Published in final edited form as:

Am J Respir Crit Care Med. 2009 October 1; 180(7): 674–683. doi:10.1164/rccm.200904-0568OC.

Dissection of Regenerating T-Cell Responses against Tuberculosis in HIV-infected Adults Sensitized by *Mycobacterium tuberculosis*

Katalin A. Wilkinson^{1,2,3}, Ronnett Seldon¹, Graeme Meintjes^{1,4}, Molebogeng X. Rangaka¹, Willem A. Hanekom^{1,5}, Gary Maartens³, and Robert J. Wilkinson^{1,2,3,4,6}

¹Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa

²National Institute for Medical Research, Mill Hill, London, United Kingdom

³Department of Medicine, University of Cape Town, South Africa

⁴Infectious Diseases Unit, GF Jooste Hospital, Cape Town, South Africa

⁵School of Child and Adolescent Health, University of Cape Town, South Africa

⁶Division of Medicine, Imperial College London, United Kingdom

Abstract

Rationale—Combination antiretroviral treatment (cART) reduces the risk of tuberculosis in HIV-infected people. Therefore a novel approach to gain insight into protection against tuberculosis is to analyze the T cells that expand in people sensitized by *Mycobacterium tuberculosis* (MTB) during cART.

Objectives—To longitudinally analyze CD4 T-cell subsets during the first year of cART, from the time of starting cART (Day 0), in 19 HIV-infected, MTB-sensitized adults.

Methods—Peripheral blood mononuclear cells were obtained on Day 0, Weeks 2, 4, 12, 24, 36, and 48 of cART and were stimulated with purified protein derivative (PPD) followed by flow cytometry to analyze surface markers and intracellular cytokines.

Measurements and Main Results—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 CD27⁺CD45RA[−] and CD27⁺CCR5[−] CD4⁺ cells expanded by 12 weeks ($P < 0.02$) followed by naive CD27⁺CD45RA⁺ cells at 36 weeks ($P = 0.02$). Terminally differentiated effector CD4⁺CD27[−]CCR7[−] cells decreased by 12 weeks ($P = 0.02$), paralleled by a

Correspondence and requests for reprints should be addressed to Katalin A. Wilkinson, Ph.D., Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Room S3.13, Wernher & Beit Building South, Observatory 7925, Republic of South Africa. katalin.wilkinson@uct.ac.za.

Conflict of Interest Statement: K.A.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.X.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.A.H. received up to \$1,000 in consultancy fees from GlaxoSmithKline Bio and up to \$1,000 in lecture fees from NFID. G.M. received up to \$1,000 from Abbott and up to \$1,000 from MSD in CME lecture fees. R.J.W. holds patents from the Institut Pasteur, United States 10/994,191, confirmation number 1197 “Recombinant adenylate cyclase of *Bordetella* sp for diagnostic and immunomonitoring uses, method of diagnosing or immunomonitoring using said recombinant adenylate cyclase, and kit for diagnosis.”

proportional decline of PPD-specific CD4⁺IFN- γ ⁺ cells ($P = 0.02$). However, the absolute numbers of PPD-specific IFN- γ -producing cells, determined by enzyme-linked immunospot assay, increased ($P = 0.02$).

Conclusions—Rapid effector responses are often measured when evaluating immunity. We show that although cART is associated with an absolute increase in effector function, the proportional response decreased and the strongest correlate of increased cART-mediated immunity in this study was the central memory response.

Keywords

bacterial infections; RNA viruses; drug therapy

Tuberculosis (TB) is the leading bacterial cause of death in Africa, with an estimated annual incidence of 940 cases per 100,000 population in South Africa (1–3). HIV infection is the greatest risk factor for developing TB, and the rate of coinfection in South Africa is estimated to be 53% (2, 4). These circumstances define the analysis of immune responses to TB and HIV as a pressing research priority. The introduction of combination antiretroviral therapy (cART) in both high- and low-income countries has transformed the prognosis of HIV-infected people in terms of mortality and morbidity (5). This is due to suppression of viral replication allowing an increase in the number of CD4 T cells, and functional restoration of antigen-specific immune responses to several pathogens (6–11).

The immune mechanisms of protection against TB are incompletely defined. In the murine model of TB obligate roles for CD4 T cells, IFN- γ , and tumor necrosis factor (TNF) in controlling acute and latent *Mycobacterium tuberculosis* (MTB) infection have been demonstrated (12–16). In humans the essential role of CD4 T cells can be inferred from the greatly increased risk of active TB in HIV-infected people (17). The protective role of IFN- γ is best demonstrated by the fact that people with mutations in the type 1 cytokine pathway are poorly able to control the replication of ordinarily nonpathogenic mycobacteria (18–20). The rapid reactivation of TB in a proportion of patients treated with the anti-TNF monoclonal antibody infliximab implicates TNF in the maintenance of latent infection (21). Despite these individually well-defined factors that mediate protection against TB, human field studies do not clearly correlate IFN- γ secretion with protection (22, 23). The lack of a definitive correlate of protection or pathology is particularly important in the field of vaccinology (24, 25). In addition, a reliable correlate of protection would help predict more accurately the risk of TB reactivation in latently infected people.

Based on surface phenotype and function, T cells can be divided into naive (which give rise to memory T cells after antigen encounter), central memory (which home to the lymph nodes and need costimulation to perform effector functions), and effector memory/terminally differentiated effector subsets (that home to disease sites and are able to perform immediate effector function without the need for costimulation) (26–31). Different pathogens pose different challenges to the immune system and variation in initial antigen load, antigen persistence, location, and pathway of presentation may all affect T-cell differentiation. HIV coinfection dramatically increases the risk of developing active TB (from 10% over a lifetime to more than 10% per annum), and this risk is increased at all

stages of HIV infection (32). The latter implies that it is not only the numerical reduction in CD4 numbers that predisposes to TB, but also the early loss of specific subsets that mediate protection (33). There is clear evidence that cART is an effective way to reduce the risk of TB in HIV-infected people (34–37). As cART has no direct antimicrobial action (it acts by improving the immune system), we hypothesized that MTB-specific T cells that expand during cART in HIV-MTB coinfecting people mediate protection. Therefore, the aim of this study was to dissect human acquired resistance to TB by characterizing, in a longitudinal analysis, the MTB-specific cell types that expand during cART, thereby rendering a highly susceptible group of people less susceptible to developing active TB.

Some of the results of these studies have been previously reported in the form of abstracts (38, 39).

METHODS

Study Site and Participants

This study was approved by the University of Cape Town Research Ethics Committee (336/2004). HIV-infected adults enrolled into the antiretroviral treatment program, provided by GF Jooste Hospital, Manenberg, Cape Town, were recruited and longitudinally followed up for one year, between April 2005 and September 2007. All patients recruited to the study were informed in their own language, provided a written consent for inclusion, and samples were identified with a study number only. Eligibility for cART was based on a CD4 count of 200 cells/ μ l or less, and/or World Health Organization clinical stage 4 disease, according to South African national guidelines. Exclusion criteria at recruitment were active TB, pregnancy, or age younger than 18 years. Exclusion criteria during the follow-up period were development of TB or immune reconstitution inflammatory disease. The standard first-line antiretroviral combination of stavudine, lamivudine, and either nevirapine or efavirenz was prescribed. The baseline of the study was the day patients started cART (Day 0); follow-up time points were at weeks 2, 4, 12, 24, 36, and 48 into cART. A total of 30 ml venous blood was drawn at each time point for immunological analyses. Ascertainment of *M. tuberculosis* sensitization was by *in vitro* reactivity to either early secretory antigenic target (ESAT)-6 or culture filtrate protein (CFP)-10 in the enzyme-linked immunospot assay (ELISpot).

Twelve HIV-uninfected, MTB-sensitized adults (as determined by either a positive tuberculin skin test or a positive response to either ESAT-6 or CFP-10 in the ELISpot assay) were recruited from the same community (five females, seven males, median age 25 yr) and served as control subjects in parts of the experiments.

Flow Cytometry and Intracellular Cytokine Assay

Peripheral blood mononuclear cells (PBMC) were separated using standard protocols and were always used fresh for flow cytometry after overnight antigen stimulation. A minimum of 2×10^5 or maximum of 5×10^5 PBMC (depending on availability) were plated in 96-U plates in 200 μ l volume RPMI culture media supplemented with 10% FCS. Antigen stimulation and staining were performed in the same 96-U plate. Tuberculin purified protein

derivative (PPD; Statens Serum Institute, Copenhagen, Denmark) was added as stimulating antigen at 10 µg/ml. Staphylococcal enterotoxin B (SEB; Sigma-Aldrich, Gillingham, Dorset, UK) was used as a positive control at 5 µg/ml, whereas the negative control wells were left unstimulated in all experiments. No costimulatory molecules were added. Brefeldin A (5 µg/ml, Sigma-Aldrich, St. Louis, MO) was added 2 hours after antigen stimulation and the plate was incubated overnight at 37°C, 5% CO₂. Staining for flow cytometry was performed a total of 18 hours from plating the cells, exactly as previously described (40, 41). Cells were first pelleted in the 96-U plate at 1,200 rpm for 5 minutes. The culture medium was removed using a multichannel pipette; the cells were loosened by gently vortexing the plate and washed with fluorescence-activated cell sorter (FACS) wash buffer (PBS/ 2% FCS/ 0.1% NaN₃). Cells were stained using the following pretitrated antibodies in various combinations (at 3 µl/well for 20 min at 4°C): CD4-PerCp, CD8-PerCp, CD19-PerCp, CD3-APC, CD27-FITC, CD69-FITC, CD25-FITC, CD62L-PE, CCR5-PE, CCR7-PE, CD45RA-PE, CD45RO-PE, CD56-PE (all from BD Pharmingen, San Diego, CA). After surface staining, cells were removed from the wells and transferred to FACS tubes in FACS fix buffer (PBS/2% FCS/ 0.1% NaN₃ containing 1.6% paraformaldehyde [PFA]). Cells intended for intracellular cytokine staining were left in the wells for permeabilization using 100 µl Cytofix/Cytoperm solution for 20 minutes at 4°C, as described in the BD Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA). After washing, the antibodies for intracellular cytokine staining (IFN γ -APC, TNF-APC, IL-2-FITC, IL-10-PE, all from BD Biosciences) were added in various combinations at pretitrated amounts for 30 minutes at 4°C. Cells were washed again, resuspended in wash buffer, and transferred to FACS tubes for acquisition. A BD-FACS Calibur Flow Cytometer was used to acquire all cells. Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. Data analysis was performed using FlowJo Cytometry Analysis software (TreeStar Inc, Stanford University, FlowJo Africa scheme) by first gating on the lymphocyte population, then selecting out the CD4⁺ cells. Further analysis was restricted to this population only. The combination of various markers was restricted by the number of PBMC available, and limited to four colors. Thus, we combined CD4/CD27/IFN- γ with either CCR5, CCR7, or CD45RA for surface phenotyping. Further combinations included CD4/CD69/CD45RO/IFN- γ , CD3/CD4/CD25/IL-10, and CD4/TNF/IL-2/IL-10 for intracellular cytokines. Cells from all donors reacted to the positive control, and the median proportion of IFN- γ -positive cells on SEB stimulation was 5.74%. The median background using unstained and unstimulated cells was less than 0.01% and was therefore not subtracted.

Measurement of IFN- γ by ELISpot and Ascertainment of *M. tuberculosis* Sensitization

ELISpot analysis was performed on batches of cryopreserved PBMC, as previously described (42–44). Antigenic stimuli were endotoxin free and included the region of difference-1 (RD1) encoded ESAT-6 (Rv3875, 5 µg/ml) and CFP-10 (Rv3874, 2.5 µg/ml), the secreted ESAT-6 homolog protein TB10.3 (Rv3019c, 2.5 µg/ml), α -crystallin (Acr)-2 (Rv0251c, 20 µg/ml), and Acr1 (Rv2031c, 1 µg/ml), as well as PPD (5 µg/ml). Control wells included phytohemagglutinin (5 µg/ml) and no antigenic stimulus.

In some experiments, CD4⁺ cells were depleted from PBMC using Dynabeads conjugated to anti-CD4 monoclonal antibody (Invitrogen Dynal AS, Oslo, Norway) as previously described (45) and set up in the ELISpot assay. Depletion of CD4⁺ cells was found to be 90 ± 5% efficient by flow cytometry. ELISpot plates were read on an Immunospot Series 3B Analyzer (Cellular Technology, Cleveland, OH) and plates were retained for visual inspection and confirmation in the case of anomaly.

Statistical Analysis

Analysis was performed using GraphPad Prism Version 5.0a for Mac OS X. The normality of data was assessed by the D'Agostino and Pearson normality test. Normally distributed data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's posttest for multiple comparisons, or by paired *t* test. Results are quoted as means ± standard deviation. The Kruskal Wallis test with Dunn's posttest correction or Wilcoxon matched pairs test was used for paired data that were not normally distributed and the Mann Whitney U test for unpaired observations. Nonparametrically distributed data are quoted as medians plus the interquartile range (IQR).

RESULTS

Baseline Characteristics and Follow-up of the Study Participants

A total of 28 patients were enrolled to the study. Nine patients were excluded from the analysis for the following reasons: two patients developed tuberculosis, three patients relocated to a different treatment center, one patient defaulted cART, one patient withdrew from the study, one patient died in a car accident, and one patient died before starting cART. The remaining 19 patients completed the study as follows: 13 attended all follow-up visits, 5 missed one follow-up visit, and 1 missed two visits. The median age of the 19 participants included in the final analysis was 35 years (IQR, 32–39); 7 were males and 12 were females. Nine patients had documented evidence of previous TB, but none of the 19 patients included in the final analysis developed active TB during the follow-up period, and none developed opportunistic infections caused by nontuberculous mycobacteria (e.g., *Mycobacterium avium* complex). The median baseline viral load was 103,419 copies/ml (IQR, 11,000–250,000), and fell to low or undetectable levels by 6 months of treatment (Table 1).

When comparing the group of patients who had previous TB (*n* = 9; 8 females) with those who did not (*n* = 10; 4 females), there were no significant differences between median age (34 and 36 yr, respectively), median nadir CD4 count (93 and 94.5, respectively), and median CD4 count at 6 months of cART (312 and 201.5, respectively). The median nadir viral load was higher in the group who had previous TB (179,759 copies/ml) compared with those who did not (67,710 copies/ml); however, the difference was not statistically significant (*P* = 0.6), and viral suppression by 6 months of cART was similar in the two groups. The number of other illnesses during cART (Table 1) was not different between the groups.

cART resulted in a steady increase in absolute CD4 count as well as the percentage of CD4⁺ T cells as determined by flow cytometry. As CD4 counts were not available for all patients

at all time points, comparisons could only be made between Week 0 ($n = 19$), Week 24 ($n = 19$), and Week 48 ($n = 18$) (Table 1). A highly significant increase in absolute CD4 count occurred over 48 weeks ($P < 0.0001$) with the median increasing from a nadir of 93 (IQR, 40–131) to 313 (IQR, 236–434) at Week 48. A similar significant increase in the percentage of CD4⁺ cells occurred ($P < 0.0001$, determined as percentage of all PBMC) from $7.3 \pm 4.8\%$ to $17.4 \pm 7.1\%$ by 48 weeks of cART (Figure 1). By contrast, the proportion of CD8⁺ cells (determined as percentage of all PBMC) did not change significantly over the 48 weeks of cART ($P = 0.94$), with median of 55% (IQR, 39–61) at Week 0 and 53% (IQR, 43–64) at Week 48.

Ascertainment of *M. tuberculosis* Sensitization

Using ELISpot analysis and the RD1-encoded antigens ESAT-6 and CFP-10, we established within the first month of starting cART that all patients enrolled in the study were sensitized by MTB. Patients who we were unable to test at Week 0 were tested at Week 2 or Week 4 of cART. Two patients who had no initial response developed a detectable IFN- γ ELISpot response by 2 weeks into cART. The median summed response to ESAT-6 and CFP-10 was 147 (IQR, 20–843) IFN- γ spot-forming cells (SFC)/ 10^6 PBMC at the time of starting cART ($n = 19$, all patients). The group of patients who had previous TB ($n = 9$) tended to have higher ESAT-6 and CFP-10 ELISpot responses at baseline compared with those who did not (median summed response at Week 0 was 223, IQR, 52–1178, and 137, IQR, 20–451, respectively) but the difference was not statistically significant ($P = 0.55$).

T-cell Phenotypes in Peripheral Blood

Naive CD4⁺ cells—After staining of PPD-stimulated PBMC as described, we gated on CD4⁺ T cells and selected the CD27⁺CD45RA⁺ population (Figure 2). This allowed us to analyze the expansion of naive CD4⁺ T cells during cART. The mean proportion of the naive CD4⁺ T-cell population was $16.1 \pm 12.8\%$ at the time of starting cART. No significant change occurred until Week 36, when it showed an increase to $25.2 \pm 15.3\%$ ($P = 0.017$; Figure 3), sustained at Week 48 ($27 \pm 15.4\%$, $P = 0.008$).

Central memory CD4⁺ cells—We analyzed central memory T cells by their expression of the costimulatory molecule CD27 and lack of expression of CD45RA or the chemokine receptor CCR5. Thus, we first gated on CD4⁺ T cells, selected the CD27⁺CD45RA[−] population, and analyzed in all patients longitudinally. An increase in the percentage of CD4⁺CD27⁺ CD45RA[−] cells from a mean of $34.2 \pm 17.8\%$ to $47.8 \pm 14.6\%$ ($P = 0.001$) occurred between Weeks 0 and 12, and remained significantly higher until Week 48 ($46.3 \pm 12.1\%$, $P = 0.002$; Figure 4A). Significance for trend was $P = 0.03$, as indicated by one-way ANOVA. This observation was corroborated by an increase in the percentage of CD4⁺CD27⁺CCR5[−] cells from $52.3 \pm 29.5\%$ at Week 0 to $58 \pm 22.2\%$ at Week 12, continuing to increase until Week 48 ($67.1 \pm 20.1\%$, $P = 0.02$; Figures 2 and 4B).

Effector CD4⁺ T cells—The chemokine receptor CCR5 is expressed on a subset of effector T cells, including effector memory and terminally differentiated effector cells (46–48). We found that the proportion of CD4⁺ cells expressing CCR5 varied widely between donors (Figure 5A). However, a gradual but steady decrease in median from 5.8% (IQR, 3–

12) at Week 0 was observed throughout, becoming significant at Week 48 of cART (2.7%, IQR, 2.1–4.8; $P = 0.02$). Next, we used the chemokine receptor CCR7, the absence of which differentiates effector cells from central memory cells that express CCR7 and are able to home to lymph nodes (27). T cells lacking CCR7 and CD27 are believed to be of the terminally differentiated phenotype that home to the disease site and exert immediate effector function without the need for costimulation (47, 49). The CD4⁺CD27[−] CCR7[−] population significantly decreased from a mean of $50 \pm 29.1\%$ at Week 0 to $38.2 \pm 20.4\%$ at Week 12 of cART ($P = 0.02$; Figures 2 and 5B). This was sustained over 48 weeks ($32 \pm 20.2\%$, $P = 0.02$ compared with Week 0), supporting the findings described above.

Activated CD4⁺ T cells—The activation markers CD69 and CD25 were also assessed on PPD-stimulated PBMC. Very low levels of CD69 expression on CD4⁺ T cells were observed throughout the study in all patients. The majority of CD4⁺ CD69⁺ cells also expressed CD45RO. Thus, the median proportion of cells positive for CD4⁺CD69⁺CD45RO⁺ was 1.67% (IQR, 1.2–3.3) at Week 0 and 1.3% (IQR, 0.8–1.6) at Week 48. Similarly, the expression of the activation marker CD25 on CD4⁺ cells was also low. The median proportion of CD4⁺CD25⁺ at Week 0 was 2.3% (IQR, 0.85–6.55), whereas at Week 48 it was 1.46% (IQR, 0.91–3.67), with no statistically significant difference between the two time points. The proportion of CD4⁺CD25⁺ cells expressing IL-10 was almost undetectable, with a median of 0.09% (IQR, 0.03–0.29) at Week 0 and 0.02% (IQR, 0.01–0.07) at Week 48 (data not shown).

Cytokine-producing CD4⁺ T Cells during cART

We identified CD4⁺ T cells that produce IFN- γ in response to PPD stimulation by intracellular staining. The median proportion of PPD-specific CD4⁺IFN- γ ⁺ T cells steadily decreased from 0.92% (IQR, 0.28–1.38) at Week 0 to 0.35% (IQR, 0.17–0.52) at Week 48 of cART ($P = 0.021$, Kruskal Wallis test; Figures 2 and 6A). The majority of CD4⁺IFN- γ ⁺ cells displayed a memory phenotype, as more than 80% were also positive for CD45RO throughout the study period (data not shown).

Measuring IFN- γ production alone underestimates the complexity of the host cytokine response to PPD stimulation and may not be an optimal readout as a correlate of protection. Besides IFN- γ production, T cells also secreting IL-2 and TNF recently have been shown to be induced by bacillus Calmette-Guérin (BCG) vaccination in infants (50) as well as in BCG-vaccinated adults boosted by recombinant MVA85A administered as a vaccine (51). We also assayed IL-10 production, which is an important regulator of effector T-cell responses against TB and induced by BCG vaccination in newborns (52). Therefore, CD4⁺ cells were stained for intracellular IL-2, IL-10, or TNF, respectively, after PPD stimulation. There was a significant decline in the proportion of CD4⁺IL-2⁺ T cells during the study period ($P = 0.006$, Kruskal Wallis test; Figure 6B), with the median falling from 0.43% (IQR, 0.27–1.3) at Week 0 to 0.2% (IQR, 0.14–0.36) at Week 48. The proportional change of CD4⁺IL-10⁺ cells was more variable, increasing from a median of 0.36% (IQR, 0.22–0.8) at Week 0 to 0.63% (IQR, 0.21–3.12) at 2 weeks into cART ($P = 0.06$; Figure 6C), but with no overall change during 48 weeks. There was also no significant change in the proportion

of CD4⁺TNF⁺ cells between Week 0 (median 0.34%; IQR, 0.16–1.1) and Week 48 (median 0.31%; IQR, 0.15–0.63) of cART ($P = 0.24$; Figure 6D).

Antigen-specific ELISpot Responses during cART

In the same samples we also enumerated the number of IFN- γ -producing cells in response to PPD by ELISpot analysis. A significant overall increase in the median number of PPD-specific IFN- γ SFC/10⁶ PBMC occurred over 48 weeks of cART ($P = 0.02$, Kruskal Wallis test; Figure 7A), from a median of 7 SFC at Week 0, to 60 at Week 48. Correction for multiple comparisons showed this increase to be most significant at 12 weeks (median 192 SFC/million PBMC, $P < 0.05$).

When summing the antigen-specific response to ESAT-6 and CFP-10 again a significant increase was seen during 48 weeks ($P = 0.04$, one-way ANOVA; Figure 7B). After correcting for multiple comparisons the difference at 36 weeks (611 SFC/million PBMC, 95% confidence limits of the difference being 0–1221) and 48 weeks (700 SFC/million PBMC, 95% confidence interval 120–1282) were most significant ($P < 0.05$), compared with Week 0. Analyzing the RD1 antigen-specific responses according to previous TB status, the increase in the summed response to ESAT-6 and CFP-10 was significantly higher at 48 weeks of cART in the group of patients who did not previously receive TB therapy ($n = 10$, median 1,793 SFC/million PBMC), compared with those who did ($n = 9$, median 548 SFC/million PBMC, $P = 0.01$, data not shown).

Additional antigens tested included two a crystallins (Acr1 encoded by Rv2031c and Acr2 encoded by Rv0251c) and the secreted ESAT-6 homolog protein TB10.3 (Rv3019c). Although there was a considerable inter- and intrasubject variability in the responses over time, the trend again indicated an increase in antigen-specific responses during cART, which were significant for Acr2 ($P = 0.02$; Figure 7C) and TB10.3 ($P = 0.04$; Figure 7D).

To ascertain whether the responding T cells in the ELISpot assay were CD4 or CD8 positive, we depleted CD4-positive cells from PBMC in four donors (after receiving 36–48 wk of cART; two had previous TB and two did not). The ELISpot assay was set up exactly as above, using the same antigens as stimulants. The effect of CD4 cell depletion was a mean decrease in ELISpot response of $82 \pm 11\%$ for ESAT-6, $90 \pm 10\%$ for CFP-10, $86 \pm 12\%$ for Acr1, $75 \pm 7\%$ for Acr2, and $86 \pm 4\%$ for TB10.3 antigens (Figure 7E). These data indicate that CD4-positive cells are the main source of IFN- γ secretion detected by the ELISpot assay in these patients.

Comparison of Cytokine-producing CD4⁺ T cells with Those from HIV-Uninfected People

Finally, we compared the proportion of cytokine-producing CD4⁺ T cells at Week 48 of cART with those seen in 12 HIV-uninfected MTB-sensitized people recruited from the same community. In general the cytokine-producing capacity of the cells from HIV-infected patients receiving cART for nearly a year (48 wk), was significantly lower than that of HIV-uninfected control subjects (Figure 8). The median proportion of CD4⁺IFN- γ ⁺ cells was 0.35% (IQR, 0.17–0.52) compared with 0.73% (IQR, 0.5–0.85) in the HIV-uninfected control subjects ($P = 0.004$). Similarly, the median proportion of CD4⁺IL-2⁺ cells was 0.2%

(IQR, 0.15–0.36) versus 0.6% (IQR, 0.43–1; $P = 0.0001$), and the median proportion of CD4⁺IL-10⁺ cells was 0.31% (IQR, 0.14–0.53) versus 0.84% (IQR, 0.6–2.4; $P = 0.001$).

DISCUSSION

We report the reconstitution of different cell types in HIV-infected, MTB-sensitized patients who started cART in a developing country setting. During 48 weeks of cART we found a decrease in viral load and an increase in CD4 counts and CD4⁺ T-cell percentages, as well as increased numbers of antigen-specific T cells by ELISpot. A significant expansion of central memory cells occurred by 12 weeks, and expansion of naive T cells by 36 weeks. The proportion of terminally differentiated effector cells, however, declined by 12 weeks and was sustained over 48 weeks. Similarly, the proportion of CD4⁺IFN- γ ⁺ and CD4⁺IL-2⁺ cells declined in response to PPD stimulation.

Because the BCG vaccine is universally administered in infancy in South Africa, and the annual rate of TB infection in Cape Town is very high, we assumed that the majority of adults recruited to this study would be sensitized by mycobacteria. Our ELISpot analysis indeed indicated that all 19 participants had *in vitro* evidence of MTB sensitization. Studies in developed countries have defined the overall change in CD4 T-cell composition occurring after the introduction of cART (8, 9, 53–57). However, *in vitro* analyses of the changes induced by cART in the antituberculous immune response have been limited to low-incidence areas (8–10, 58) and no systematic analysis in a very high incidence area has been conducted. Our detailed phenotypic characterization of the PPD-stimulated lymphocytes showed that central memory cells expanded first during cART. CD4⁺ T cells also expressing the costimulatory molecule CD27 and lacking CD45RA expanded by a median of 29%, whereas the expansion of CD4⁺CD27⁺CCR5[–] cells was more modest, albeit also significant. These data point to an early expansion of central memory T cells by 3 months into cART using two different gating strategies and two different combinations of cell surface markers characteristic of central memory T cells.

By contrast the frequency of CD4⁺CCR5⁺ effector cells proportionally declined during 48 weeks of cART, and the frequency of terminally differentiated CD4⁺CD27[–]CCR7[–] effector cell population also significantly declined in the first 12 weeks of cART. Similarly, the proportion of CD4⁺ cells producing IFN- γ in response to stimulation with PPD also declined over 48 weeks of cART. However, at the same time the actual number of PPD-specific T cells as determined by ELISpot analysis significantly increased. The ELISpot assay is based on rapid IFN- γ release (within 6 h of antigen contact), enumerating a population of memory CD4⁺ and CD8⁺ T cells that exhibit rapid effector function without needing to divide and differentiate over several days (42, 59). Our cell depletion experiments demonstrate that CD4⁺ T cells are the main source of IFN- γ secretion in response to stimulation with various MTB antigens in blood, as well as at the site of disease, as shown previously (42). In summary, within the expanding CD4⁺ T cell pool (from 7.3–17.4%) the effector cell population proportionally declines. This trend is also reflected by analyzing PPD-specific effector cells: the median number of PPD-specific cells increases from 7 to 60 (as measured by ELISpot), but proportionally within the whole expanding CD4⁺ population falls from median 0.92 to 0.35% (as measured by flow cytometry) in 48 weeks of cART. These data

reflect a reconstituting host immune response, in which it can be envisaged that a smaller proportion of the host's efforts have to be directed toward a single antigenic target (a concept diagram explaining this is shown in Figure 9). However, when comparing the cytokine-producing capacity of the HIV-infected patients receiving cART for 48 weeks with HIV-uninfected control subjects recruited from the same community, we found that the capacity of the reconstituting CD4⁺ T cells to secrete IFN- γ , IL-2, or IL-10 was still lower than that seen in the healthy control subjects ($P < 0.01$ for all cytokines). These data support earlier findings indicating impaired restoration of fully functional MTB-specific CD4 T-cell responses despite long-term antiretroviral treatment (60, 61). It may also help to explain why TB incidence rates among people receiving cART in Cape Town remains higher than the rate among HIV-uninfected people living in the same community (62, 63).

The naive CD4⁺CD27⁺CD45RA⁺ population expanded significantly by 36 weeks of cART. The major site of T-cell production is the thymus, which is believed to involute after puberty (64). However, HIV-infected patients treated with highly active antiretroviral therapy show a progressive increase in naive T-cell numbers (8, 65). The origin of these naive T cells could be *de novo* thymic synthesis or expansion in the periphery. Studies quantifying thymic output by measuring T-cell receptor excision circles (TRECs, which are formed during T-cell ontogeny in the thymus) have shown that although thymic function declines with age, substantial output is maintained into late adulthood (66). Furthermore, these adult patients have a rapid and sustained increase in thymic output showing that *de novo* synthesis can play a part in naive T-cell reconstitution (67), but peripheral expansion may also contribute (68).

Our study has several limitations. (1) The absence of an HIV-infected group without latent TB infection. Although such a control group would be of interest, it would have been difficult to define such a group in this study as the prevalence of latent tuberculosis among the HIV-infected young adults of this community ranges between 70 and 90% (44, 69, 70). Repeating the study in a low TB incidence area would introduce differences in the genetic background of the studied populations, which would preclude meaningful immunological comparisons. In addition there is no definitive test that can exclude MTB sensitization in HIV-infected people. Work by Autran and colleagues (8) documented the T-cell reconstitution in eight HIV-infected patients in a low TB incidence area. (2) Our experimental protocol was based on 18-hour incubation with PPD that might have resulted in some MTB-specific surface marker expression compared with unstimulated cells. However, we aimed to determine the MTB-specific reconstitution of the different T-cell types in a TB-endemic area, where infection pressure, and thus antigen pressure, is high. (3) Nine of the 19 patients in our study group had documented evidence of previous TB in the years preceding initiation of cART. These patients add a degree of heterogeneity to the study, having received anti-TB therapy previously. Their lower response to the RD1 antigens ESAT-6 and CFP-10 at 48 weeks of cART suggests that their bacterial load is lower, compared with those patients who did not previously benefit from anti-TB therapy. Lower numbers of circulating RD1 antigen-specific cells detectable by the ELISpot assay relate to a lower bacterial load (71). Our data thus fit well with the recent suggestion to consider TB as a spectrum from immunity to disease based on bacterial load (72) and

support implementation of isoniazid preventive therapy in HIV-infected adults. Importantly, there were no differences between the proportions of central memory T cells or cytokine-producing CD4 T cells, as determined by flow cytometry, between the two groups either at Week 0 or at Week 48 of cART. Thus, previous TB disease, which was efficiently treated, does not seem to negatively influence the cART-induced T-cell recovery.

Although rapid effector responses are often measured when evaluating immunity, these correlate poorly with protection in field studies of TB (22, 23). More recently, a poor correlation was described between vaccination-induced T-cell responses and protection against tuberculosis, showing that IFN- γ production by CD4 T cells reflected MTB antigen load rather than protection in mice (73). Human studies in adults and infants indicate that vaccination induces T cells with complex phenotypic and polyfunctional cytokine secreting profiles, of which the less-differentiated central memory phenotype secreting IL-2 is more likely to be associated with protection (50, 51). Further, these polyfunctional MTB-specific T cells have been detected in HIV-infected people, and were inversely correlated with HIV viral load (74). Our data presented here provide insight into the regeneration of the presumed protective immunity during cART in MTB-sensitized patients, showing that cART is associated with an absolute increase in effector function while the proportional response declines. The strongest correlate of increased cART-mediated immunity in this study was the central memory response. This has important implications for vaccine design as well as monitoring vaccine efficiency.

Acknowledgments

The authors thank Priscilla Mouton for her involvement in the recruitment and follow-up of patients, and Dr A. O'Garra (National Institute for Medical Research, Mill Hill, London) for helpful comments on the manuscript.

References

1. Harrison, SBR.; Ntuli, A., editors. South African health review 2007. Health Systems Trust; Durban: 2007.
2. WHO. [Accessed 2008 Dec 28] Global tuberculosis control—surveillance, planning, financing. 2008. Available from: http://www.who.int/tb/publications/global_report/2008/en/
3. Statistics SA. Mortality and causes of death in South Africa, 1997–2003: findings from death notification. Statistics SA, Private Bag X44, Pretoria 0001. 2005
4. John MA, Menezes CN, Chita G, Sanne I, Grobusch MP. High tuberculosis and HIV coinfection rate, Johannesburg. *Emerg Infect Dis*. 2007; 13:795–796. [PubMed: 18044049]
5. Braitstein P, Brinkhof MW, Dabis F, Schechter M, Boulle A, Miotti P, Wood R, Laurent C, Sprinz E, Seyler C, et al. Mortality of HIV-1-infected patients in the first year of antiretroviral therapy: comparison between low-income and high-income countries. *Lancet*. 2006; 367:817–824. [PubMed: 16530575]
6. Li TS, Tubiana R, Katlama C, Calvez V, Ait Mohand H, Autran B. Long-lasting recovery in CD4 T-cell function and viral-load reduction after highly active antiretroviral therapy in advanced HIV-1 disease. *Lancet*. 1998; 351:1682–1686. [PubMed: 9734884]
7. Komanduri KV, Viswanathan MN, Wieder ED, Schmidt DK, Brecht BM, Jacobson MA, McCune JM. Restoration of cytomegalovirus-specific CD41 T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. *Nat Med*. 1998; 4:953–956. [PubMed: 9701250]

8. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R, Katlama C, Debre P, Leibowitch J. Positive effects of combined antiretroviral therapy on CD41 T cell homeostasis and function in advanced HIV disease. *Science*. 1997; 277:112–116. [PubMed: 9204894]
9. Wendland T, Furrer H, Vernazza PL, Frutig K, Christen A, Matter L, Malinverni R, Pichler WJ. Haart in HIV-infected patients: restoration of antigen-specific CD4 T-cell responses in vitro is correlated with CD4 memory T-cell reconstitution, whereas improvement in delayed type hypersensitivity is related to a decrease in viraemia. *AIDS*. 1999; 13:1857–1862. [PubMed: 10513643]
10. Schluger NW, Perez D, Liu YM. Reconstitution of immune responses to tuberculosis in patients with HIV infection who receive antiretroviral therapy. *Chest*. 2002; 122:597–602. [PubMed: 12171838]
11. Battegay M, Nuesch R, Hirschel B, Kaufmann GR. Immunological recovery and antiretroviral therapy in HIV-1 infection. *Lancet Infect Dis*. 2006; 6:280–287. [PubMed: 16631548]
12. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne mycobacterium tuberculosis infection in mice. *J Exp Med*. 2001; 193:271–280. [PubMed: 11157048]
13. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to mycobacterium tuberculosis infection. *J Exp Med*. 1993; 178:2249–2254. [PubMed: 7504064]
14. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989; 56:731–740. [PubMed: 2647299]
15. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor necrosis factor-alpha is required in the protective immune response against mycobacterium tuberculosis in mice. *Immunity*. 1995; 2:561–572. [PubMed: 7540941]
16. Botha T, Ryffel B. Reactivation of latent tuberculosis infection in TNF-deficient mice. *J Immunol*. 2003; 171:3110–3118. [PubMed: 12960337]
17. Maartens G, Wilkinson RJ. Tuberculosis. *Lancet*. 2007; 370:2030–2043. [PubMed: 17719083]
18. Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, Drysdale P, Jouanguy E, Doffinger R, Bernaudin F, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science*. 1998; 280:1432–1435. [PubMed: 9603732]
19. de Jong R, Altare F, Haagen IA, Elferink DG, Boer T, van Breda Vriesman PJ, Kabel PJ, Draaisma JM, van Dissel JT, Kroon FP, et al. Severe mycobacterial and salmonella infections in interleukin-12 receptor-deficient patients. *Science*. 1998; 280:1435–1438. [PubMed: 9603733]
20. Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R, Levin M. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med*. 1996; 335:1941–1949. [PubMed: 8960473]
21. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med*. 2001; 345:1098–1104. [PubMed: 11596589]
22. Ellner JJ, Hirsch CS, Whalen CC. Correlates of protective immunity to mycobacterium tuberculosis in humans. *Clin Infect Dis*. 2000; 30:S279–S282. [PubMed: 10875800]
23. Hoft DF, Worku S, Kampmann B, Whalen CC, Ellner JJ, Hirsch CS, Brown RB, Larkin R, Li Q, Yun H, et al. Investigation of the relationships between immune-mediated inhibition of mycobacterial growth and other potential surrogate markers of protective mycobacterium tuberculosis immunity. *J Infect Dis*. 2002; 186:1448–1457. [PubMed: 12404160]
24. Reece ST, Kaufmann SH. Rational design of vaccines against tuberculosis directed by basic immunology. *Int J Med Microbiol*. 2008; 298:143–150. [PubMed: 17702652]
25. Fletcher H, McShane H. Tuberculosis vaccines: current status and future prospects. *Expert Opin Emerg Drugs*. 2006; 11:207–215. [PubMed: 16634697]
26. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotypic and functional separation of memory and effector human CD81 T cells. *J Exp Med*. 1997; 186:1407–1418. [PubMed: 9348298]

27. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999; 401:708–712. [PubMed: 10537110]
28. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, Appay V, Rizzardi GP, Fleury S, Lipp M, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature*. 2001; 410:106–111. [PubMed: 11242051]
29. Appay V, Dunbar PR, Callan M, Klennerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, et al. Memory CD81 T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med*. 2002; 8:379–385. [PubMed: 11927944]
30. Seder RA, Ahmed R. Similarities and differences in CD41 and CD81 effector and memory T cell generation. *Nat Immunol*. 2003; 4:835–842. [PubMed: 12942084]
31. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*. 2004; 22:745–763. [PubMed: 15032595]
32. Wood R, Maartens G, Lombard CJ. Risk factors for developing tuberculosis in HIV-1-infected adults from communities with a low or very high incidence of tuberculosis. *J Acquir Immune Defic Syndr*. 2000; 23:75–80. [PubMed: 10708059]
33. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. Massive infection and loss of memory CD41 T cells in multiple tissues during acute siv infection. *Nature*. 2005; 434:1093–1097. [PubMed: 15793563]
34. Kirk O, Gatell JM, Mocroft A, Pedersen C, Proenca R, Brettle RP, Barton SE, Sudre P, Phillips AN, EuroSIDA study group jd. Infections with mycobacterium tuberculosis and mycobacterium avium among HIV-infected patients after the introduction of highly active antiretroviral therapy. *Am J Respir Crit Care Med*. 2000; 162:865–872. [PubMed: 10988097]
35. Jones JL, Hanson DL, Dworkin MS, DeCock KM. HIV-associated tuberculosis in the era of highly active antiretroviral therapy. The adult/adolescent spectrum of HIV disease group. *Int J Tuberc Lung Dis*. 2000; 4:1026–1031. [PubMed: 11092714]
36. Badri M, Wilson D, Wood R. Effect of highly active antiretroviral therapy on incidence of tuberculosis in South Africa: A cohort study. *Lancet*. 2002; 359:2059–2064. [PubMed: 12086758]
37. Golub JE, Saraceni V, Cavalcante SC, Pacheco AG, Moulton LH, King BS, Efron A, Moore RD, Chaisson RE, Durovni B. The impact of antiretroviral therapy and isoniazid preventive therapy on tuberculosis incidence in HIV-infected patients in Rio de Janeiro, Brazil. *AIDS*. 2007; 21:1441–1448. [PubMed: 17589190]
38. Wilkinson, KA. Highly active antiretroviral therapy and the restoration of protective responses against tuberculosis in HIV infected people [abstract]; Keystone Symposia: Challenges of Global Vaccine Development; Cape Town, South Africa. 2007;
39. Wilkinson, KA. Combined antiretroviral therapy and the restoration of protective responses against tuberculosis in HIV infected people [abstract]; Seventh International Conference on the Pathogenesis of Mycobacterial Infections; Saltsjobaden, Stockholm, Sweden. 2008;
40. Amyes E, Hatton C, Montamat-Sicotte D, Gudgeon N, Rickinson AB, McMichael AJ, Callan MF. Characterization of the CD41 T cell response to Epstein-Barr virus during primary and persistent infection. *J Exp Med*. 2003; 198:903–911. [PubMed: 12975456]
41. Amyes E, McMichael AJ, Callan MF. Human CD41 T cells are predominantly distributed among six phenotypically and functionally distinct subsets. *J Immunol*. 2005; 175:5765–5773. [PubMed: 16237068]
42. Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, Klennerman P, Maskell N, Davies R, Pasvol G, Lalvani A. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. *Clin Infect Dis*. 2005; 40:184–187. [PubMed: 15614710]
43. Wilkinson KA, Kon OM, Newton SM, Meintjes G, Davidson RN, Pasvol G, Wilkinson RJ. Effect of treatment of latent tuberculosis infection on the T cell response to mycobacterium tuberculosis antigens. *J Infect Dis*. 2006; 193:354–359. [PubMed: 16388482]
44. Rangaka MX, Diwakar L, Seldon R, van Cutsem G, Meintjes GA, Morroni C, Mouton P, Shey MS, Maartens G, Wilkinson KA, et al. Clinical, immunological, and epidemiological importance

- of antituberculosis T cell responses in HIV-infected Africans. *Clin Infect Dis*. 2007; 44:1639–1646. [PubMed: 17516410]
45. Wilkinson KA, Simsova M, Scholvinck E, Sebo P, Leclerc C, Vordermeier HM, Dickson SJ, Brown JR, Davidson RN, Pasvol G, et al. Efficient ex vivo stimulation of mycobacterium tuberculosis-specific T cells by genetically detoxified *Bordetella pertussis* adenylate cyclase antigen toxoids. *Infect Immun*. 2005; 73:2991–2998. [PubMed: 15845506]
 46. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(1) T cells. *J Exp Med*. 2001; 194:1711–1719. [PubMed: 11748273]
 47. Campbell JJ, Murphy KE, Kunkel EJ, Brightling CE, Soler D, Shen Z, Boisvert J, Greenberg HB, Vierra MA, Goodman SB, et al. CCR7 expression and memory T cell diversity in humans. *J Immunol*. 2001; 166:877–884. [PubMed: 11145663]
 48. Mackay CR. Dual personality of memory T cells. *Nature*. 1999; 401:659–660. [PubMed: 10537102]
 49. Fritsch RD, Shen X, Sims GP, Hathcock KS, Hodes RJ, Lipsky PE. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J Immunol*. 2005; 175:6489–6497. [PubMed: 16272303]
 50. Soares AP, Scriba TJ, Joseph S, Harbacheuski R, Murray RA, Gelderbloem SJ, Hawkrigge A, Hussey GD, Maecker H, Kaplan G, et al. *Bacillus Calmette-Guerin* vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J Immunol*. 2008; 180:3569–3577. [PubMed: 18292584]
 51. Beveridge NE, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, Ambrozak DR, Precopio ML, Scheinberg P, Alder NC, et al. Immunisation with BCG and recombinant MVA85a induces long-lasting, polyfunctional mycobacterium tuberculosis-specific CD41 memory T lymphocyte populations. *Eur J Immunol*. 2007; 37:3089–3100. [PubMed: 17948267]
 52. Hanekom WA. The immune response to BCG vaccination of newborns. *Ann N Y Acad Sci*. 2005; 1062:69–78. [PubMed: 16461790]
 53. Evans TG, Bonnez W, Soucier HR, Fitzgerald T, Gibbons DC, Reichman RC. Highly active antiretroviral therapy results in a decrease in CD81 T cell activation and preferential reconstitution of the peripheral CD41 T cell population with memory rather than naive cells. *Antiviral Res*. 1998; 39:163–173. [PubMed: 9833957]
 54. Amendola A, Poccia F, Martini F, Gioia C, Galati V, Pierdominici M, Marziali M, Pandolfi F, Colizzi V, Piacentini M, et al. Irhan study group. Decreased CD95 expression on naive T cells from HIV-infected persons undergoing highly active anti-retroviral therapy (HAART) and the influence of IL-2 low dose administration. *Clin Exp Immunol*. 2000; 120:324–332. [PubMed: 10792383]
 55. Landay AL, Bettendorf D, Chan E, Spritzler J, Schmitz JL, Bucy RP, Gonzalez CJ, Schnizlein-Bick CT, Evans T, Squires KE, et al. Evidence of immune reconstitution in antiretroviral drug-experienced patients with advanced HIV disease. *AIDS Res Hum Retroviruses*. 2002; 18:95–102. [PubMed: 11839142]
 56. Lange CG, Lederman MM, Madero JS, Medvik K, Asaad R, Pacheko C, Carranza C, Valdez H. Impact of suppression of viral replication by highly active antiretroviral therapy on immune function and phenotype in chronic HIV-1 infection. *J Acquir Immune Defic Syndr*. 2002; 30:33–40. [PubMed: 12048361]
 57. Mussini C, Pinti M, Borghi V, Nasi M, Amorico G, Monterastelli E, Moretti L, Troiano L, Esposito R, Cossarizza A. Features of “CD4-exploders,” HIV-positive patients with an optimal immune reconstitution after potent antiretroviral therapy. *AIDS*. 2002; 16:1609–1616. [PubMed: 12172082]
 58. Pontesilli O, Kerkhof-Garde S, Pakker NG, Notermans DW, Roos MT, Klein MR, Danner SA, Miedema F. Antigen-specific T-lymphocyte proliferative responses during highly active antiretroviral therapy (HAART) of HIV-1 infection. *Immunol Lett*. 1999; 66:213–217. [PubMed: 10203057]
 59. Lalvani A, Brookes R, Hambleton S, Britton WJ, Hill AV, McMichael AJ. Rapid effector function in CD81 memory T cells. *J Exp Med*. 1997; 186:859–865. [PubMed: 9294140]

60. Sutherland R, Yang H, Scriba TJ, Ondondo B, Robinson N, Conlon C, Suttill A, McShane H, Fidler S, McMichael A, et al. Impaired IFN-gamma-secreting capacity in mycobacterial antigen-specific CD4 T cells during chronic HIV-1 infection despite long-term HAART. *AIDS*. 2006; 20:821–829. [PubMed: 16549965]
61. Mendonca M, Tanji MM, Silva LC, Silveira GG, Oliveira SC, Duarte AJ, Benard G. Deficient in vitro anti-mycobacterial immunity despite successful long-term highly active antiretroviral therapy in HIV-infected patients with past history of tuberculosis infection or disease. *Clin Immunol*. 2007; 125:60–66. [PubMed: 17631053]
62. Lawn SD, Wood R. Tuberculosis control in South Africa—will HAART help? *S Afr Med J*. 2006; 96:502–504. [PubMed: 16841129]
63. Lawn SD, Badri M, Wood R. Tuberculosis among HIV-infected patients receiving HAART: long term incidence and risk factors in a South African cohort. *AIDS*. 2005; 19:2109–2116. [PubMed: 16284460]
64. Mackall CL, Gress RE. Pathways of T-cell regeneration in mice and humans: implications for bone marrow transplantation and immunotherapy. *Immunol Rev*. 1997; 157:61–72. [PubMed: 9255622]
65. Notermans DW, Pakker NG, Hamann D, Foudraire NA, Kauffmann RH, Meenhorst PL, Goudsmit J, Roos MT, Schellekens PT, Miedema F, et al. Immune reconstitution after 2 years of successful potent antiretroviral therapy in previously untreated human immunodeficiency virus type 1-infected adults. *J Infect Dis*. 1999; 180:1050–1056. [PubMed: 10479130]
66. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. 1998; 396:690–695. [PubMed: 9872319]
67. Ribeiro RM, de Boer RJ. The contribution of the thymus to the recovery of peripheral naive T-cell numbers during antiretroviral treatment for HIV infection. *J Acquir Immune Defic Syndr*. 2008; 49:1–8. [PubMed: 18667918]
68. Kaufmann GR, Zaunders J, Murray J, Kelleher AD, Lewin SR, Solomon A, Smith D, Cooper DA. Relative significance of different pathways of immune reconstitution in HIV type 1 infection as estimated by mathematical modeling. *AIDS Res Hum Retroviruses*. 2001; 17:147–159. [PubMed: 11177394]
69. Connell TG, Shey MS, Seldon R, Rangaka MX, van Cutsem G, Simsova M, Marcekova Z, Sebo P, Curtis N, Diwakar L, et al. Enhanced ex vivo stimulation of mycobacterium tuberculosis-specific T cells in HIV-infected persons via antigen delivery by the *Bordetella pertussis* adenylate cyclase vector. *Clin Vaccine Immunol*. 2007; 14:847–854. [PubMed: 17522328]
70. Rangaka MX, Wilkinson KA, Seldon R, Van Cutsem G, Meintjes GA, Morroni C, Mouton P, Diwakar L, Connell TG, Maartens G, et al. Effect of HIV-1 infection on T-cell-based and skin test detection of tuberculosis infection. *Am J Respir Crit Care Med*. 2007; 175:514–520. [PubMed: 17158278]
71. Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, Pasvol G, Hill AV, Lalvani A. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol*. 2001; 167:5217–5225. [PubMed: 11673535]
72. Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. *Trends Microbiol*. 2009; 17:183–188. [PubMed: 19375916]
73. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, Mex P, Miekley D, Kaufmann SH. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci USA*. 2007; 104:12434–12439. [PubMed: 17640915]
74. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, Walker BD. Detection of polyfunctional mycobacterium tuberculosis-specific T cells and association with viral load in HIV-1-infected persons. *J Infect Dis*. 2008; 197:990–999. [PubMed: 18419535]

AT A GLANCE COMMENTARY**Scientific Knowledge on the Subject**

Antiretroviral treatment reduces the risk of tuberculosis (TB) in HIV-infected subjects, but mechanisms of protective cellular immunity to TB remain ill understood. Longitudinal analysis of immunocompromised HIV-infected subjects who undergo immune-reconstituting antiretroviral therapy represents a novel approach to understanding protective mechanisms in human TB.

What This Study Adds to the Field

We show that in the context of overall improved TB antigen-specific T-cell responses it is the central memory rather than effector memory response that best correlates with decreased susceptibility. This has important implications for TB vaccine design and efficacy.

Figure 1. Expansion of CD4⁺ T cells during combination antiretroviral therapy (cART). Scatter dot plots indicate the mean of CD4⁺ T cells as determined by flow cytometry during the 48 weeks of follow-up, which increased over time (one-way analysis of variance, $P < 0.0001$).

Figure 2. Representative scatter dot blots indicating the gating strategy used throughout the study.

(A) Selection of CD4-positive cells: a scatter blot using side scatter (SSC) and FL3 (CD4-PerCp) was applied and CD4-positive cells were selected by gating. (B) CD4-positive cells were further shown as SSC-FL4 (IFN- γ -APC) to select for CD4⁺IFN γ ⁺ cells. A similar gating strategy was applied for CD4⁺IL2⁺, CD4⁺IL10⁺, CD4⁺TNF⁺ cells (not shown). (C) CD4-positive cells were analyzed for their surface expression of CD27, CCR7, CCR5, CD45RA, or CD45RO (not shown) (D) Representative example of the positive control staphylococcal enterotoxin B-stimulated cells stained for IFN- γ or tumor necrosis factor.

Figure 3. Expansion of naive CD4⁺ cells.

The CD4⁺CD27⁺CD45RA⁺ naive T-cell population, determined by flow cytometry, significantly expanded by Week 36 ($P = 0.017$), and was sustained at Week 48 ($P = 0.008$). Results are presented as scatter dot plot indicating the mean.

Figure 4. Expansion of central memory CD4⁺ cells.

(A) Scatter dot plot with the mean of the CD4⁺CD27⁺CD45RA⁻ population, determined by flow cytometry, showing significant increase by 12 weeks ($P = 0.001$), that remained significantly higher until Week 48 (one-way analysis of variance, $P = 0.03$). (B) Scatter dot plot with the mean of the CD4⁺CD27⁺CCR5⁻ population that also proportionally increased by 12 weeks ($P = 0.01$) and remained significantly higher until Week 48 ($P = 0.02$).

Figure 5. Proportional decline of effector CD4⁺ T cells.

(A) Scatter dot plot with horizontal lines indicating the median percent determined by flow cytometry, showing gradual decrease in median percent of CD4⁺CCR5⁺ cells from Week 0 to Week 48 of combination antiretroviral therapy (cART) ($P = 0.02$). (B) Scatter dot plot indicating the mean of the CD4⁺CD27⁻CCR7⁻ population, which significantly decreased from Week 0 to Week 12 of cART ($P = 0.02$). This was sustained over 48 weeks ($P = 0.02$ compared with Week 0), supporting the findings presented in A.

Figure 6. Cytokine-producing CD4⁺ T cells determined by flow cytometry.

Box and whisker plots with horizontal lines indicating the median percent, the whiskers the minimum and maximum values, and the boxes the interquartile range for (A) Purified protein derivative-specific CD4⁺IFN γ ⁺ T cells showing a significant decrease during combination antiretroviral therapy (cART) ($P = 0.02$). (B) CD4⁺IL2⁺ T cells also significantly decreased during cART ($P = 0.006$), whereas (C) CD4⁺IL-10⁺ and (D) CD4⁺TNF⁺ cells showed no overall significant change during the 48 weeks of follow-up.

Figure 7. Antigen-specific enzyme-linked immunospot assay (ELISpot) responses during combination antiretroviral therapy (cART).

A, C, and D show box and whisker plots with horizontal lines indicating the median, the whiskers the minimum and maximum values, and the boxes the interquartile range of the purified protein derivative (PPD), α crystalline (Acr)-2, and TB10.3 antigen-specific IFN- γ ELISpot responses, respectively. The general trend indicated a significant increase in antigen-specific responses to PPD ($P = 0.02$), Acr2 ($P = 0.02$), and TB10.3 ($P = 0.04$). The combined response to the two region of difference-1 antigens ESAT-6 and CFP-10 (shown on the bar graph B indicating the mean and 95% confidence intervals) also significantly increased over time ($P = 0.04$). E shows the effect of CD4⁺ cell depletion on the ELISpot response: the mean reduction in IFN- γ -producing cell numbers in four donors is indicated for each antigen, showing that the main source of IFN- γ in these experiments is the CD4⁺ T cells.

Figure 8. Comparison of HIV-infected people on combination antiretroviral therapy (cART) for 48 weeks and HIV-uninfected people recruited from the same community.

The cytokine-producing capacity of cells from HIV-infected patients on cART for 48 weeks (solid circles), was significantly lower than that of the HIV-uninfected control subjects (open circles): CD4⁺IFN γ ⁺ ($P = 0.004$), CD4⁺IL-2⁺ ($P = 0.0001$), CD4⁺IL-10⁺ ($P = 0.001$).

Figure 9. Concept diagram explaining the dynamics of the reconstituting T cells during combination antiretroviral therapy (cART), corrected for CD4 counts.

The proportion of naive ($CD4^+CD27^+CD45RA^+$), central memory ($CD4^+CD27^+CD45RA^-$), and terminally differentiated effector ($CD4^+CD27^-CCR7^-$) T cells was summed at each time point for each patient, normalized to 100% and multiplied by the CD4 count of each respective patient at that time point. The diagram illustrates that although effector T cells proportionally decline during cART, the actual numbers increase.

TABLE 1
BASELINE CHARACTERISTICS AND FOLLOW-UP OF THE STUDY
PARTICIPANTS

No.	Patient code	Age	Previous TB	Other Illnesses during the 48-wk Follow-up	Viral Load		CD4 Count	
					Nadir	6 mo	Nadir	6 mo
1	RECON1	41	Yes	None	1030	LDL	141	183
2	RECON2	35	No	None	32000	230	161	302
3	RECON3	46	Yes	PNP	21000	LDL	63	353
4	RECON4	25	Yes	None	180000	LDL	23	295
5	RECON5	44	Yes	Hepatomegaly, persistent pleural effusion, but good adherence, good viral load suppression and CD4 count increased	n/a	280*	147	567
6	RECON6	37	No	None	500000	<50	22	126
7	RECON7	32	Yes	None	250000	LDL	309	356
8	RECON8	38	Yes	Cryptococcal meningitis	102888	<50	93	179
9	RECON10	35	No	Anemia	2400	LDL	108	104
10	RECON11	30	Yes	Some diarrhea, nausea	500000	<50	131	535
11	RECON13	38	No	Bactrim-induced neutropenia, off Bactrim	11000	<50	86	295
12	RECON14	40	No	PNP	103419	52	103	336
13	RECON17	32	No	None	n/a	LDL	61	117
14	RECON20	39	No	Drug-induced hepatitis, latent syphilis, DVT	380000	<50	118	248
15	RECON21	38	No	None	140000	20000*	99	194
16	RECON22	30	No	None	2400	LDL	90	140
17	RECON24	26	No	None	n/a	LDL	11	209
18	RECON25	32	Yes	None	179759	87	40	115
19	RECON28	34	Yes	Previous PCP	n/a	LDL	3	312

Definition of abbreviations: DVT = deep vein thrombosis; LDL = lower than detectable level; n/a = not available; PCP = Pneumocystis pneumonia; PNP = peripheral neuropathy; TB = tuberculosis.

* LDL at 1-year follow-up.