs and it has been found that the viability of PBMCs greater than or equal to 70% was correlated with consistent proliferative response and suitable for functional analysis [55]. The viability of PBMCs in our study was consistently greater than 90% immediately following isolation of PBMCs as well as after short term cryopreservation and thawing. Therefore, it is possible do batch analysis of samples after cryopreservation of PBMCs at -80^0 C freezer for at least two weeks. However, cryopreservation of PBMCs for more than two weeks at -80^0 C, (for example one month) has to be validated and long term storage of samples should be done in liquid nitrogen.

In addition to the viability we have also analyzed the effect of short-term cryopreservation on the function of CD4 T cells. As we were mainly interested on characterizing cytokine production by CD4 T cells in HIV patients and healthy controls, we evaluated the effect of cryopreservation on cytokine production by CD4 T cells. Measurement of IFN-γ production by antigen-specific T cells is usually used as a surrogate marker of infections that require cellular immune response for protection or induction of immunity following vaccination. Therefore, to determine the effect of short-term cryopreservation on IFN-γ production by CD4 T cells we compared the frequency of IFN-γ producing CD4 T cells before and after one week cryopreservation in -80^0C freezer and we found comparable frequency of responding CD4. Hence this method can be used for isolation

and cryopreservation of PBMCs for analysis of cytokine production by CD4 T cells after short term cryopreservation in -80^{0}C freezer.

Next we established an *in vitro* assay to characterize mycobacteria specific memory CD4 T cell function. Mycobacteria specific memory T cells could be generated following infection with mycobacteria species such as *M. tuberculosis* or *M. avium*, or could be induced by vaccination. Even though, the donors in our study do not have history of tuberculosis, all of them were vaccinated with bacille Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, which is the only approved vaccine against TB until now and reported to have variable level of protection against tuberculosis in different populations [56]. This vaccine has been administered in Norway until 2009 to all secondary school children at about the age of twelve [57]. Therefore, the mycobacteria specific memory T cell response is most likely due to this vaccine, although it could also be attributed to environmental mycobacteria exposure.

Subsequent to recognition of mycobacterial antigens in the context of major histocompatibility complex class II (MHC II), mycobacteria specific memory CD4 T cells will be activated and produce cytokines and proliferate[24, 53]. Since we found very low frequency of responding CD4 T cells after short term stimulation with mycobacterial antigens in the donors we tested, to increase frequencies of mycobacteria-specific T cells, antigen specific T cells were expanded for one week with heat-killed *M. avium* bacteria or MTb and/or *M. avium* PPD. The expanded T cells were then used in different assays such as for cytokine analysis by ICS and for determining the growth inhibition capacity of expanded T cells in infected MDM. One week expanded mycobacteria specific T cells showed frequencies of cytokine producing cells up to 11 % (CD4+ IFN-γ+) following overnight re-stimulation (Figure 11 A).

Initially to determine the optimal time for measuring cytokines we compared the frequency of responding CD4 T cells (IFN-γ production) after six hour and overnight re-stimulation with different mycobacterial antigens including MTb PPD, *M. avium* PPD and live *M. avium* bacteria. We found higher frequencies of responding CD4 T cells after overnight incubation, which is in line with a recent study [58] that showed initial release of cytokines by activated T cells varied temporally and concentrates at approximately 2-6 hour and 12-16 hour following stimulation.

However, integrated measurement of cytokines by ICS does not reveal information regarding the evolution and persistence of cytokine production. Even though we got higher frequency of cytokine producing CD4 T cells after overnight stimulation compared to six hour stimulation, it was not clear whether the cytokine expression was persistent or short lived. To determine the life time of single as well as double cytokine expression, cytokine secretion was inhibited after two hour and in last four hour of total incubation time (20hr). The frequency of single IFN-γ and TNF-α as well as double positives (simultaneously producing IFN-γ and TNF-α) diminished on average by two and three fold, respectively, after 16 hour of stimulation. Although we didn't measure cytokine at several time points previous study[59] showed that single IFN-γ and TNF-α production by CD4 T cells reached peak at 16 and 8 hours of incubation, respectively, and then started to decline. The life time of simultaneous multiple cytokines production by CD4 T cells was shown to be transit and it was demonstrated that multifunctional response occurred mainly as a result of sequential rather than simultaneous release of cytokines[58]. Il-2 also has similar pattern of cytokine section with IFN-γ [58]. Even though there was no single optimal time for measuring multiple cytokines, we decided to use overnight stimulation (16 hours) for determining intracellular cytokine production by flow-cytometry, since most antigen specific CD4 T cells could be detected with in this window.

In addition to kinetics of cytokine production by antigen expanded CD4 T cells, we also evaluated antigen dose dependent response and donor to donor variation following overnight re-simulation of expanded T cells. The frequency of effector cytokine producing CD4 T cells in response to overnight re-stimulation with different doses of live or heat-killed mycobacterial antigens presented from MDM were higher compared to the short term stimulation (unexpanded T cells) and the frequency of responding CD4 T cells increased in proportion to the dose of bacteria. The highest frequencies of responding CD4 T cells observed in MDM infected with live *M. avium* at MOI 10X, that suggests most of MDM were infected and served as antigen presenting cells at this MOI. There was also considerable donor to donor variation in cytokine production.

Apart from cytokine production we also evaluated the ability of mycobacteria specific expanded effector CD4 T cells to inhibit the growth of live *M. avium* bacteria in infected MDM. From previous study[53] on growth inhibition ability of expanded T cells as well as from our study on

cytokine analysis from antigen expanded T cells following stimulation with different doses live *M. avium* bacteria (section 4.2.1.3), we found that MOI 5x and MOI 10x were suitable for this assay. Infected MDM at MOI (5 & 10x) were cultured for 72 hours with and without expanded T cells and then the growth inhibition of bacteria was determined with a luminometer. Infected MDM co-cultured with expanded effector T cells had enhanced ability to inhibit the growth of live *M. avium* bacteria compared with infected MDM without T cell co-culture as shown by decreased intensity of light in co-cultured wells. Even though this experiment was done on samples from healthy controls, the assay has paramount importance in studying co-infection with mycobacteria and HIV.

More than 10 million individuals are estimated to be co-infected with MTb and HIV worldwide and are at higher risk of developing active tuberculosis compared with people without HIV [43]. As both mycobacteria and HIV infects macrophages studying the interaction between HIV and mycobacteria in the macrophages may lead to better understanding of the increased susceptibility of co-infected individuals.

A recent *in vitro* study on the effect of HIV-1 on the growth of *M. tuberculosis* in primary human alveolar macrophages and monocytes obtained from HIV-1 infected individuals and healthy controls found out no difference in the bacterial load in the two groups [60]. In contrast, another study using mycobacteria with different propensities of causing disease including *M. tuberculosis*, *M. avium* and *M paratuberculosis* demonstrated that coinfection with HIV was associated with increased bacterial burden in MDM compared to singly infected MDM, with the highest mycobacterial load being observed in MTb/HIV coinfected MDM, followed by *M. avium* and *M paratuberculosis* [61].

The difference in these two studies could be due to the use of different time point for determining the bacterial load, while in the first study the bacterial load was measured after 96 hours, in the second study it was measured after 7 days of coinfection. However, both studies do not precisely reflect what is happening *in vivo*, as the co-infection does not occur in isolated macrophages, instead there would be dynamic interaction between the infected macrophages and other cells of the innate immune system as well as cells of adaptive immune system.

On the other hand, studying the impact of HIV on mycobacteria in co-infected macrophages co-cultured with and without T cells might mirror what is happening *in vivo* and could lead to better understanding of mycobacterial pathogenesis in co-infected individuals. Hence, this assay could be an important tool for *in vitro* study HIV/mycobacteria co-infection; even though we didn't use it yet in our study due to small sample volume obtained from the two HIV patients in our study.

Tuberculosis, a disease caused by an intracellular pathogen is relatively well controlled by cell mediated immunity in healthy individuals, but occurs at higher frequency in HIV infected patients. In order to determine the impact of HIV infection on mycobacteria specific polyfunctional CD4 T cells we compared mycobacteria specific memory CD4 T cell response in HIV patients and healthy individuals.

Initially to determine whether the HIV patients in our study have comparable CD4 T cells with the healthy controls, we compared the proportion of CD3 T cells expressing CD4 molecule in the two groups and we found lower proportion of CD4 T cells in HIV patients in comparison with healthy control. This was in spite of the fact that the two HIV patients have been on a combination of antiretroviral therapy for long periods of time. Even though HAART does not result in complete clearance of the virus from the body, it is associated with considerable immune reconstitution. However, not all individuals achieve complete recovery of the immune system [54], small proportion of individuals receiving HAART shows impaired recovery of CD4 T cells despite suppression of viral replication. Several factors are associated with impaired CD4 T cell reconstitution including lower baseline CD4 count, higher baseline plasma viral load, and older age [62, 63].

In order to determine whether this was also reflected in the overall frequency of mycobacteria specific CD4 T cell response we compared the frequency of cytokine producing CD4 T cells in both groups. In the two patients we found lower frequency of responding CD4 T cells in HIV-1 infected patients. This finding is consistent with previous studies which demonstrated that mycobacteria specific CD4 T cells were depleted in the blood [64] as well as in the lung [60] of HIV-1 infected patients as measured by IFN-γ production. In the former study [64] mycobacteria specific CD4 T cells in latently infected individuals were depleted rapidly following infection with HIV (with in 1 year of seroconversion) and irrespective of the total CD4 count, compared to latently infected HIV uninfected individuals. Interestingly, mycobacteria

specific CD4 T cells from latently infected HIV uninfected individuals expressed high levels of CCR5 which is a coreceptor for HIV virus and together with the CD4 molecule allows entry of virus into CD4 T cells. This could explain early infection and depletion of mycobacteria specific CD4 T cells. However, mycobacteria specific CD4 T cells were detectable in HIV patients who developed active tuberculosis [64], suggesting mycobacteria specific CD4 T cells were not completely deleted as a result of HIV infection, but their function seems to be impaired even after long term antiretroviral therapy in HIV-1 infected patients as measured by IFN-γ production [65].

Multiple cytokine producing cells instead of monofunctional T cells has recently been associated with protection against intracellular pathogens that requires T cell mediated immunity[32]. To determine how the underlying HIV infection increase the susceptibility of infected individuals, we assessed the impact of HIV infection on functional profile of mycobacteria specific CD4 T cells and found a lower proportion of mycobacteria specific polyfunctional CD4 T cells in HIV infected patients compared with healthy individuals. Since CD4 T cells play a central role for protection against mycobacterial infections it is obvious that the depletion of these cells in HIV infected individuals could lead to increased susceptibility to several opportunistic infections including tuberculosis. However, unlike other opportunistic infections tuberculosis can occur early in the course of HIV infection before significant loss of CD4 T cells [46] as well as in patients taking antiretroviral therapy [45], suggesting functional impairment of CD4 T cells. Our preliminary finding supports the hypothesis that polyfunctional CD4 T cells are selectively depleted by the virus. This may account for the increased risk of tuberculosis in HIV-1 infected patients, among other factors including impaired apoptosis mediated killing of *M. tuberculosis* by alveolar macrophages in HIV infected patients [66]. Although we didn't analyze mycobacteria specific polyfunctional CD4 T cells at the site of disease manifestation, available evidence indicates that this cells are also depleted in the lung of HIV infected patients with no evidence of tuberculosis[60].

Even though the protective immune response against tuberculosis is not completely understood, recent studies shows that indeed polyfunctional CD4 T cells could represent correlates of protection [40, 41] despite studies opposing this finding are also available [38, 39]. Our finding of lower proportion of polyfunctional CD4 T cells in HIV patients, who are at high risk of

developing tuberculosis compared to healthy individuals, suggests polyfunctional CD4 T cells might be important for the protection of tuberculosis. In addition, the reappearance of mycobacteria specific polyfunctional CD4 T cells in HIV patients after onset of antiretroviral therapy who are relatively less susceptible to tuberculosis compared to untreated patients, further consolidates the importance of polyfunctional CD4 T cells in protection against tuberculosis [67].

5.1 Limitation of the study

In this study we compared mycobacteria specific CD4 T cell function in HIV patients and healthy controls. By comparing mycobacteria specific CD4 T cell function in the two groups we got some clues on how the underlying HIV infection might increase the susceptibility of HIV infected patients to tuberculosis, although our study has some limitation.

The first is in fact low sample size of our study participants, which happened mainly due to late ethical approval, that prevents us from doing rigorous analysis of mycobacteria specific immune response at different stages of HIV infection and with sufficient sample size that could have given us better insight on how the mycobacteria specific T cell response is affected by HIV at different stages of infection as well as before and after onset of antiretroviral therapy. The second limitation in this study was the use of unmatched healthy control. Initially our intension was to recruit matched healthy control whenever we get blood sample from HIV infected patients, however, for the same reason of late ethical approval we couldn't be able to get matched volunteer healthy control at the time we got blood sample from HIV patients. The HIV patients are relatively older than the healthy controls participated in our study.

5.2 Conclusion

The role of polyfunctional CD4 T cells in protection against human tuberculosis is not resolved yet, even though this information is required to develop an optimal vaccine against this deadly disease. Recent studies on protective cell mediated immune response in humans have reached on different conclusion regarding the importance of polyfunctional CD4 T cells in human pulmonary tuberculosis. While some studies associated this subset of cells with protection, others have concluded that they have no role in protection.

To address this problem we studied mycobacteria specific polyfunctional CD4 T cell response in HIV infected patients in comparison with healthy control. As HIV patients are more vulnerable to tuberculosis even with relatively normal CD4 T cell count as well as after onset of antiretroviral therapy compared to healthy individuals, we hypothesize that the underlying HIV infection might impair the quality of mycobacteria specific CD4 T cell response in addition to its effect on the magnitude of the response.

Before we characterize mycobacteria specific immune response in HIV patients and healthy controls, we established method for biobanking of PBMCs as well as an *in vitro* assay for analysis of mycobacteria-specific T cell function using samples from healthy BCG vaccinated individuals. Due to the nature of the project, in which the HIV patients visit the clinic less frequently (every three month), we planned to analyze patient sample in batch every two weeks. For this purpose we used slightly modified cryopreservation protocol and assessed the impact of short term cryopreservation on the viability and functional response of CD4 T cells. We found comparable result before and after freezing of PBMCs for one and two weeks in -80^{o}C freezer. Beside Biobanking we also established an *in vitro* assay for evaluating the ability of mycobacterial antigen expanded T cells to inhibit the growth of slightly modified *M. avium* bacteria in infected monocyte derived macrophages and we found enhanced killing of this bacterium in infected MDM cocultured with antigen expanded T cells compared with infected MDM alone. This assay is particularly important for elucidating the pathogenesis of mycobacteria/HIV coinfection, even though we didn't use it here due to small sample volume obtained from patients. We also established an *in vitro* assay for analysis of mycobacteria specific polyfunctional CD4 T cell function after overnight stimulation with MTb and *M. avium*

derived purified proteins. The method was optimized to give low background so as to detect antigen specific T cells that occur at lower frequency in the peripheral blood.

Subsequent to establishing the method we used the assay to compare mycobacteria specific T cell function in HIV patients and healthy controls. Despite being on antiretroviral therapy for long period of time, the two HIV patients in our study had lower overall frequency of mycobacteria specific CD4 T cell response. Moreover, the polyfunctional response is severely impaired in these patients. Therefore, the increased susceptibility of HIV patients to tuberculosis might be attributed to both decreased magnitude as well as quality of mycobacteria specific CD4 T function.

The selective depletion of polyfunctional CD4 T cells in HIV patients who are at high risk of developing tuberculosis, suggests that these cells might be particularly important in protection against tuberculosis. However, due to small number of participants in our study it was not possible to do thorough analysis of how the mycobacteria specific CD4 T cell function is affected by HIV virus at different stages of infection. Hence further studies involving patients at different stages of HIV infection as well as before and after onset of antiretroviral therapy and also TB/HIV coinfected patients, might lead to better understanding of how the underlying HIV infection affects mycobacteria specific CD4 T cells and it would give us indirect evidence of the role of polyfunctional CD4 T cells in protection against tuberculosis.

5.3 Future perspective

New anti-tuberculosis vaccines are urgently needed to combat the global pandemic. In line with this elucidation of biomarkers that predict protection or susceptibility to disease are shown to be more critical for the success of effective vaccine development against tuberculosis[68]. In this project we have developed tools to characterize mycobacteria specific CD4 T cell function in HIV patients, who are at higher risk of developing tuberculosis, using samples from healthy controls. Had we got sufficient number of samples from HIV patients, we would have been able to analyze mycobacteria specific CD4 T cell function at different stages of HIV infection in comparison with matched healthy controls that would have given us better insight on the mechanism of disease susceptibility and the role of polyfunctional CD4 T cells in protection against tuberculosis. However, due to the reason mentioned before, we were able to get sample from only two HIV patients.

From our preliminary study we got some indication that the underlying HIV infection might impair mycobacteria-specific CD4 T cell response. Extending the experiments to different groups as well as a sufficient number of study subjects allowing statistical analysis are required in the future course of the project. A suggestion for a possible study design could be to compare mycobacteria specific immunity in healthy controls with HIV patients before and after the onset of treatment as it has been described [44] that more susceptible HIV patients become relatively less susceptible to tuberculosis after the onset of antiretroviral therapy. The work in this thesis established the experimental basis for future studies and yielded some indication that pursuing the planned experiments seems quite promising. Results from further studies might contribute to a better understanding of the protective immune response to mycobacteria, how HIV increases the susceptibility of these patients to tuberculosis. Moreover, in future studies one could think about inclusion of memory markers in addition to the surface and functional markers used in our study, as this will enable us to dissect which memory phenotype is associated with protection or disease susceptibility. Besides the tools to study frequencies of mycobacteria specific T cells in different groups of donors, this study established assays that allow studying the effect of mycobacteria- specific T cells on survival of mycobacteria inside the infected macrophages. Also established method to expand mycobacteria specific T cells and then analyze differences in T cell-activation and effector functions when mycobacterial antigen is presented from *in vitro*-

generated macrophages. These tools are open for a wide variety of different analysis such as studies with modified macrophages (e.g. siRNA knock-down of proteins), modified mycobacteria (e.g. less and more virulent strains, engineered strains), macrophages with and without HIV and mycobacterial co-infection. Studies using these tools may lead to better understanding of mycobacterial pathogenesis in coinfected individual and enlighten mechanisms that might result in better T cell immunity.

Finally, having the methods at hand we would expect to find better insight on how the underlying HIV infection affect mycobacteria specific T cell function and makes this patients more vulnerable to tuberculosis and also the role of polyfunctional CD4 T cells in protection against tuberculosis, provided that we got sufficient number of study participants.

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7. APPENDIX I: DETAILED PROCEDURES FOR DONOR SAMPLES

The procedures described here are for analysis of samples from healthy donors. Special protocol avaliable for work with samples acquired from HIV patients, but not described here. Samples from HIV patients almost exclulsively processed in BL3 (Virus lab) at Gastro center (IKM). Basically same procedures used apart from some special prequations done when samples are taken out of the virus lab.

1. Sample collection and processing SOP

Purpose

Determine the procedure for collection, handling, isolation, and cryopreservation of peripheral blood mononuclear cells (PBMC) from whole blood.

Background

Freshly collected or cryopreserved PBMCs are used for the evaluation of vaccine or ART induced cellular immune response, HIV associate changes in immune response. These assays require PBMC that have been isolated and cryopreserved under strictly defined conditions that ensure optimal recovery, viability, and functionality **[69-72]**.

Specimen

Patient preparation

- None

Specimen type

- Na-heparin anti-coagulated whole blood

Minimum specimen volume

- 20ml

Specimen handling

- Fresh anti-coagulated blood should be stored at room temperature until it is processed.
- Total time from collection to freezing should be 8 hr. or less and total processing time from isolation to freezing should be less than 4 hrs.

Specimen rejection criteria

- Clotted specimen

  Remove the clot and process as usual. If the cell yield is less than the expected, contact the nurses for possible replacement of specimen.
- Hemolysed specimen

  Process as usual and contact the nurses for specimen replacement if the cell yield is significantly less than the expected yield.
- Unacceptable specimen

  Unlabeled or mislabeled specimen

PBMC isolation and processing procedure

- Allow the density gradient media to come to room temperature
- Dilute blood sample 1:1 with PBS in 50ml tube.
- Overlay the diluted sample with a volume of Ficoll® that is equal to the original sample volume.
- Centrifuge at 800 x g (approximately 2000RPM) for 20 minutes at room temperature (approximately 20˚C) in a swing-out rotor. If the blood is stored for more than 2 hours, increase the centrifugation time to 30 minutes.
- Harvest PBMC located at the interface of the PBS and Ficoll® layers into a fresh tube.
- Fill the tube with PBS to wash the cells.
- Centrifuge cell suspension 4-5 minutes (300-400xg approx. 1500RPM) at 4°C, discard supernatant (2X).
- Resuspend the cell pellet in (5ml) 2%FCS in PBS and perform a cell count.

Cryopreservation

Reagents

10% DMSO in FBS (heat inactivated)

- Resuspend isolated PBMC in ice-cold fetal bovine serum with 10% DMSO.
- Transfer aliquot cell suspension (0.5-1ml) to cryovial that had been pre-chilled to -20^0 C.
- Immediately after placing the cell suspension in cryovial, transfer the specimen to precooled controlled rate freezing container and place it to -80^0 C freezer.

Thawing

- Thaw frozen specimen using 37^0 C water bath with gentle agitation until completely melted and then put it on ice for 2 minute
- Dilute the thawed cell suspension slowly with RPMI 1640 medium supplemented with 10% human A+ serum and 25 mM HEPES buffer at room temperature.
- Make final volume 10 ml and spin it. Repeat 1x.
- Resuspend the pellet in the required volume of media (10%A+) and perform cell count and viability analysis.

2. Stimulation mycobacteria specific-T cells(short term)

- Prepare 4 million PBMCs/ml in 10%A+(heat inactivated) in RPMI
- Add 100µl of cell suspension /well in96 well plate (number of cells per well 0.4 million PBMC)
- The cells were stimulated in duplicate as follows:
    - Unstimulated PBMC + media alone
    - Avium PPD 10µg/ml
    - MTb PPD 10µg/ml
    - Live 5x
    - Live 10x
    - PMA/ionomycin @ 1x 2µl/ml
- Add anti-CD28 & anti-CD49d @ final concentration 1 µg/test to all wells
- Incubated for 2hr @ 37 ^{0}C 5% CO_2
- Add protein transport inhibitor cocktail @ final concentration (1x) 2µl/ml after 2 hr. incubation @ 37 ^{0}C 5% CO_2, and incubated overnight (16hr)
- After overnight (about 16hr hour) incubation transfer stimulated cells to tubes wash with pure PBS and stain for surface and intracellular cytokines, and live dead stain

Surface Staining

Antibody	Fluorochrome	Amount added
Anti-CD4	FITC	1 µg/test
Anti-CD3	eFLOR 450(pacific blue)	1 µg/test
Live/dead	APC CY7	1µl/ml

- LIVE dead add 1 µL/mL of cells resuspended at 1-10x10^6 cells per mL in azide-free and serum/protein-free PBS. All tubes were resuspended in pure PBS for the sake of live dead stain, not in flow buffer.
- Incubate in the dark for 30 minutes at 4^0C
- Add 1ml of 2% FCS in PBS Buffer and spin the cells (300-400xg=1500RPM) at 4°C for 5 minute (wash 2X).

Fixation

- Fix the cells by adding 100µl 4%PFA (keep total volume app-200µl & incubate it in the dark @ room temperature for 20 minutes.
- Without washing add 2ml of 0.5% saponin in 2%FCS, PBS & centrifuge samples @ 1500RPM @ room temperature for 5 minutes, discard supernatant & resuspend it in 100µl of 0.5% saponin.

Intracellular staining

Antibody	Fluorochrome	Amount added
Anti-IFN-γ	PE	1 µg/test
Anti-TNF-α	APC	1 µg/test
Anti-IL-2	PE CY7	1 µg/test

- Incubate in the dark at room temperature for 20 minutes.

- Add 1ml of 0.5% saponin & centrifuge samples @ 1500RPM @ room temperature for 5 minutes, discard supernatant.
- Add 2ml of 2%FCS in PBS & centrifuge samples @ 1500RPM @room temperature for 5 minutes discard supernatant & resuspend in 250µl of 2%FCS in PBS.
    - ❖ A test is defined as the amount (µg) of antibody that will stain a cell sample in a final volume of 100 µL. Cell number should be determined empirically but can range from 10^5 to 10^8cells/test.

Flow cytometric acquisition

- Draw gate on lymphocytes population based on forward sideward scatter properties. Acquire at least 100,000 events for each sample.

3. Expansion of mycobacteria specific T cells

- Prepare 4 million PBMC/ml in 10%A+ in RPMI
- Add 0.5ml of cell suspension /well in 5 wells in 24 well plate & make it 1ml by adding 10%A+ in RPMI (number of cells per well 2 million PBMC)
- Stimulate the cells were with heat-killed *M. avium* MOI 5X. Heat killing 70^0C for 30 minute in heating block.

Assumption: monocyte number 10% of total PBMC i.e. 200,000 monocyte/well. Therefore 1 million bacteria added per well except to unstimulated well.

- Incubated for 6 days @ 37 ^{0}C 5% CO_2
- Add 10 IU IL-2 @ day 2 (stock diluted 1:100 & 10 µl added to each well)
- Transfer the expanded PBMCs to tubes; wash 1x with 10%A+ in RPMI and count and use for downstream application.

4. Preparation of MDM (Autologous)

- Prepare 4 million PBMC/ml in 10%A+ in RPMI
- Add 100µl of cell suspension/well in 96 microtiter plate (number of cells per well 0.4 million)

- Incubate @ 37 ^{0}C 5% CO_2 for 1 hour
- Remove non adherent cells & wash twice with warm sterile PBS
- Add 100 µl of 30%A+ in RPMI & keep it in 37^{0}C 5%CO_2 incubator for 6 days
- Use them for downstream assays.

5. Compensation

Compensation setup was done for all colors using polymeric beads except for live dead staining. For live dead staining PBMCs were used. Small aliquot of cells treated with heat, 65°C for 1 minute then immediately place on ice for 1 minute. After this treatment, the heat-killed cells combined 1:1 with live cells and then stained with the Fixable Viability Dye.

The cells were gated on lymphocytes based on forward sideward scatter properties. 50,000 events were acquired for each sample**.**

Procedure for compensation (BD CompBeads)

- Vortex BD™ CompBeads thoroughly before use.
- Label a separate 12 x 75 mm sample tube for each fluorochrome-conjugated mouse Ig, κ antibody to be used on a given experiment.
- Add 100 µl of 2%FCS in PBS to each tube.
- Add 1 full drop (approximately 60 µl) of the BD CompBead Negative Control (FBS) and 1 drop of the BD™ CompBead Anti-mouse κ beads to each tube and vortex.
- Add 1 µl of each antibody stock to the appropriately-labeled tube. (Make sure the antibody is deposited to the bead mixture, then vortex.)
- Incubate 15 - 30 minutes at room temperature. Protect from exposure to direct light.
- Acquire samples on flow cytometer, make the necessary adjustment (e.g. PMT voltage) and save the compensation result calculated by the instrument and apply to subsequent experiments.

6. Sample processing work sheet

Sample processing worksheet					
Participant ID:			Specimen ID:		
Collection date:		Time:		Collected by:	
Processing start da		Time:		Processed by:	
Data to be collected during processing				**sample**	
Sample tube type (circle one)				NaHep / ACD / EDTA other:	
Blood condition				NORM / HEMO/ CLOTTED	
Usable whole blood volume				mL	
Counting Method (name of instrument or manual count)					
Counting re-suspension volume				mL	
Cell count average concentration (C)				x 106 cells/mL	
Total cell number (T) = C x V				x 106 cells	
Calculate cell yield/mL of whole blood(QC check)= (T/Usable Whole Blood Volume				x 106 cells/mL	
Freezing Date and Time (Place note in comments if not within 4 hours of processing start time)					

8. APPENDIX II:
ABSTRACT SUBMITTED FOR THE 7TH CONFERENCE FOR GLOBAL HEALTH AND VACCINATION RESEARCH, 26 TO 27 SEPTEMBER 2012, TRONDHEIM

Analysis of mycobacteria-specific T cell function in HIV-infected individuals and healthy controls

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Objective

Tuberculosis caused by *Mycobacterium tuberculosis* (MTb) represents a major public health problem worldwide and it is the main cause of death among people living with HIV/AIDS. Specific T cells play an important role in the control of mycobacterial infection and seem to be impaired in HIV-patients co-infected with mycobacteria including those receiving highly active antiretroviral therapy. The aim of this project is to establish and use *in vitro* assays to characterize mycobacteria-specific effector T cell functions in HIV-1 infected patients in comparison with healthy controls.

Methods

For establishing the methods we used blood samples from healthy donors and isolated peripheral blood mononuclear cells (PBMC) by density gradient centrifugation. To analyze frequencies of mycobacteria-specific T cells in peripheral blood, PBMCs were stimulated overnight with different mycobacterial antigens. After intracellular staining, effector cytokine production (IFN-γ, TNF and IL-2) from T cells was analyzed by multicolor flow-cytometry.

Results

An assay was established to analyze polyfunctional mycobacteria-specific CD4+ and CD8+ effector T cell responses from freshly isolated blood cells, which occur at very low frequencies in peripheral blood. The assay was optimized to give low background and allow sensitive and reproducible T cell analysis. Stimulation was tested with live *Mycobacterium avium* (*M. avium*), causing opportunistic infections in HIV-patients, as well as purified antigens from MTb and *M. avium*. In preliminary results we see the highest frequency of overall T cell response to stimulation with live *M. avium*; there seem to be differences in polyfunctionality of the T cell responses.

Conclusion

The techniques will be used to compare T cell responses towards MTb and *M. avium* antigens as well as live *M. avium* in healthy donors as well as in HIV patients at different stages of infection. Future applications will include analysis of T cell functions in response to antigen presented from macrophages after *in vitro* expansion of mycobacteria-specific T cells.