Asymptomatic HIV-infected Individuals on Antiretroviral Therapy Exhibit Impaired Lung CD4⁺ T-Cell Responses to Mycobacteria

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Abstract

Rationale: HIV-infected persons on antiretroviral therapy (ART) remain at higher risk of pulmonary tuberculosis (TB) than HIV-uninfected individuals. This increased susceptibility may be caused by impairment of alveolar macrophage (AM) function and/or mycobacteria-specific alveolar CD4⁺ T-cell responses observed in HIV-infected ART-naïve adults.

Objectives: To determine whether ART was associated with improvement in both AM function, assessed by phagosomal proteolysis, and alveolar CD4⁺ T-cell responses to *Mycobacterium* in HIV-infected individuals.

Methods: Peripheral blood was drawn and bronchoalveolar lavage (BAL) performed on healthy, 35 HIV-uninfected, 25 HIV-infected ART-naïve, and 50 HIV-infected ART-treated asymptomatic adults. Phagosomal proteolysis of AM was assessed with fluorogenic beads. Mycobacteria-specific CD4⁺ T-cell responses were measured by intracellular cytokine staining.

Measurements and Main Results: HIV-infected adults on ART exhibited lower plasma HIV viral load and higher blood CD4⁺ T-cell count than ART-naïve adults. AM proteolysis and total mycobacteria-specific Th1 CD4⁺ T-cell responses in individuals on ART for greater than or equal to 4 years were similar to HIV-uninfected control subjects but those on ART for less than 4 years had impaired responses. Total influenza-specific alveolar Th1 CD4⁺ T-cell responses were intact in all individuals receiving ART. In contrast, BAL and blood mycobacteria-specific polyfunctional CD4⁺ T-cell responses were impaired in adults on ART irrespective of duration.

Conclusions: AM and mycobacteria-specific alveolar CD4⁺ T-cell responses in HIV-infected adults on ART for less than 4 years are impaired and may partly explain the high risk of TB in HIV-infected individuals on ART. Strategies to augment ART to improve lung immune cell function and reduce the high incidence of TB in HIV-infected adults who initiate ART should be investigated.

Keywords: HIV; T cells; macrophages; *Mycobacterium tuberculosis*; ART

Pulmonary tuberculosis (TB) causes high morbidity and mortality in HIV-infected individuals (1). Unlike other opportunistic infections, the risk of developing active TB is greatly increased in persons even before

significant depletion of CD4⁺ T cells in peripheral blood (2). In high transmission regions, most TB cases in HIV-infected individuals result from recent *Mycobacterium tuberculosis* (Mtb) infection indicating

that in these individuals the lung is more permissive for the establishment or progression of primary infections (3). Recent studies have reported that alveolar macrophage (AM) proteolytic function

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At a Glance Commentary

Scientific Knowledge on the

Subject: The use of potent antiretroviral therapy (ART) has led to a dramatic fall in the burden of tuberculosis (TB) among HIV-infected individuals, but the incidence of TB still remains higher in HIV-infected adults receiving ART compared with HIV-uninfected individuals.

What This Study Adds to the

Field: We show that HIV-infected adults on ART have impaired alveolar macrophage function and mycobacteria-specific alveolar CD4⁺ T-cell responses, most markedly during early ART treatment. These findings underscore the need for strategies to augment ART to improve lung immune cell function and reduce the high incidence of TB. The development and use of subunit vaccines that promote repopulation of the lung with crucial mycobacteria-specific polyfunctional CD4⁺ T-cell subsets and the use of prophylactic TB chemotherapy in HIV-infected adults, especially during the early years of ART when they are most vulnerable, should be explored.

and mycobacterial antigen-specific Th1 CD4⁺ T-cell responses in the lung are impaired in asymptomatic HIV-infected,

antiretroviral therapy (ART)–naïve persons (4–6). These findings indicate that HIV alters both innate and adaptive immunity in the lung to render HIV-infected individuals incapable of mounting adequate pulmonary immune responses to control Mtb.

Potent ART inhibits HIV replication to allow immune recovery. Several previous studies have profiled the kinetics of peripheral blood CD4⁺ T-cell count as a surrogate marker of immune recovery during ART. These studies reported a rapid increase in CD4⁺ T cells during the first few months of treatment, followed by a more gradual increase during the subsequent months or years before reaching a plateau after approximately 4 years of ART (7, 8). ART-associated immune recovery reduces morbidity and mortality caused by HIV-related opportunistic infections (9). For example, a 65% reduction in the incidence of TB in HIV-infected adults on ART irrespective of the CD4⁺ T-cell count has been reported previously (10). However, despite the benefits of ART, TB incidence rates in HIV-infected individuals who received ART for more than 3 years remain 5- to 10-fold higher than HIV-uninfected persons (11).

The development of strategies to reduce the burden of TB in HIV-infected individuals treated with ART requires a better understanding of the host immune factors that underlie their persistent risk of TB. To this end, we investigated the association between use of ART and the restoration of immune cell function in the lung. We conducted a prospective

cross-sectional study in healthy, HIV-1-uninfected and asymptomatic HIV-1-infected adults to determine whether ART was associated with improvement in both AM function, assessed by phagosomal proteolysis, and alveolar CD4⁺ T-cell responses to *Mycobacterium* in HIV-infected individuals.

Methods

Subjects

The study was conducted at the Queen Elizabeth Central Hospital, a large teaching hospital in Blantyre, Malawi. Participants were recruited from the hospital's voluntary counseling and testing and ART clinics. They were healthy, asymptomatic adults (≥ 18 yr) comprising HIV-1-uninfected and HIV-1-infected volunteers with no clinical evidence of active disease, willing to undergo bronchoscopy and bronchoalveolar lavage (BAL) for research purposes. HIV testing was performed on whole blood using two commercial point-of-care rapid HIV test kits, Determine HIV 1/2 kit (Abbott Diagnostic Division, Abbott Park, IL) and Unigold HIV 1/2 kit (Trinity Biotech Inc., Bray, Ireland). A participant was considered HIV-uninfected if the test was negative by both kits or HIV-infected if the test was positive by both kits. If Determine and Unigold results were discordant, a third rapid test using Bioline HIV 1/2 kit (Standard Diagnostics Inc., Gyeonggi-do, Republic of Korea) was performed to resolve the discordance.

Table 1. Demographics of Study Participants

	HIV-uninfected (n = 35)	HIV-infected ART-Naïve (n = 25)	HIV-infected ART < 4 yr (n = 28)	HIV-infected ART $\geq$ 4 yr (22)
Age, yr, median (range)	30 (18–45)	29 (20–44)	32 (20–50)	37 (21–46)
Sex (M:F)	24:11	9:16	8:20	7:15
Blood CD4 count, cells/ μ l, median (IQR)	799 (642–942)	395 (249–558)	450 (331–640)	445 (286–604)
Alveolar macrophage count, 10 ⁶ cells/100 ml, median (IQR)	12.4 (10.4–19.1)	15.1 (10.0–21.8)	17.2 (10.0–28.3)	22.0 (15.2–27.3)
BAL lymphocyte count, 10 ⁶ cells/100 ml, median (IQR)	5.8 (3.6–11.7)	8.7 (4.9–17.5)	10.0 (5.6–16.4)	7.2 (4.8–16.5)
BAL CD4 count, 10 ⁶ cells/100 ml, median (IQR)	3.0 (1.5–6.2)	2.5 (1.7–5.1)	2.7 (1.5–5.3)	2.6 (1.6–4.2)
BAL CD8 count, 10 ⁶ cells/100 ml, median (IQR)	1.8 (0.9–4.3)	3.5 (1.2–4.8)	5.3 (2.0–7.0)	3.2 (2.2–8.0)
Plasma viral load, copies/ml, median (IQR)	N/A	28,131 (618–262,032)	0 (0–149)	0 (0–75)
Years on ART, mean (range)	N/A	N/A	1.6 (0.3–3.9)	6.8 (4.9–8.9)
Stavudine/lamivudine/nevirapine	N/A	N/A	16	22
Tenofovir/lamivudine/efavirenz	N/A	N/A	12	0

Definition of abbreviations: ART = antiretroviral therapy; BAL = bronchoalveolar lavage; IQR = interquartile range; N/A = not applicable.

Figure 1. Impaired alveolar macrophage proteolysis function in HIV-infected adults on antiretroviral therapy (ART) for less than 4 years. Adherent alveolar macrophages were incubated with DQ-BSA reporter beads for 10 minutes or 240 minutes to measure phagosomal bulk proteolysis. The cells were acquired on a flow cytometer and the activity index was determined. Data were log transformed. Gray bars represent geometric mean and black horizontal lines represent confidence intervals. Data were analyzed using one-way analysis of variance and Student *t* test (HIV⁺ ART naive, *n* = 18; HIV⁺ ART < 4 yr, *n* = 15; HIV⁺ ART ≥ 4 yr, *n* = 16; HIV⁻, *n* = 19).

HIV-1-infected participants were divided into three subgroups based on the time on ART at the time of recruitment:

(1) ART-naïve, (2) on ART for less than 4 years, and (3) on ART for greater than or equal to 4 years. First-line ART consisted

of stavudine, lamivudine, and nevirapine, or tenofovir, lamivudine, and efavirenz (following a change in the national guidelines in 2011). Exclusion criteria for the study were current or past history of smoking, use of immunosuppressive drugs, severe anemia (hemoglobin < 8 g/dl), and known or suspected pregnancy. Peripheral blood CD4⁺ T-cell counts were measured in all participants, and HIV plasma viral load measurements were performed in HIV-infected participants only. The research =ethics committee of the Malawi College of Medicine approved the study and all participants provided written-informed consent.

Collection and Processing of BAL and Blood Samples

BAL samples were collected at bronchoscopy and processed as previously described (5, 12). AM were isolated from BAL cells by adherence to plastic (6) and adherent cells were used for assessment of AM phagosomal proteolysis; nonadherent BAL cells, which were predominantly lymphocytes, were used to assess intracellular

Figure 2. Representative flow cytometry dot plot from an HIV-uninfected adult showing multiple subsets of antigen-specific CD4⁺ T cells in bronchoalveolar lavage (BAL). Nonadherent BAL cells and peripheral blood mononuclear cells were stimulated overnight with purified protein derivative or influenza vaccine for 18 hours and CD4⁺ T-cell responses were measured by intracellular cytokine staining. The dot plots were obtained by gating on singlets, lymphocytes, live cells, CD3⁺ cells, CD4⁺ cells, and combination of three cytokines. They show the frequency (percentage) of IFN-γ-, tumor necrosis factor (TNF)-α-, and/or IL-17-producing CD4⁺ T cells in BAL, in an unstimulated negative control and cells stimulated with PMA/ionomycin (positive control), mycobacteria, and influenza virus antigens. PMA = phorbol myristate acetate.

cytokine production by alveolar CD4⁺ T cells. Peripheral blood samples were also collected from participants. Plasma samples were used for measurements of HIV viral load, whereas peripheral blood mononucleated cells were used to assess intracellular cytokine production by blood CD4⁺ T cells. Because of limitations in cell numbers, not all the assays were performed on cells obtained from every participant.

Measurement of AM Phagosomal Proteolysis

Phagosomal proteolysis in AM was measured using a flow cytometry-based

reporter bead assay as described previously (6, 13). The readout for the assay was Activity Index, which was calculated by first determining the ratio of median fluorescence intensity of the reporter over calibration flour at 10-minute and 240-minute time points, and then dividing the ratio at 240 minutes by ratio at 10 minutes.

Intracellular Cytokine Staining

Antigen-specific CD4⁺ T-cell responses were measured as previously described (5). In brief, peripheral blood mononucleated cells (1×10^6 cells per well) and nonadherent BAL cells (0.5×10^6 cells per well)

suspended in 200 μ l of complete media were cultured in 96-well plates and stimulated with purified protein derivative (10 μ g/ml; Statens Serum Institut, Copenhagen) and influenza vaccine (0.45 μ g/ml; Sanofi Pasteur MSD, United Kingdom). Cells were stained with Violet Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, United Kingdom), surface markers (anti-CD3 phycoerythrin [PE]-Cy5, anti-CD4 allophycocyanin [APC]-H7, and anti-CD8 PE-Cy7 antibodies; BD Bioscience, United Kingdom), and intracellular markers (anti-IFN- γ APC, anti-tumor necrosis factor [TNF]- α Alexa Fluor 488, and anti-IL17 PE antibodies;

Figure 3. Impaired total mycobacteria-specific alveolar Th1 CD4⁺ T-cell responses in HIV-infected adults on antiretroviral therapy (ART) for less than 4 years. Nonadherent bronchoalveolar lavage (BAL) cells were stimulated overnight with purified protein derivative and influenza vaccine. Cells were analyzed by flow cytometry for IFN- γ and tumor necrosis factor production by the CD4⁺ T-cell population. (A and C) Mycobacterium-specific responses in BAL and peripheral blood mononuclear cells (PBMCs). (B and D) Influenza-specific responses in BAL and PBMCs. The data were log transformed and are shown as the total frequency of antigen-specific Th1 IFN- γ - and tumor necrosis factor-producing CD4⁺ T cells. The horizontal bars represent geometric mean and confidence interval. Data were analyzed using one-way analysis of variance and Student *t* test (HIV⁻, *n* = 32; HIV⁺ ART naive, *n* = 25; HIV⁺ ART < 4 yr, *n* = 28; HIV⁺ ART ≥ 4 yr, *n* = 22).

BD Bioscience). Approximately 50,000 events were acquired in the CD4⁺ gate using a CyAn ADP 9 color flow cytometer (Beckman Coulter). Flow cytometry data were analyzed using FlowJo software (TreeStar).

Statistical Analyses

Statistical analyses and graphical presentation were performed using GraphPad Prism 5 (GraphPad Software) or PESTLE 1.7 and SPICE 5.3 (both NIAID). The programs PESTLE and SPICE were kindly provided by Mario Roederer, Vaccine Research Center, NIAID, National Institutes of Health. Flow cytometry data were log transformed and analyzed using one-way analysis of variance and Student *t* test. SPICE histograms were analyzed using Student *t* test. Results are given as geometric mean with confidence intervals (CI), except SPICE histograms, which are given as mean and SEM. Differences were considered statistically significant when *P* less than 0.05.

Results

Participant Characteristics

We recruited 35 HIV-uninfected and 75 HIV-infected adults of whom 25 were ART-naïve, 28 had received ART for less than 4 years, and 22 had received ART for greater than or equal to 4 years. All study participants had received bacillus Calmette-Guérin vaccination during childhood (purified protein derivative skin testing or γ -IFN release assays were not performed). Their characteristics are summarized in Table 1.

AM Phagosomal Proteolysis Function Is Impaired in HIV-infected Adults on ART during the Early Years of Treatment

The digestion of particulate matter and microbes by proteolysis following phagocytosis is required for both microbicidal activity and antigen presentation (13, 14). Recently, we reported that AM phagosomal proteolysis function is impaired in asymptomatic HIV-infected ART-naïve adults (6). To determine whether use of ART is associated with improvement in this functional defect, we used the flow cytometry-based reporter bead assay to measure phagosomal proteolysis in AM

Figure 4. Impaired polyfunctional mycobacteria-specific alveolar CD4⁺ T cells in HIV-infected adults on antiretroviral therapy (ART). Nonadherent bronchoalveolar lavage (BAL) cells were stimulated overnight with (A) purified protein derivative and (B) influenza vaccine. Cells were analyzed by flow cytometry for IFN- γ , tumor necrosis factor (TNF), and IL-17 production by the CD4⁺ T-cell population. Data are shown as relative distribution of IFN- γ , TNF, and IL-17-producing CD4⁺ T-cell subsets within the total response. Bar charts represent the mean and SEM of the contribution of the indicated subset (x axis) toward the total antigen-specific CD4⁺ T-cell response against the indicated participant groups (color coded as shown). *P* values were computed using Student *t* test (denoted as "+" if *P* < 0.05), comparing each group against HIV-uninfected adults. Each pie chart represents the

from HIV-uninfected and HIV-infected ART-naïve adults and those on ART for less than or greater than or equal to 4 years. We found that AM proteolysis was lower in HIV-infected ART-naïve adults (geometric mean log activity index, 0.09 [CI, 0.07–0.12] vs. 0.16 [CI, 0.13–0.19]; $P = 0.004$) and HIV-infected individuals who had been on ART less than 4 years (geometric mean log activity index, 0.09 [CI, 0.06–0.14] vs. 0.16 [CI, 0.13–0.19]; $P = 0.05$) compared with HIV-uninfected participants (Figure 1). However, there was no difference in AM proteolysis between HIV-infected individuals who had been on ART for greater than or equal to 4 years and HIV-uninfected participants (geometric mean log activity index, 0.17 [CI, 0.14–0.21] vs. 0.16 [CI, 0.13–0.19]; $P = 0.39$) (Figure 1). These findings suggest that in asymptomatic HIV-infected individuals on ART, AM phagosomal proteolysis function is impaired during the early years of treatment.

Low Frequency of Total Mycobacteria-Specific Cytokine-Producing Alveolar Th1 CD4⁺ T Cells in HIV-infected Adults on ART during the Early Years of Treatment

Next, we investigated Th1 and Th17 mycobacteria- and influenza-specific CD4⁺ T-cell responses in BAL and peripheral blood using an intracellular cytokine-staining assay. Th1 CD4⁺ T cells were defined by their production of IFN- γ and/or TNF, whereas Th17 cells were defined by their production of IL-17. A representative flow cytometry dot plot of BAL CD4⁺ T-cell responses from an HIV-uninfected adult is shown in Figure 2.

We found that the frequencies of total mycobacteria- and influenza-specific cytokine-producing alveolar Th1 CD4⁺ T cells were lower in HIV-infected ART-naïve adults compared with HIV-uninfected adults (mycobacteria, 0.5% [0.2–1.3] vs. 1.7% [1.0–2.8], $P = 0.01$; influenza, 0.2% [0.1–0.6] vs. 0.9% [0.4–1.6], $P = 0.02$) (Figures 3A and 3B). In HIV-infected participants receiving ART, the frequency of total mycobacteria-specific cytokine-producing alveolar Th1 CD4⁺ T cells was lower in those on ART for

less than 4 years compared with HIV-uninfected individuals (0.5% [0.2–1.3] vs. 1.7% [1.0–2.8]; $P = 0.02$), but was not different between individuals on ART for greater than or equal to 4 years and HIV-uninfected adults (1.6% [0.7–3.9] vs. 1.7% [1.0–2.8]; $P = 0.90$) (Figure 3A).

In contrast, there was no difference in the frequency of total influenza-specific cytokine-producing alveolar Th1 CD4⁺ T cells between HIV-infected participants on ART and HIV-uninfected individuals (ART < 4 yr, 1.1% [0.7–1.8] vs. 0.9% [0.4–1.6], $P = 0.58$; ART $\geq$ 4 yr, 0.9% [0.7–1.4] vs. 0.9% [0.4–1.6], $P = 0.83$) (Figure 3B). There was no significant difference in the frequencies of mycobacteria- and influenza-specific cytokine-producing alveolar Th17 CD4⁺ T cells between HIV-uninfected and HIV-infected ART-naïve adults (mycobacteria, 0.10% [0.05–0.17] vs. 0.08% [0.03 vs. 0.19], $P = 0.89$; influenza, 0.05% [0.02–0.09] vs. 0.02% [0.01–0.04], $P = 0.14$) or between HIV-uninfected adults and HIV-infected participants on ART (mycobacteria: ART < 4 yr, 0.10% [0.05–0.17] vs. 0.16% [0.07–0.39], $P = 0.30$; ART $\geq$ 4 yr, 0.10% [0.05–0.17] vs. 0.16% [0.05–0.54], $P = 0.24$) (influenza: ART < 4 yr, 0.05% [0.02–0.09] vs. 0.08% [0.04–0.18], $P = 0.23$; ART $\geq$ 4 yr, 0.05% [0.02–0.09] vs. 0.08% [0.03–0.23], $P = 0.26$) (see Figures E1A and E1B in the online supplement).

In blood, we found no difference in the frequencies of total Th1 and Th17 mycobacteria- and influenza-specific cytokine-producing CD4⁺ T cells between HIV-uninfected and HIV-infected adults in the three subgroups (all $P > 0.05$) (Figures 3C and 3D; see Figures E1C and E1D). Furthermore, the frequencies of total antigen-specific Th1 CD4⁺ T cells in BAL and peripheral blood did not correlate with total CD4⁺ T-cell counts in BAL and blood, respectively (see Figure E2). Our data suggest that HIV-associated depletion of mycobacteria- and influenza-specific Th1 CD4⁺ T cells differs between the lung and peripheral blood compartments. They also suggest that in asymptomatic HIV-infected adults on ART, the frequency of total mycobacteria-specific cytokine-producing alveolar Th1 CD4⁺ T cells is low during the early years of ART, which is in contrast to

the normal frequency of total influenza-specific cytokine-producing alveolar Th1 CD4⁺ T cells seen in individuals on ART.

Low Mycobacteria-Specific Polyfunctional Alveolar and Blood Th1 CD4⁺ T-Cell Response in HIV-infected Adults on ART

The previous analysis compared the total Th1 and Th17 populations of mycobacteria- and influenza-specific cytokine-producing alveolar and blood CD4⁺ T cells between HIV-infected and HIV-uninfected adults but did not determine changes in individual antigen-specific CD4⁺ T-cell subsets. To assess the impact of HIV infection on antigen-specific cytokine-producing CD4⁺ T-cell subsets, we used the intracellular cytokine-staining assay to determine the relative contribution of mycobacteria- and influenza-specific CD4⁺ T-cell subsets in the total responses, identified by the ability to produce IFN- γ , TNF, and IL-17 in different combinations. The analysis allowed identification of seven different subsets: (1) IFN- γ ⁺TNF⁺IL17⁺, (2) IFN- γ ⁺TNF⁺IL17[−], (3) IFN- γ ⁺TNF[−]IL17⁺, (4) IFN- γ [−]TNF⁺IL17⁺, (5) IFN- γ ⁺TNF[−]IL17[−], (6) IFN- γ [−]TNF[−]IL17[−], and (7) IFN- γ [−]TNF[−]IL17⁺ cells.

First, we determined the dominant mycobacteria- and influenza-specific cytokine-producing CD4⁺ T-cell subsets in BAL and blood from HIV-uninfected adults. In BAL, we found that IFN- γ ⁺TNF⁺ double-producing (DP; *red*), IFN- γ ⁺ single-producing (SP; *pink*), and TNF⁺ SP (*purple*) cells were the dominant mycobacteria- and influenza-specific CD4⁺ T-cell subsets (mycobacteria, IFN- γ ⁺TNF⁺ DP 36%, IFN⁺ SP 22%, and TNF⁺ SP 24%; influenza, IFN- γ ⁺TNF⁺ DP 20%, IFN⁺ SP 48%, and TNF⁺ SP 18%) (Figures 4A and 4B). In blood, IFN- γ ⁺TNF⁺ DP and TNF⁺ SP cells were the dominant mycobacteria- and influenza-specific CD4⁺ T-cell subsets (mycobacteria, IFN- γ ⁺TNF⁺ DP 50% and TNF⁺ SP 22%; influenza, IFN- γ ⁺TNF⁺ DP 44% and TNF⁺ SP 38%) (Figures 5A and 5B). These data show that cells that simultaneously produce IFN- γ and TNF (IFN- γ ⁺TNF⁺ DP) form an integral part of the mycobacteria- and influenza-specific CD4⁺ T-cell

Figure 4. (Continued). mean distribution across subjects of different antigen-specific cytokine-producing CD4⁺ T-cell subsets (color coded as shown) within the total response in a particular group. (C) Relative distribution of polyfunctional IFN- γ ⁺ and TNF-producing CD4⁺ T cells within the total response. (HIV[−], $n = 32$; HIV⁺ ART naïve, $n = 25$; HIV⁺ ART < 4 yr, $n = 24$; HIV⁺ ART $\geq$ 4 yr, $n = 15$).

responses in BAL and peripheral blood in HIV-uninfected adults.

Next, we compared the relative contribution of mycobacteria- and influenza-specific cytokine-producing $CD4^+$ T-cell subsets within the total responses between HIV-infected and HIV-uninfected adults. We found that the relative contribution of mycobacteria-specific $IFN-\gamma^+TNF^+ DP CD4^+$ T cells in the total response was lower in HIV-infected ART-naïve compared with HIV-uninfected adults, both in BAL (20% vs. 36%; $P = 0.017$) and blood (26% vs. 50%; $P = 0.006$) (Figures 4C and 5C). The relative contribution of influenza-specific $IFN-\gamma^+TNF^+ DP CD4^+$ T cells in the total response was lower in HIV-infected ART-naïve compared with HIV-uninfected adults, both in BAL (8% vs. 20%; $P = 0.018$) and blood (26% vs. 44%; $P = 0.006$) (Figures 4C and 5C). These findings suggest that mycobacteria- and influenza-specific polyfunctional $CD4^+$ T-cell responses are impaired in the lung and peripheral blood after HIV infection.

Following the observation that mycobacteria- and influenza-specific polyfunctional $CD4^+$ T-cell responses are impaired in HIV-infected ART-naïve adults, we investigated whether they are also impaired in individuals on ART. We found that the relative contribution of mycobacteria-specific $IFN-\gamma^+TNF^+ DP CD4^+$ T cells in the total response was lower in HIV-infected individuals on ART compared with HIV-uninfected adults, both in BAL (<4 yr ART, 20% vs. 36%, $P = 0.017$; ≥ 4 yr ART, 23% vs. 36%, $P = 0.046$) and blood (<4 yr ART, 30% vs. 50%, $P = 0.023$; ≥ 4 yr ART, 26% vs. 50%, $P = 0.005$) (Figures 4C and 5C). However, the relative contribution of influenza-specific alveolar $IFN-\gamma^+TNF^+ DP CD4^+$ T cells in the total response was lower in BAL in HIV-infected individuals on ART compared with HIV-uninfected adults (<4 yr ART, 10% vs. 20%, $P = 0.045$; ≥ 4 yr ART, 4% vs. 10%, $P = 0.001$), but was normalized in peripheral blood in both ART groups (all $P > 0.05$) (Figures 4C and 5C). These data suggest that asymptomatic HIV-infected adults on ART have impaired mycobacteria-specific polyfunctional $CD4^+$ T-cell responses in BAL and peripheral blood irrespective of duration of treatment. They also suggest differences in antigen-specific polyfunctional $CD4^+$ T-cell responses between pathogens, which in the case of

Figure 5. Impaired polyfunctional mycobacteria-specific blood $CD4^+$ T cells in HIV-infected adults on antiretroviral therapy (ART). Peripheral blood mononuclear cells (PBMCs) were stimulated overnight with (A) purified protein derivative and (B) influenza vaccine. Cells were analyzed by flow cytometry for IFN- γ , tumor necrosis factor (TNF), and IL-17 production by the $CD4^+$ T-cell population. Data are shown as relative distribution of IFN- γ , TNF, and IL-17-producing $CD4^+$ T-cell subsets within the total response. Bar charts represent the mean and SEM of the contribution of the indicated subset (x axis) toward the total antigen-specific $CD4^+$ T-cell response against the indicated participant groups (color coded as shown). P values were computed using Student t test (denoted as “+” if

influenza-specific polyfunctional CD4⁺ T-cell responses seems compartmentalized.

Discussion

HIV impairs pulmonary innate and antigen-specific adaptive immune responses leading to increased risk to lower respiratory tract infections (LRTI). Although morbidity and mortality caused by LRTI has fallen dramatically among HIV-infected adults on ART (7–10), they still remain at higher risk of TB than their HIV-uninfected counterparts (11). In this study, we investigated whether impaired pulmonary innate and antigen-specific adaptive immune responses persist in HIV-infected adults on ART to explain, at least in part, their increased susceptibility to pulmonary TB. Although our findings do not directly demonstrate causality, they are consistent with a delay in the reversal of impaired pulmonary innate immune responses and an incomplete reconstitution of mycobacteria-specific CD4⁺ T-cell responses.

We have shown that compared with HIV-uninfected adults, AM phagosomal proteolysis was not impaired in HIV-infected adults who had been on ART for greater than or equal to 4 years but was impaired in those on ART for less than 4 years. The temporal association between recovery of AM proteolysis and duration of ART is consistent with previous studies that profiled the kinetics of peripheral blood CD4⁺ T-cell count recovery during ART (7, 8). AM are key effectors of innate immunity in the lung (15) and constitute over 90% of immune cells in BAL from healthy HIV-uninfected adults (16). AM also serve as APCs (17), although they are less efficient than pulmonary dendritic cells (18). In the alveolar space, primary CD4⁺ T-cell immune responses are initiated by dendritic cells, whereas secondary responses are initiated by both dendritic cells and AM (17, 18). Costimulation is essential for initiation of primary CD4⁺ T-cell responses (19). AM do not initiate these responses due to defective costimulation via the CD28 pathway because they do not express B7-1 or B7-2 antigens (20).

Secondary CD4⁺ T-cell responses, however, are less dependent on accessory costimulatory signals (19); consequently, AM are able to initiate these responses (17). We speculate that impaired AM proteolysis affects antigen processing and presentation (21), resulting in suboptimal initiation of antigen-specific alveolar CD4⁺ T-cell responses. Impaired AM innate immune functions may, therefore, leave HIV-infected individual on ART vulnerable to LRTI.

Antigen-specific CD4⁺ T-cell responses are important in defense against common respiratory pathogens including Mtb (22–24) and influenza virus (25). Consistent with our previous observations (5) and those of others (4), we found low frequencies of total mycobacteria- and influenza-specific cytokine-producing Th1 CD4⁺ T cells in BAL but not peripheral blood in asymptomatic HIV-infected adults. However, the frequencies of total antigen-specific Th1 CD4⁺ T cells were normalized in individuals on ART, with influenza-specific CD4⁺ T cells normalizing earlier than mycobacteria-specific CD4⁺ T cells. Together, these data suggest that impaired respiratory pathogen-specific CD4⁺ T-cell responses are compartmentalized in the lung in HIV-infected adults, and normalization of the total Th1 mycobacteria-specific alveolar CD4⁺ T cells requires longer duration on ART.

Although the frequency of total mycobacteria- and influenza-specific cytokine-producing Th1 CD4⁺ T cells in BAL was normal in HIV-infected adults who had been on ART for greater than or equal to 4 years, the proportion of some CD4⁺ T-cell subsets within the total population were altered. Specifically, the proportion of mycobacteria- and influenza-specific IFN-γ⁺TNF-γ⁺ DP CD4⁺ T cells were lower than those observed in HIV-uninfected adults. IFN-γ and TNF play important roles in defense against Mtb (1, 9, 22) and influenza virus infections (26, 27). Our findings suggest preferential depletion of these antigen-specific polyfunctional CD4⁺ T cells by HIV and incomplete recovery during long-term

ART. Polyfunctional CD4⁺ T cells correlate with control of viral pathogens in humans (28, 29) and protection against disease progression in murine models of bacterial pathogens (30), including Mtb (31). Given the importance of IFN-γ and TNF in host defense against Mtb and influenza virus, it is plausible that incomplete restoration of mycobacteria- and influenza-specific IFN-γ and TNF-producing alveolar CD4⁺ T-cell responses early during ART would put the host at increased risk of developing active TB (11) and influenza-associated pathology (32). In support of this hypothesis, the highest mortality caused by HIV-related LRTI occurs during the early years following initiation of ART (33–35).

We also explored the impact of HIV infection and ART on antigen-specific cytokine-producing Th17 CD4⁺ T cells in BAL and blood. Previous studies reported lower mycobacteria-specific cytokine-producing peripheral blood Th17 CD4⁺ T cells in TB patients compared with persons with latent TB infection. Furthermore, patients with severe TB had significantly lower Th17 response than those with mild disease (36). However, there are limited data on IL-17 in the alveolar compartment because previous studies were hampered by difficulties in detecting alveolar Th17 cells because of their very low frequencies (37) and low concentrations of IL-17 in diluted BAL fluid (38). Consistent with the findings of Semple and coworkers (39), we detected low frequencies of mycobacteria-specific cytokine-producing Th17 CD4⁺ T cells in BAL and found no significant difference between healthy HIV-uninfected and asymptomatic HIV-infected adults, irrespective of their ART duration status. These findings suggest that susceptibility to TB in HIV-infected adults on ART may not be caused by depletion of mycobacteria-specific alveolar Th17 CD4⁺ T cells.

The cross-sectional nature of this study constitutes a limitation. Ideally, a longitudinal study sampling at different time points following initiation of ART would have allowed more detailed comparisons at the level of individual participants. However, given the invasive

Figure 5. (Continued). $P < 0.05$), comparing each group against HIV-uninfected adults. Each pie chart represents the mean distribution across subjects of different antigen-specific cytokine-producing CD4⁺ T-cell subsets (color coded as shown) within the total response in a particular group. (C) Relative distribution of polyfunctional IFN-γ- and TNF-producing CD4⁺ T cells within the total response. (HIV⁻, n = 32; HIV⁺ ART naive, n = 25; HIV⁺ ART < 4 yr, n = 28; HIV⁺ ART ≥ 4 yr, n = 22).

nature and the associated risk of bronchoscopy, especially in HIV-infected persons, a longitudinal study was judged not to be justifiable. Furthermore, the alveolar lymphocyte counts from HIV-uninfected individuals were higher than has been reported previously (4, 5, 40). Household indoor air pollution is highly prevalent in Malawi (41) and it is possible that this group of individuals had ongoing alveolar inflammation. Nonetheless, the abnormal alveolar lymphocyte cell count did not correlate with frequency of mycobacteria- and influenza-specific alveolar Th1 CD4⁺ T cells, and does not alter the main findings and conclusion of the study.

In conclusion, we have shown that asymptomatic HIV-infected adults on ART have impaired AM phagosomal proteolysis and low frequencies of total mycobacteria-specific cytokine-producing alveolar Th1 CD4⁺ T cells, most marked during the first

few years of treatment. These defects are not present in those who receive ART for greater than or equal to 4 years. In contrast, mycobacteria antigen-specific polyfunctional alveolar and blood CD4⁺ T-cell responses are impaired in HIV-infected adults on ART irrespective of the duration of treatment. Persistence of these immune defects particularly early during ART may in part explain the high susceptibility to pulmonary TB among HIV-infected individuals on ART.

These findings have important public health implications for developing strategies to reduce the high burden of TB in patients initiating ART. Use of TB preventive chemoprophylaxis during the first 4 years of ART has recently been shown to be a possible intervention strategy in a clinical trial (42). Although the trial did not address the underlying reasons for the high TB incidence, it showed that isoniazid prophylaxis reduced the

incidence of TB when used in combination with ART. To the best of our knowledge, our study is the first to show that HIV-infected adults on ART still have impaired anti-TB adaptive immune responses in the lung. Alternatively, the development of subunit vaccines that promote repopulation of the lung with crucial mycobacteria-specific polyfunctional CD4⁺ T-cell subsets ought to be considered. Irrespective of the strategy, this study identifies early ART as a period of particular vulnerability in the treatment of HIV infections. ■

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