ANTIMYCOBACTERIAL IMMUNE RESPONSES IN HIV-INFECTED CHILDREN STARTING ANTIRETROVIRAL THERAPY IN LUSAKA, ZAMBIA

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1.0 INTRODUCTION

1.1 Background

An estimated 250,000 children died of AIDS in 2005 (WHO, 2006) and 370,000 children under the age of 15 became infected with HIV in 2007 (UNAIDS, 2008). Globally over 2.0 million children were estimated to be living with HIV in 2007 (UNAIDS, 2008). Almost 90% of these children live in sub-Sahara Africa (UNAIDS, 2008). Over 250,000 children develop TB and 100,000 children die each year from TB (WHO, 2006).

Tuberculosis is among the top 10 causes of death in children worldwide. The burden of TB is higher in the developing countries compared to the developed countries. (Swaminathan S, Rekha B, 2010). Tuberculosis is a common and often deadly infectious disease caused by *Mycobacterium tuberculosis*. It usually attacks the lungs (as pulmonary tuberculosis) but can also affect other organs (Chintu C, Mwaba P, 2005).

In HIV-infected children, the risk of developing TB is high (WHO, 2006). With the scaling up of ART in sub-Saharan Africa, and Zambia in particular, more HIV-infected children will have access to treatment (Avert, 2008). Effective therapy should result in improved immune responses to opportunistic infections such as tuberculosis (Edmonds et al, 2009). The immune response to TB is complex and the outcome is greatly influenced by the mycobacterium itself. Hence it has been difficult to determine the requirements for a protective immune response to TB, but this knowledge is essential for development of improved vaccine strategies especially for HIV-infected children (Sutherland et al, 2010).

Globally the number of children receiving ART increased from about 75,000 in 2005 to almost 200,000 in 2007 (WHO, 2008). In Zambia, 11,602 children were receiving ART by 2007 (UNAIDS, 2008).

1.2 Statement of the problem

Tuberculosis is the leading bacterial cause of death in Africa (Wilkinson et al, 2009). It is one of the most common infections in Zambian adults and children infected with HIV (Chintu et al, 1993). A study carried out by the Virology Laboratory, UTH (1995)

revealed that the prevalence of HIV in children aged 1- 4 years in an urban community was 4.1 percent while in the age group of less than one year prevalence was 18 – 22 percent.

According to a study by Chintu et al (1993), the seroprevalence of HIV-1 in 237 hospitalised children aged 1 month to 14 years with clinical diagnosis of TB was 37% while it was 10.7% among the control group (242 children). This indicates that children with HIV infection are highly susceptible to TB infection.

Bhat et al (1993) suggested that HIV seropositive children have an eight–fold higher risk of TB infection than those who are HIV seronegative. However, this was at a time when there was no ART for HIV- infected children in Zambia.

1.3 Study justification

Although there have been studies in adults suggesting that ART can prevent the development of tuberculosis in HIV-infected individuals, the mechanism is not fully understood and very little information exists in children . Studies that have focused on the quality of immune reconstitution in HIV-infected children who are on ART with respect to immunity against tuberculosis (TB) are limited.

Previous studies in Belgium have shown that upon immune reconstitution, there is an increase in the naïve T cell count in children (Rossum et al, 2001; Hainaut et al, 2000). However, there is no documentation on the change in the proportion of circulating naïve T cells in Zambian children on ART. Furthermore, no studies have documented naïve T cells, activated T cells and memory T cells in Zambian children on ART.

The importance of this research is to better understand pathogen-specific immune responses after ART, particularly to *Mycobacterium tuberculosis*. This may also help assess the effect of ART in Zambian children.

2.0 LITERATURE REVIEW

There are limited studies that have been done in Zambia on immune reconstitution after initiation of ART in children; therefore most of the literature review is based on studies from overseas and the African region.

Immune Reconstitution in Children

A Spanish study by Franco et al (2000) investigated CD4 and CD8 T cell regeneration after ART in HIV-infected children and adult patients. This study showed that the kinetics of naïve T cell reconstitution after ART is different in adults and children. Children had an early and strong increase in both naïve CD4 and CD8 T cells. In contrast, adults showed a lower increase of these cells.

Another study by Resino et al (2001) at hospitals in Madrid and Seville, Spain focused on naïve and memory CD4 T cells and T cell activation markers in HIV-1 infected children on HAART. They found that elevated percentages of CD8 T cells were associated with increased memory CD4 T cells. In contrast, naïve and activated CD4 T cells negatively correlated with CD8 T cell percentage. This may be partly due to the fact that CD8 T cells eliminate infected cells. No association between viral load and naïve CD4 T cells was observed.

A Dutch study by Rossum (2001) found that children with HIV infection have a greater capacity to reconstitute their naïve CD4 T cells when compared to HIV-infected adults treated with similar antiretroviral therapy. This study showed that both the absolute CD4 T cell count and CD4 T cell percentages increased significantly from a median of 471 cells/mm3 and 17%, to 939 cells/mm3 and 32% respectively after 48 weeks of ART. In all age groups, the increase of total CD4 T cells was caused by an increase of naïve CD4 T cells.

In Belgium a study by Hainaut et al (2000) looked at immune reconstitution in children after initiating ART. They found that CD4 T cell counts increased significantly with HAART (294 cells/ml, 459 cells/ml and 619 cells/ml at 0, 3 and 6 months, respectively).

The naïve CD4 T cell subsets increased from 113 cells/ml before treatment to 269 cells/ml at 3 months and 403 cells/ml at 6 months.

Another study by Hainaut et al, (2003) which evaluated age-related immune reconstitution during highly active antiretroviral therapy in HIV-infected children, found that naïve and memory CD4 T cell percentages were already significantly increased after 3 months of HAART. In contrast to memory CD4 percentage, naïve CD4 T cell percentages continued to rise until 12 months. They also observed a decrease in memory and activated CD8 T cells after 3 months. The study concluded that the recovery of naïve CD4 T cells occurs more rapidly in children if treatment is started at a younger age, but after 1 year of viral replication control, patients of all ages achieved the same level of restoration.

From the above studies, it has been shown that after ART there is an early and strong increase of both naïve CD4 T cells and naïve CD8 T cells in children. There is also an increase of memory CD4 T cells after 3 months of ART, but a decrease of memory CD8 T cells and activated CD8 T cells.

A study undertaken in Uganda by Ruel T. et al (2009) focused on dynamics of T cell activation accompanying CD4 recovery in antiretroviral-treated HIV-infected children. They sought to determine if treatment with ART would reduce levels of T cell activation (as defined by CD38 and HLA-DR co-expression) in 291 HIV-infected Ugandan children. Their data suggested that significant decreases in T cell activation accompany CD4 T cell recovery in ART- treated HIV infected children to levels that approach but do not reach those of uninfected children.

Impact of ART on Immune Responses to *Mycobacterium tuberculosis*

The impact of ART on immune responses to *Mycobacterium tuberculosis* may take a long time to study *in-vivo*, but *in-vitro* assays that mimic *in-vivo* conditions may help us to understand what happens when *Mycobacterium tuberculosis* invades the human body.

A study by Kampmann et al (2006) in South Africa investigated whether changes in mycobacterial-specific immune responses can be demonstrated in children after starting antiretroviral therapy. This prospective cohort study of 15 bacillus Calmette-Guerin (BCG)–vaccinated HIV-infected children evaluated *in-vitro* antimycobacterial immune responses before and during the first year of ART. They measured mycobacterial growth *in-vitro* using a novel whole blood assay employing a reporter–gene tagged BCG. Before ART, blood from children showed limited ability to restrict the growth of mycobacteria in a functional whole blood assy. After ART, a decrease in the growth of mycobacteria was observed, implying there was a rapid and sustained reconstitution of specific antimycobacterial immune responses upon introduction of ART. Although interferon gamma (IFN-γ) levels in culture supernatants did not reflect this response, there was a decline in tumor necrosis factor-alpha (TNF–α) levels. TNF–α induces apoptotic cell death and its reduction implies more immune cells survived.

Another South African study by Wilkinson K. et al (2009) longitudinally analysed CD4 T cell subsets during the first year of ART, from the time of starting ART (Day 0), in HIV infected, *Mycobacterium tuberculosis* sensitized adults. PBMCs were obtained on Day 0, weeks 2, 4, 12, 24, 36 and 48 of ART and were stimulated with PPD followed by flow cytometry to analyse surface markers and intracellular cytokines. They found that CD4 T cells significantly increased during follow-up and the viral load fell to undetectable levels in each patient, indicating successful immune restoration. Central memory cells expanded by 12 weeks, followed by naïve T cells at 36 weeks. Effecter T cells decreased by 12 weeks, paralleled by a proportional decline of PPD specific IFN-γ producing CD4 T cells. However the absolute numbers of PPD specific producing cells determined by enzyme-linked immunospot assay increased. This study showed that in the context of overall improved TB antigen-specific T cell responses, the central memory rather than effector memory response best correlates with decreased susceptibility.

A study in Gambia by Sutherland J. et al (2010), assessed polyfunctional (IFN-γ, IL-2, TNF –α) T cell responses to TB antigens (including PPD) in three groups of HIV-infected adult patients dependent on their TB status, CD4 T cell counts and antiretroviral

exposure. A total of 41 patients were recruited. They found that the proportion of T cells secreting IFN-γ in response to TB antigens were higher in HIV-positive patients with lower CD4 T cell counts but that this response was reliant on CD8 T cells. They also found that after PPD stimulation no difference in the proportion of CD4 T cells secreting IFN-γ was observed in HIV patients with TB compared to HIV patients without TB. They found that polyfunctional response after stimulation showed very similar profiles between HIV-infected patients with TB and those without TB for both CD4 and CD8 T cells. These findings indicate that changes in the cellular response to PPD (TB antigen) relate more to CD4 T cell count than TB status. After 12 months of ART the overall polyfunctionality of cells was restored and primarily involved effector memory CD4 T cells.

Edmonds et al (2009) carried out a study to estimate the effect of antiretroviral therapy (ART) on incident tuberculosis (TB) in a cohort of HIV-infected children. They analyzed data from ART-naïve, TB-free children enrolled in an HIV care program in Kinshasa, Democratic Republic of Congo, for 4 years. Of 364 children enrolled, 242 (66.5%) initiated ART and 81 (22.3%) developed TB during follow up. At TB diagnosis, 41 (50.6%) were receiving ART. The TB incidence rate in those receiving ART was 10.2 per 100 person-years compared with 20.4 per 100 person-years in those receiving only primary HIV care. The TB incidence decreased with time on ART, from 18.9 per 100 person-years in the first 6 months to 5.3 per 100 person-years after 12 months of ART. This study concluded that ART reduces the hazard of developing TB by 50% for HIV infected children in TB-endemic areas.

These studies suggest that children who begin ART can more effectively respond to TB exposure, have fewer activated immune cells, and have a lower incidence of TB infection than children who are not on ART.

3.0 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

The proportion of children with a positive IFN-γ response (as defined by an increase over baseline levels) following PPD stimulation of whole blood will increase after 3 months of antiretroviral therapy.

3.2 General objective

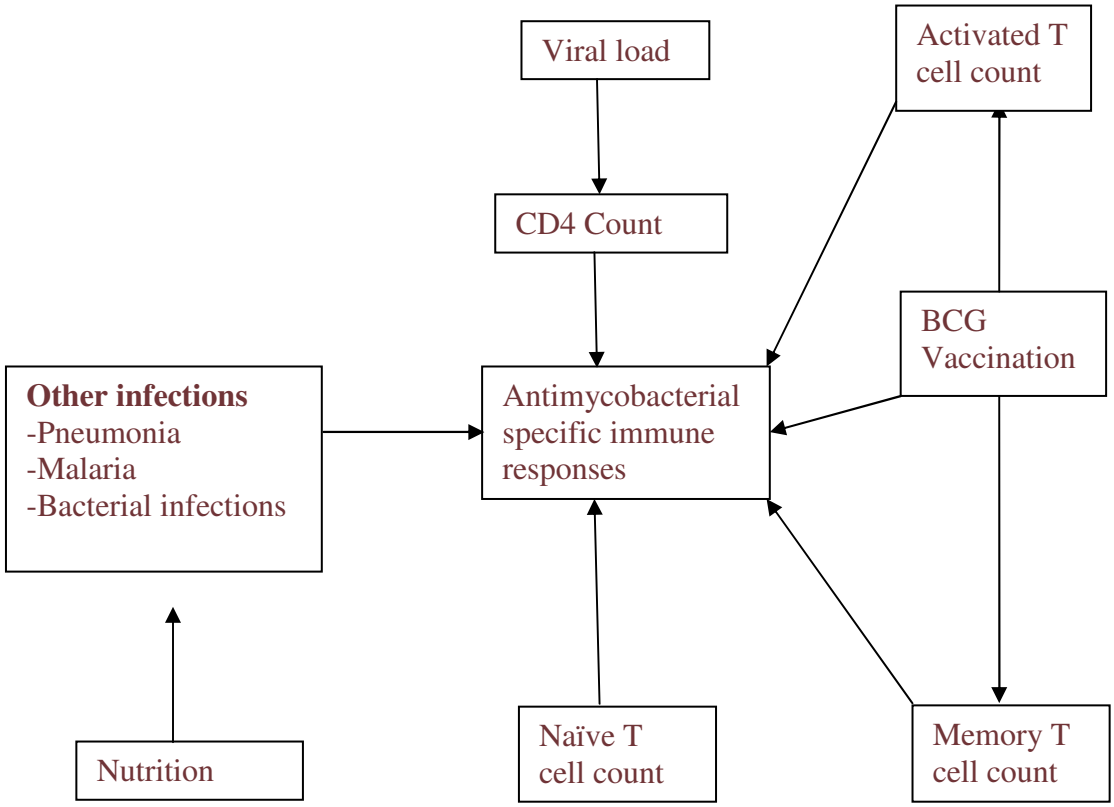
To determine the magnitude and quality of immune reconstitution in HIV-infected children on ART and pathogen–specific immune reconstitution by measuring immune responses to *Mycobacterium tuberculosis*.

3.3 Specific objectives

- To determine and compare the CD4 and CD8 T cell percentages before and after starting ART.
- Measure antimycobacterial-specific immune memory responses by *in-vitro* measurement of intracellular IFN-γ in HIV-infected children before and after starting ART.
- Determine if antimycobacterial specific immune response is associated with changes in the proportion of naïve, activated, or memory T cells.

4.0 RESEARCH METHODOLOGY

4.1 Conceptual framework



From the above framework, the independent variables considered in this study are CD4 T cell count, activated memory T cells and naïve T cells, while the dependent variable is antimycobacterial immune responses by measuring T cells expressing IFN-γ.

Table 1: Table of Variables

Dependent variable	Independent variables
Antimycobacterial specific immune responses (Percentage of T cells expressing IFN-γ after stimulation with PPD)	Percentage of CD4 T cells
	Percentage of activated T cells
	Percentage of memory T Cells
	Percentage of naïve T cells
	Time on ART

4.2 Study design

This was a prospective cohort study focusing on antimycobacterial immune responses in HIV-infected children initiating ART.

4.3 Study setting and study population

The study participants were recruited from Matero Reference Clinic in Lusaka, Zambia. Children 9 months to 5 years of age initiating ART who have a history of BCG vaccination were eligible for analysis. All the laboratory tests for this study were done at the Virology Laboratory at the University Teaching Hospital. For each child, 3 blood samples were drawn, 1 before starting ART, another after 3 months of ART and the last one at 6 months of ART.

4.4 Inclusion criteria

- Between 9 months and 5 years of age.

- Had parents or guardians who were willing to give informed consent for participation in all aspects of the study.
- Had a confirmed HIV test result and eligible for ART.
- Had parents or guardians who were willing to bring them for follow up.
- Had parents or guardians who were willing to have blood specimen collected from the child at three and six monthly intervals for the purpose of the study.

4.5 Exclusion criteria

- Child less than 9 months or above 5 years of age.
- Not residing within the catchment area at the time of enrollment.
- Unwillingness by parents or guardians for follow-up

4.6 Sampling and sample size

Samples were drawn from children initiating ART at Matero Reference Clinic in Lusaka, Zambia. Our main outcome variable was the proportion of IFN-γ producing T cells after stimulation with PPD from paired samples before and six months after starting antiretroviral therapy. We estimated that the difference in the response before and after starting ART would be normally distributed with a standard deviation of 10 and the difference in the mean response of matched pairs would be 5% of IFN-γ producing T cells. This required 44 subjects to be able to reject the null hypothesis that this response is zero with probability (power) of 0.9. The Type 1 error probability associated with the test of this null hypothesis is 0.05. Taking into account potential lost-to-follow-up an additional 10% was added; this brought the sample size to 48 individuals.

4.7 Data collection techniques

Questionnaires were administered to collect demographic and clinical information. Blood samples were drawn (3-5 ml) on the first study visit and every three months for laboratory tests.

Flow cytometry was performed using a 4-color BD FACSCalibur at Virology Laboratory at the University Teaching Hospital.

4.8 Laboratory Investigations

General markers of immune reconstitution

CD4 and CD8 T cells in HIV-infected children were measured before and after initiation of ART. Peripheral blood was collected at study entry (before starting ART), at 3 months and at 6 months, and the percentages of CD4 and CD8 T cells were measured at each visit.

Naïve and memory subsets of CD4 and CD8 T cells were measured by flow cytometry. Naïve T cells were defined by the presence of surface markers CD45RA and CCR7, while memory T cells were defined by the absence of surface maker CD45RA and the presence of surface marker CCR7.

Activated CD4 and CD8 T cells were determined by the presence of surface makers CD38 and HLA- DR.

Table 2: Table of T cell surface markers

T cell subsets	Surface markers
Naïve T cells	$CD45RA^{+}/CCR7^{+}$
Central memory T cells	$CD45RA^{-}/CCR7^{+}$
Effector T cells	$CD45RA^{+}/CCR7^{-}$
Effector memory T cells	$CD45RA^{-}/CCR7^{-}$
Activated T cells	$CD38^{+}$/HLA-DR^{+}

Mycobacterial-specific cellular immune responses

Mycobacterial-specific cellular immune responses were measured using flow cytometry to quantify intracellular IFN-γ production by T cells following *in vitro* stimulation with PPD.

4.9 Data quality control

- Nurses on the study were trained on how to complete, review and correct study forms.
- Standard operating procedures (SOPs) were used at every stage of the study.

5.0 DATA PROCESSING AND ANALYSIS

Flow cytometry data was collected using the BD FACSCalibur with CellQuest Pro and then analyzed using FlowJo v7.5 to determine T cell proportions. Data were analyzed using Statistical Package for Social Sciences (SPSS) version 18. The normality of data was assessed; some of the data was not normally distributed. The non-parametric Wilcoxon signed rank test was used to test for significance differences between paired data. The median percentage and interquartile range (IQR) were computed for T cell subsets. A p value of <0.05 was considered statistically significant.

6.0 **ETHICAL CONSIDERATION**

This study is part of a larger study called the Measles Immune Reconstitution Study, which has been approved by the University of Zambia Research Ethics Committee (UNZAREC) and the committee for Human Research at the Johns Hopkins University Bloomberg School of Public Health. Clearance for this component was sought and obtained from the UNZAREC.

Informed consent was obtained from parents or guardians and confidentiality was assured and maintained at all times.

7.0 **RESULTS**

A total of 59 children were enrolled in the study, two dropped out, three were lost to follow-up and 13 died. This left us with a total of 41 children. Blood samples were collected before starting ART (per-ART), after 3 months and after 6 months of ART for enumeration of T-cell subsets and intracellular cytokine stimulation and staining.

Table 1: Sex and Age Distribution

	Frequency	Percent (%)	Median age (months)	Interquartile Range(IQR)
Male	21	51	23	19 - 32
Female	20	49	21	16 - 34
Total	41	100	22	15 – 33

The table shows that out of a total of 41 children who participated in the study, 21 (51%) were males while 20 (49%) were females. The median age for the children was 22 months, with an IQR of 15 to 33 months.

Table 2: Median percentage of T- cell subsets for pre-ART, 3 months and 6 months on ART

T Cell Subsets	CD4 %	CD8 %	Naive CD4%	Naive CD8%	Activated CD4%	Activated CD8%	CM CD4%	CM CD8%	EM CD4%	EM CD8%
Pre-ART	9.7 (IQR, 7.9-17.7)	42.8 (IQR, 37.4-50.6)	21 (IQR, 5.7-40.7)	3.5 (IQR, 0.8-7.6)	14.8 (IQR, 12.5-21.0)	37.9 (IQR, 31.0-56.3)	3.2 (IQR, 1.7-5.1)	0.05 (IQR, 0.02-0.11)	32 (IQR, 17.7-48.8)	39.1 (IQR, 24.4-50.2)
3months on ART	19.4 (IQR, 14.0-30.1)	38.1 (IQR, 30.4-47.7)	21 (IQR, 5.4-43.9)	5.7 (IQR, 1.7-12.3)	9.6 (IQR, 6.3-14.0)	24 (IQR, 16.6-38.6)	3.4 (IQR, 2.1-7.1)	0.12 (IQR, 0.04-0.30)	28.6 (IQR, 13.7-42.7)	24 (IQR, 13.8-41.8)
6months on ART	25.9 (IQR, 17.7-31.2)	36.5 (IQR, 31.1-42.0)	24 (IQR, 4.0-52.4)	9.5 (IQR, 2.9-22.9)	7.1 (IQR, 4.4-10.8)	22.2 (IQR, 10.7-35.8)	4.7 (IQR, 1.7-10.0)	0.15 (IQR, 0.06-0.49)	28.4 (IQR, 17.3-44.1)	23.7 (IQR, 13.9-38.0)
p value (Pre-ART Vs. 3 months)	<0.001	0.026	0.719	0.048	<0.001	0.001	0.179	0.020	0.694	<0.001
p value (Pre-ART Vs. 6 months)	<0.001	0.010	0.708	0.038	<0.001	<0.001	0.029	0.021	0.793	0.006

CM CD4% = Central memory CD4 T Cells
CM CD8% = Central memory CD8 T Cells
EM CD4% = Effector memory CD4 T Cells
EM CD8% = Effector memory CD8 T Cells
IQR = Interquartile range

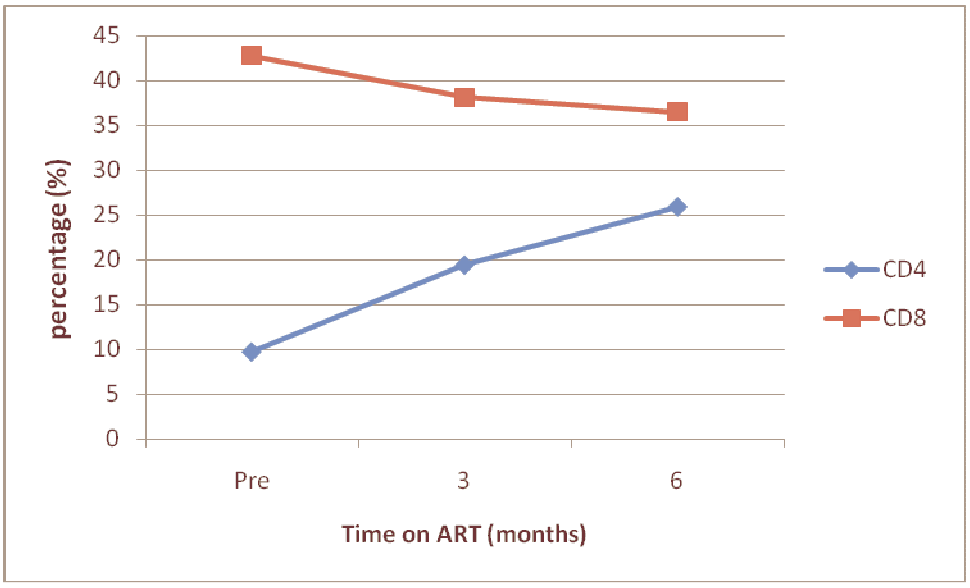
Table 3: Median percentage of T- cells expressing INFγ after stimulation with PPD

T Cells	INFγ CD4%	INFγ CD8%
Pre-ART	1.1 **(IQR, 0.5-1.7)**	0.8 **(IQR, 0.4-1.3)**
3 months on ART	0.7 **(IQR, 0.3-1.2)**	1.1 **(IQR, 0.6-1.5)**
6 months on ART	1.1 **(IQR, 0.5-2.2)**	0.8 **(IQR, 0.6-1.4)**
p value (Pre-ART Vs. 3 months)	0.033	0.355
p value (Pre-ART Vs. 6months)	0.717	0.619

INFγ CD4% = CD4 T Cells expressing INFγ after stimulation with PPD
INFγ CD8% = CD8 T Cells expressing INFγ after stimulation with PPD
IQR = Interquartile range

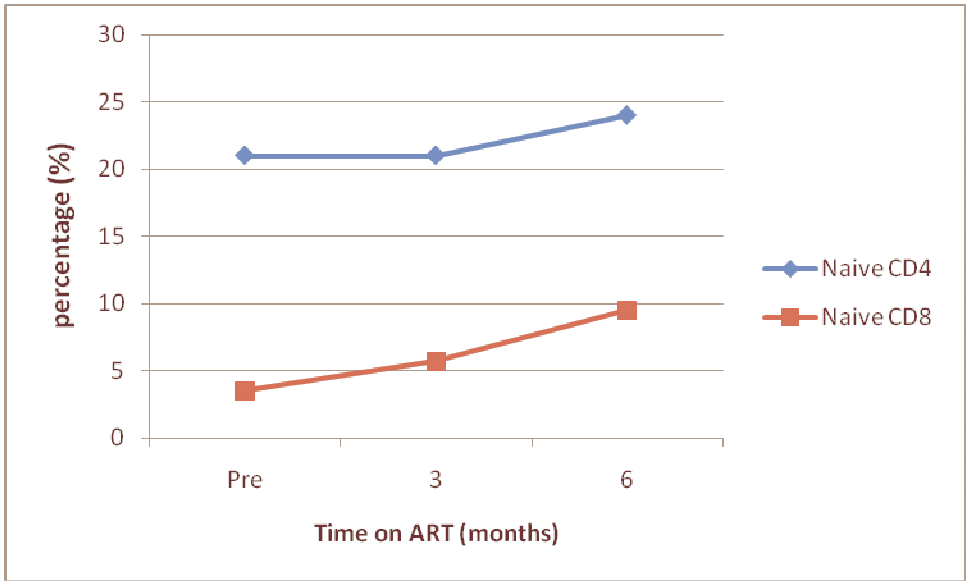
CELL SURFACE STAINING RESULTS:

Figure 1: CD4 and CD8 T cell median percentages before and after ART



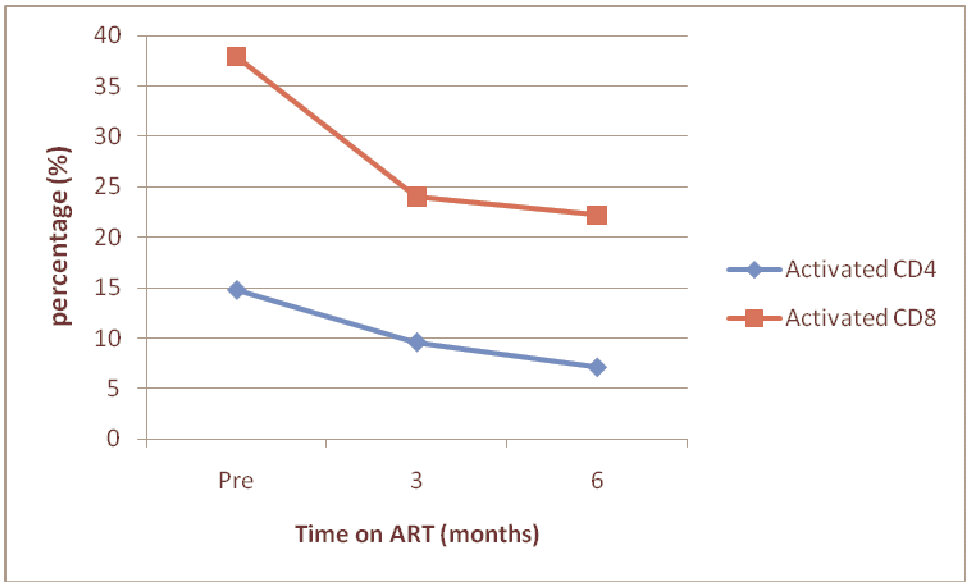
There was a statistically significant increase in CD4 T cell percentage from pre-ART (9.4%) to 3 months (19.4%; p<0.001) and 6 months (25.9%; p<0.001). On the other hand, there was a significant decrease in CD8 T cell percentage from pre-ART (42.8%) to 3 months (38.1%; p=0.026) and 6 months on ART (36.5%; p=0.01).

Figure 2: Naïve CD4 and CD8 T cell median percentages before and after ART



Naive CD4 T cell percentages from pre-ART to 3 months and 6 months were 21.0%, 21.0% and 24.0%, respectively (Figure 2). No significant difference was observed at all time points. Naïve CD8 T cell percentages for per-ART, 3 months and 6 months were 3.5%, 5.7% and 9.5%, respectively. This was a statistically significant difference in naïve CD8 T cell percentage between per ART and 3 months after ART (p=0.048) and between pre- ART and 6 months after ART (p=0.038).

Figure 3: Activated CD4 and CD8 T cell median percentages before and after ART



Activated CD4 T cell percentages decreased significantly from pre-ART (14.8%) to 3 months (9.6%; p<0.001) and 6 months (7.1%; p<0.001). Similarly, there was a decrease in the activated CD8 T cell percentage from pre-ART (37.9%) to 3 months (24.0%; p<0.001) and 6 months (22.2%; p<0.001).

Central memory CD4 and CD8 T cell median percentages before and after ART

Central memory CD4 T cell percentages for pre-ART, 3 months and 6 months were 3.2%, 3.4% and 4.7%, respectively. There was no statistically significant difference in central memory CD4 T cell percentage between pre-ART and 3 months (p = 0.179). However, there was a significant difference in central memory CD4 T cell percentages between pre-ART and 6 months (p = 0.029).

Conversely, central memory CD8 T cell percentages were significantly different at both 3 months (0.12%; p=0.020) and 6 months (0.15%, p=0.021) when compared to pre-ART (0.05%).

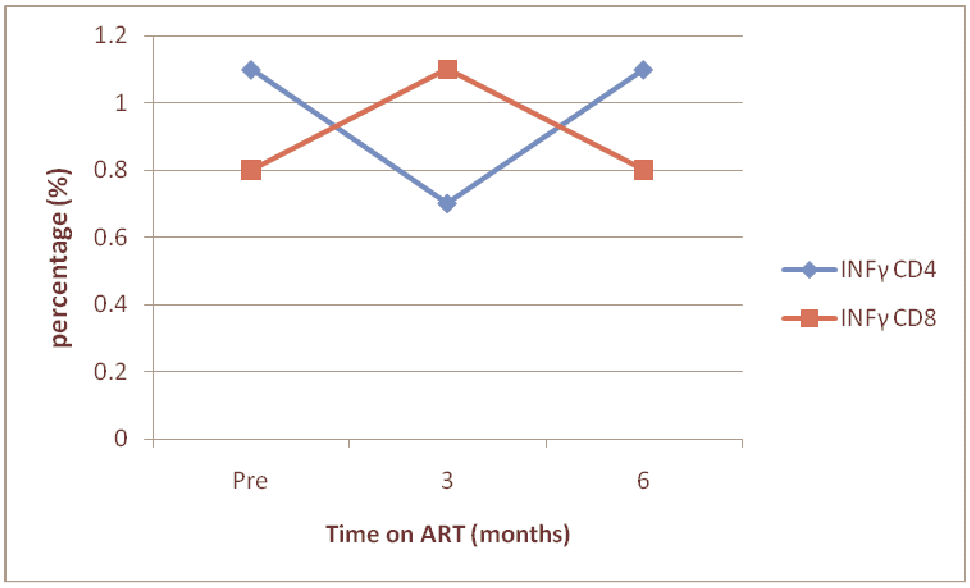
Effector memory CD4 and CD8 T cell median percentages before and after ART

Effector memory CD4 T cell percentages for pre-ART, 3 months and 6 months were 32.0%, 28.6% and 28.4%, respectively. There was no significant difference in effector memory CD4 T cell percentage at either time point after ART ($p = 0.694$ for pre ART vs. 3 months and $p = 0.793$ pre ART vs. 6 months).

There was a significant decrease in effector memory CD8 T cell percentage from pre-ART (39.1%) to 3 months (24.0%; $p<0.001$) and 6 months (23.7%; $p=0.006$).

INTRACELLULAR CYTOKINE STAINING RESULTS AFTER STIMULATION WITH PPD

Figure 4: CD4 and CD8 T cells expressing IFNγ before and after ART



The CD4 T cell percentage that expressed IFNγ for pre-ART, 3 months and 6 months after ART were 1.1%, 0.7% and 1.1%, respectively. There was a significant difference from pre-ART to 3 months ($p = 0.033$), but the percentage at 6 months returned to pre-ART levels.

Similarly, the percentages of CD8 T cells expressing IFNγ at pre-ART, 3 months and 6 months were 0.8%, 1.1% and 0.8%, respectively. There were no statistically significant differences at either time point after ART compared to pre-ART ($p = 0.355$ for pre ART vs. 3months).

8.0 DISCUSSION

The discussion is divided in two parts; the first part (cell surface staining) focuses on the general markers of immune reconstitution were T cell subsets are discussed with regard to changes in percentage from the time children start ART to 6 months of ART. The second part (intracellular cytokine staining after stimulation with PPD) focuses on antimycobacterial specific immune responses of CD4 and CD8 T cells after invitro stimulation with PPD.

Cell surface staining

CD4 and CD8 T cells in general

Immune responses in HIV-infected children on ART were investigated with particular attention to antimycobacterial–specific immune responses. A total of 41 children aged 9 months to 60 months were enrolled in the study, 21 (51%) of whom were male while 20 (49%) were female.

A statistically significant increase of CD4 T cell percentages was demonstrated after starting antiretroviral therapy. After 6 months of ART, the CD4 T cell percentage increased from 9.7% to 25%. These findings are consistent with a Belgium study by Hainaut et al (2000) on children, which found that there was a significant increase in CD4 T cell counts after ART from 294 cells /ml to 459 cells /ml after 3 months and 619 cells / ml after 6 months. This clearly demonstrated general immune reconstitution after commencement of ART. CD4 T cell percentage is used as a marker of immune reconstitution in HIV infected children, therefore the results obtained in this study supports the idea that ART improves immunity and this in turn helps to prevent TB.

On the other hand, there was a decrease in CD8 T cell percentage after starting ART. Before ART, the median CD8 T cell percentage was 42.8%, but after 6 months of ART, it decreased to 36.5%. A study by Wherry and Ahmed (2004) suggested that during viral infection CD8 T cells undergo an expansion phase, leading to the generation of effector CD8 T cells. The expansion phase is followed by a death phase, when 90 - 95% of the

effector T cells die. This may explain the high levels of CD8 T cells before ART followed by the decline after ART initiation.

Naive CD4 and CD8 T cells

Focusing on T cell subsets, there was an increase in both naïve CD4 and CD8 T cells after starting ART. Although naïve CD4 T cells did not show a statistically significant increase, naïve CD8 T cells increased significantly after 6 months. This agrees with a study by Franco et al (2000) which showed an increase in both naïve CD4 and CD8 T cells after ART. Partly, this may explain the increase in both total CD4 T cells and total CD8 T cells.

Activated CD4 and CD8 T cells

Our results show that there were more activated CD4 T cells and CD8 T cells before starting ART compared to after 3 and 6 months of ART. This result agrees with a study by Hainaut et al (2003) that found a decrease in activated CD8 T cells after three months of ART in HIV infected children. High levels of activated T cells before commencement of ART may be attributed to high viral load. After ART, there is a decrease in viral load resulting in the reduction of activation of T cells (Montaner et al, 2010).

Memory CD4 and CD8 T cells

Before starting ART, there were more effector memory T cells as compared to central memory T cells. A significant increase was observed in the central memory CD4 T cell percentage after 6 months of ART. On the other hand, effector memory CD4 T-cells decreased over the same period of time. The picture is similar when we compare central memory CD8 T cells to effector memory CD8 T cells. Central memory CD8 T–cells increase while effector memory CD8 cells decrease over time. This is consistent with the literature, as central memory T cells are longer lived and confer long-term memory while effector memory T cells can rapidly mature into effector T cells and secrete cytokines such as IFNγ and IL4 early after restimulation (Franco et al, 2000; Resino et al, 2001). A study by Resino et al (2001) found that elevated percentages of total CD8 T –cells were

associated with increased memory CD4 T-cells. If this is true, then one may conclude that most of these memory CD4 T-cells are actually effector memory CD4 T-cells.

Intracellular cytokine staining after stimulation with PPD

Antimycobacterial-specific immune responses

After 3 months of ART, CD4 T cells that produced IFNγ in response to stimulation with PPD decreased significantly. This agrees with a study by Wilkinson et al (2009) where IFNγ responses were found to decrease after 3 months of ART in HIV infected adults. This may be explained by a study by Sutherland et al (2010), which found that the proportion of CD4 T cells producing IFNγ in response to TB antigens was higher in HIV-infected patients with lower CD4 counts but the ability to produce higher IFNγ levels relied on CD8 T cells. However, our study found that after 6 months of ART, the percentage of CD4 T cells producing IFNγ went back to pre ART levels. To the author's knowledge, no study has ever documented this phenomenon, except it can be speculated that this may be due to the increase by CD4 T cells percentage after ART and the significant increase in the percentage of central memory CD8 T cells may play a role as well. For CD8 T cells, there was no statistically significant difference by IFNγ-producing cells in response to stimulation to PPD after ART. This may reflect unmeasured factors in these children or the time period was too short to measure substantial changes in IFNγ producing CD8 T cells.

According to a study by Kampmann et al (2006), a rapid and sustained reconstitution of specific antimycobacterial immune response was observed upon the introduction of ART. Decreased growth of mycobacteria was observed by an *in-vitro* assay. However, IFNγ levels in culture supernatants did not reflect this response. While culture supernatants can be used to measure the production of a cytokine, this method does not identify which cells produce it. In our case, we used intracellular cytokine staining, which measures the specific cell subsets that are expressing IFNγ but does not detect the amount of the cytokine that is actually released from the cells. Therefore, each of these methods has

their strengths and weaknesses. Also, it should be noted that though IFNγ is crucial in the protective immune response to TB, it is not enough on its own for an effective immune response (Sutherland et al, 2010).

Limitations of the study

- Cellular subsets and cytokine levels were not measured in HIV uninfected children, which eliminate the ability to compare responses with those on ART. This would be useful to determine whether immune reconstitution after ART approaches normal levels.
- The study period was relatively short and does not provide information on the long term impact of ART on immune reconstitution of cellular subsets and IFNγ levels.

9.0 CONCLUSION

Children in this study responded well to ART as indicated by increases in CD4 T cell percentages after 6 months of ART. This demonstrated that ART has a positive impact since CD4 T cells are central to an effective immune response against HIV and this in turn will reduce the risk of tuberculosis in HIV infected children. Also, CD8 T cells decreased in the same time period, supporting the fact that there was general immune reconstitution since fewer CD8 T cells are needed to kill infected cells as the immunity builds up. Overall, there was no significant difference in T cells expressing IFNγ upon stimulation with PPD after 6 months of ART, however the response before ART seem to be primarily driven by effector memory T cells as evidenced by their high percentage while that after ART by central memory T cells . This suggests that in the context of improved TB antigen specific T cell responses, central memory T cells are the primary cells in restoring immune response. These findings have important implications in vaccine development strategies for TB in HIV infected children.

10.0 RECOMMENDATIONS

- Long-term studies are needed to ascertain the impact of ART on general immune reconstitution in children.
- Although IFNγ is crucial in the protective immune response to TB, it is not sufficient on its own; therefore, studies that will assess other factors alongside IFNγ are needed.
- This study supported the idea of immune reconstitution after ART initiation and that this in turn will help prevent TB. Therefore, increased access to ART is needed for more children to benefit from the treatment. As of 2008, out of the 95,000 of HIV infected children in Zambia, only 11, 602 were receiving ART.

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Appendix I
Entry Questionnaire

Identification Information

1. Study Number _____
2. Clinic Number _____
3. Child's name _____
4. Date of Birth ____ / _____ /_____

DD MM YYYY

5. Sex Tick only one

Male	
Female	

6. Respondent's relationship to the child

Tick only one

Mother	
Father	
Grandparent	
Aunt or Uncle	
Sibling	
Other relative	
Friend	

7. If respondent is not mother: Where is the mother?

Tick only one

At home	
Leaving away from home	
Mother died	
Do not know	
Other	

8. If mother died, when? ____ / _____ /_____

DD MM YYYY

9. If respondent is not mother or not father, is the child's father alive?

Tick only one

Yes	
No	
Don't know	

10. For how many years did the child's mother go to school?

11. What is the child's mother highest level of education completed (1= Never attended, 2= Grade 1-4, 3= Grade 5-7, 4=Grade 8-9, 5= Grade 10-12, 6= Higher)

12. For how many years did the child's father go to school?

13. What is the child's father highest level of education completed? (1= Never attended, 2= Grade 1-4, 3= Grade 5-7, 4=Grade 8-9, 5= Grade 10-12, 6= Higher)

Immunization

14. Tick were appropriate

Type of immunization	No	Yes	Don't know	Date of immunization (DD/MM/YY)
BCG				
OPV0				
OPV1				
OPV2				
OPV3				
DPT1				
DPT2				
DPT3				
Hib1				
Hib2				
Hib3				
HepB1				
HepB2				
HepB3				
measles				

Past Medical History

15. Has the child been hospitalized before?

	Tick only one
Yes	
No	
Don't know	

16. If yes. How many times?

Weight and Height

17. Weight in kilograms

18. Height in centimeters

Clinical Diagnoses

Was the child diagnosed with any of the following today?
Tick were appropriate

Diagnosis	No	Yes
19. Pneumonia		
20. Diarrhoea		
21. Serious bacterial infection		
22. Tuberculosis		
23. Malaria		

Thank you for your time. Your participation is appreciated.

Appendix II
Follow-up Questionnaire

Identification Information

1. Study Number _____
2. Clinic Number _____
3. Child's name _____
4. Date of Birth ____ / _____ /_____

DD MM YYYY

5. Sex Tick only one

Male	
Female	

6. Respondent's relationship to the child

Tick only one

Mother	
Father	
Grandparent	
Aunt or Uncle	
Sibling	
Other relative	
Friend	

7. If respondent is not mother: Where is the mother?

Tick only one

At home	
Leaving away from home	
Mother died	
Do not know	
Other	

Interim Medical History

8. Has the child been hospitalized since the last clinic visit?

Tick only one

Yes	
No	
Don't know	

9. If yes. How many times?

10. For what illnesses was the child hospitalized?

Tick were appropriate

Diagnosis	No	Yes
Diarrhoea		
Pneumonia		
Malaria		
Tuberculosis		
Measles		

11. Did the child receive any immunizations since the last clinic visit?

Tick only one

Yes	
No	
Don't know	

If YES, record new immunizations in the Table for Q12.
If the child did not have the immunization card at the study entry visit, complete Q12.

12. Tick were appropriate

Type of immunization	No	Yes	Don't know	Date of immunization (DD/MM/YY)
BCG				
OPV0				
OPV1				
OPV2				
OPV3				
DPT1				
DPT2				
DPT3				
Hib1				
Hib2				
Hib3				
HepB1				
HepB2				
HepB3				
measles				

Weight and Height

13. Weight in kilograms

14. Height in centimeters

Clinical Diagnoses

Was the child diagnosed with any of the following today?
Tick were appropriate

Diagnosis	No	Yes
15. Pneumonia		
16. Diarrhoea		
17. Serious bacterial infection		
18. Tuberculosis		
19. Malaria		

Thank you for your time. Your participation is appreciated.

Appendix III

Consent Form

Title of Study

Antimycobacterial Immune Responses in HIV-infected children on Antiretroviral Therapy in Lusaka, Zambia.

Investigator

Hope .C. Nkamba, Biomedical scientist, University Teaching Hospital, Virology Laboratory, Lusaka.
Cell Number: 0977743889.

Background Information

This study will look at the ability of children on antiretroviral therapy to protect themselves against TB infection. Although there are studies that have shown that effective ART should result in improved immune responses to opportunistic infections such as tuberculosis, little is known on the magnitude and quality of immune reconstitution in HIV infected children infected children on ART with respect to immunity against tuberculosis. This is very important especially that diagnosis of TB in children can be difficult.

Procedures

If you agree for your child to participate in this study, the following things will happen.

1. We will ask you some questions your health and the health of your child, including illnesses your child has had and medicines and vaccines your child has received. This will take about 10 minutes.
2. We will take a small sample of blood (one teaspoon) from your child. The blood will be collected at the same time your child has blood drawn for treatment and will be used to measure the body's responses to mycobacterium tuberculosis.
3. Your child's weight and height will be measured and your child will be examined for signs of illness.
4. If your child is ready to start antiretroviral therapy, we ask that every three months you bring your child for routine care at this Clinic and give us about 15 minutes of your time to answer some questions and allow us to take a small sample of blood (one teaspoon) from your child when blood is drawn to monitor the treatment.

Risks

There may be some mild pain when drawing blood for the test.

Benefits

By participating in the study your child may benefit from additional attention provided by the study team and the assistance they provide to the clinic staff.

Confidentiality

All information obtained in this study will be considered confidential and used only for research purposes. You and your child will not be named in any reports of the study.

Payment(s) for Participating

You will not be paid for participating in this study.

Voluntariness

Your participation in this study is entirely voluntary and you have the right to refuse to take part or withdraw at any time. Even if you refuse to take part or withdraw from the study, you will still receive the same medical care available to you at the clinic.

Contact Persons

In the event that you become injured during the course of the study or you think you have not been treated fairly or think you have been hurt by joining the study or you have any other questions about the study, you should contact the investigator at the Virology Lab, UTH on 0977743889 or the chairperson of the research Ethics Committee at the University of Zambia, School of Medicine on telephone number 256067.

Consent

I agree for my child to participate in this study. I have been given a chance to read this document and it has been read and explained to me.

Name of participant:_____

Signature or thumbprint of legally Authorized representative: _____Date___/___/___

Signature of person obtaining consent: _____Date____/___/___

Witness (Name and Signature): _____Date___/___/___