ORIGINAL PAPER

Antimycobacterial immune responses in HIV-infected children starting antiretroviral therapy in Lusaka, Zambia

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ABSTRACT

Background: Children infected with HIV are at risk of developing tuberculosis (TB). Antiretroviral therapy has been linked to improved immune responses to TB. The objectives of the study were to determine the magnitude and quality of immune reconstitution in HIV-infected children receiving antiretroviral therapy (ART) and to determine pathogen-specific immune reconstitution to *Mycobacterium tuberculosis*.

Methods: A total of 59 children of age 9 months to 5 years initiating ART with a history of BCG vaccination from Matero Reference Clinic in Lusaka were enrolled in a prospective cohort study. Demographic and clinical data were collected using questionnaires. Blood samples were drawn before starting ART, at 3 months and 6 months for measurement of T cell subsets and PPD stimulation for intracellular cytokine staining.

Results: After 6 months of ART, the median CD4 T cell percentage increased from 9.4% at baseline to 25.9% ($p < 0.001$). Total CD8 T cell percentage decreased from 42.8% pre-ART to 36.5% after 6 months of ART ($p = 0.010$). However, naïve CD8 T cells increased within the same period ($p = 0.038$). Both activated CD4 and CD8 T cells decreased after 6 months of ART ($p < 0.001$). On the other hand, both central memory CD4 and CD8 T cells increased after 6 months of ART ($p = 0.029$ and 0.021, respectively), while effector memory CD8 T cells decreased ($p = 0.006$). After 3 months of ART, CD4 T cells expressing IFN-γ decreased ($p = 0.033$) but after 6 months of ART the percentage increased to pre-ART levels.

Conclusion: ART has a positive impact on HIV-infected children, likely reducing the risk of tuberculosis as evidenced by the increases in CD4 T cells critical to an effective immune response against TB. Before starting ART, anti-mycobacterial immune responses seem to be primarily driven by effector memory T cells while after ART by central memory T cells. Therefore, central memory T cells appear to be the primary cells in restoring specific immune responses. These findings have valuable implications for TB vaccine development strategies in HIV-infected children.

INTRODUCTION

With the scaling up of antiretroviral therapy (ART) in sub-Saharan Africa and Zambia in particular, more HIV infected children will have access to treatment[1]. An estimated 250,000 children died of AIDS in 2005 and 2.3 million children were estimated to be living with HIV/AIDS globally. Almost 90% of these children live in sub-Sahara[2]. In HIV infected children, the risk of developing tuberculosis (TB) is high[3,4,5]. Over 250,000 children develop TB and 100,000 children will continue to die each year from TB[3]. However, effective therapy

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should result in improved immune responses to opportunistic infections such as TB[6]. Globally the number of children receiving ART increased from about 75,000 in 2005 to almost 200,000 in 2007[7]. In Zambia, out of the estimated 34,000 children in need of ART 18,040 were receiving ART by 2008[2].

According to a study by Chintu et al[9], 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). 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 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[10,11]. However, no studies have documented naïve T cells, activated T cells and memory T cells in Zambian children on ART.

METHODS

Study design

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

Study setting and population

The study participants were recruited from Matero Reference Clinic in Lusaka, Zambia. The Biomedical Research Ethics Committee of the University of Zambia granted ethical approval for the study. Children 9 months to 5 years of age initiating ART with a history of BCG vaccination were eligible for analysis. Informed consent was obtained from parents or guardians and confidentiality was assured and maintained at all times. All the laboratory tests were done at the Virology Laboratory at the University Teaching Hospital. Blood samples were collected before starting ART (pre-ART), after 3 months and after 6 months of ART for enumeration of T-cell subsets and intracellular cytokine stimulation and staining.

Laboratory Investigations

General markers of immune reconstitution

CD4 and CD8 T cell percentages were obtained using Becton Deckinson (BD) monoclonal antibodies to CD4, CD8 and CD3. T cell activation markers $CD38^+$ / $HLA\text{-}DR^+$ were used to identify activated T cells. For naïve T cells $CD45RA^+$ / $CCR7^+$, for central memory T cells $CD45RA^-$ / $CCR7^+$ while for effector memory T cells $CD45RA^-$/ $CCR7^-$ monoclonal antibodies were used. Acquisition was done using a 4- color BD FACSCalibur with CellQuest Pro software and then analysed FACS plots with FlowJo version7.5.

Mycobacterial specific immune responses

Overnight antigen stimulation: Cells were stimulated with tuberculin purified protein derivative (PPD; Statens Serum Institute, Denmark). Stimulation with Staphylococcal enterotoxin B (SEB; Sigma-Aldrich, UK) was done as positive control while the negative control was left unstimulated. The mixture of cells and antigens was incubated overnight at 37°C (16 hours).

Intracellular cytokine staining: After overnight stimulation, cell surface staining using monoclonal antibodies for CD3, and CD4 or CD8 (BD Pharmingen) was done. This was followed by intracellular cytokine staining with IFN-γ monoclonal antibodies (BD Pharmingen). Acquisition was done using the BD FACSCalibur.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 18. 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.

RESULTS

Participation and distribution

A total of 59 children were enrolled in the study, 2 withdrew, 3 were lost to follow-up and 13 died before review at 3 months. This left us with a total of 41 children. Out of 41 children who participated in the study, 21 (51%) were males while 20 (49%) were females. The median age for the males was 23 (Q_1= 19, Q_3= 32) months while for the females it was 21(Q_1= 16, Q_3=34) months. The median age for all the children was 22 (Q_1= 15, Q_3= 33) months.

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 1A).

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 1B).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).

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), (Figure 1C).

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%), (Table 1).

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), (Table 1).

CD4 and CD8 T cells expressing IFNγ before and after ART

The CD4 T cell percentage that expressed IFN-γ 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 (Figure 1D).

Similarly, the percentages of CD8 T cells expressing IFN-γ 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).

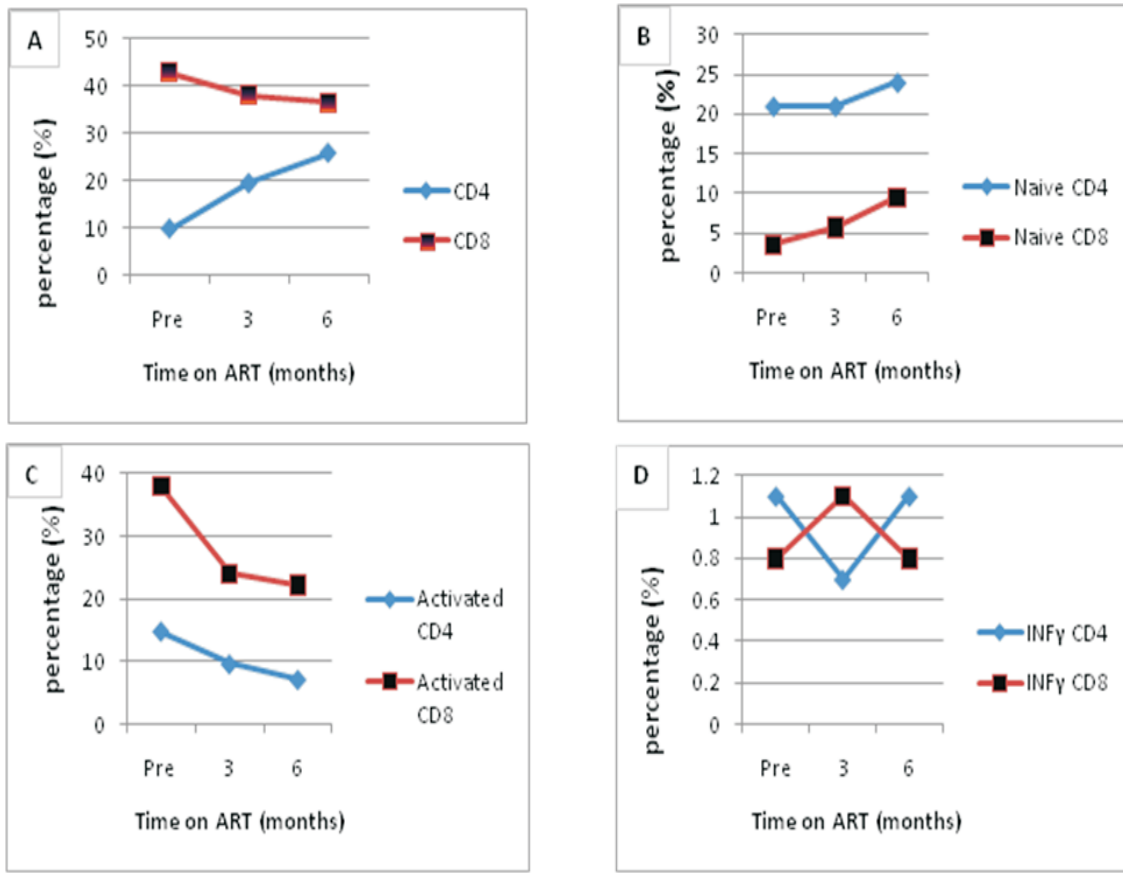

Figure 1

A illustrates the increase in CD4 T cells with a conversary decrease in CD8 T cells as the immunity builds up after starting ART. B shows increase in naïve T cells while C illustrates the reduction in activated T cells. D shows specific immune response after stimulation with PPD at each time point. CD4 T cells expressing IFN-γ after 3 months of ART decreased significantly while at 6 months reverted to pre-ART levels. Although there seem to be no difference in T cells expressing IFN-γ between pre-ART and 6 months, results suggest that the primary T cell subsets driving the response at these time points are different.

Table 1: 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 2: 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

DISCUSSION

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 25.0%. These findings are consistent with a Belgium study by Hainaut et al[11] 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. 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 suggested that during viral infection CD8 T cells undergo an expansion phase, leading to the generation of effector CD8 T cells[12]. 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.

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[13] 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.

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 [14,15] 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[16].

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[13,17]. A study by Resino et al[17] 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.

Focusing on antimycobacterial-specific immune response, CD4 T cells that expressed IFNγ in response to stimulation with PPD decreased significantly after 3 months of ART. This agrees with a study by Wilkinson et al[18] where IFNγ responses were found to decrease after 11 months of ART in

HIV infected adults. This may be explained by a study by Sutherland et al[19], 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 CD8 T cells expressing IFNγ. It should be noted that although IFNγ is crucial in the protective immune response to TB, it is not enough on its own for an effective immune response[19].

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. 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.

COMPETING INTEREREST S

The authors declare that they have no competing interests in whatever form.

AUTHOR'S CONTRIBUTIONS

HCN designed the study, acquired flow cytometry data, conducted the statistical analysis, interpreted the results and drafted the manuscript. KRL participated in flow cytometry data analysis and review of the manuscript. WM and SS guided the study design, the statistical analysis, and the interpretation of results and critically reviewed the manuscript.

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