MAJOR ARTICLE

Interferon γ Responses to Mycobacterial Antigens Protect against Subsequent HIV-Associated Tuberculosis

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Background. The cellular immune responses that protect against tuberculosis have not been identified.

Methods. We assessed baseline interferon γ (IFN-γ) and lymphocyte proliferation assay (LPA) responses to antigen 85 (Ag85), early secretory antigenic target 6 (ESAT-6), and *Mycobacterium tuberculosis* whole cell lysate (WCL) in human immunodeficiency virus (HIV)–infected and bacille Calmette-Guérin (BCG)–immunized adults with CD4 cell counts of ⩾200 cells/μL who received placebo in the DarDar tuberculosis vaccine trial in Tanzania. Subjects were followed prospectively to diagnose definite or probable tuberculosis.

Results. Tuberculosis was diagnosed in 92 of 979 subjects during a mean follow-up of 3.2 years. The relative risk of tuberculosis among subjects with positive IFN-γ responses to Ag85 was 0.51 (95% confidence interval [CI], 0.26–0.99; $P = .049$), to ESAT-6 was 0.44 (95% CI, 0.23–0.85; $P = .004$), and to WCL was 0.67 (95% CI, 0.49–0.88; $P = .002$). The relative risk of tuberculosis was not significantly associated with baseline LPA responses. In a multivariate Cox regression model, subjects with IFN-γ responses to ESAT-6 and WCL had a lower hazard of developing tuberculosis, with a hazard ratio for ESAT-6 of 0.35 (95% CI, 0.16–0.77; $P = .009$) and a hazard ratio for WCL of 0.30 (95% CI, 0.16–0.56; $P < .001$).

Conclusions. Baseline IFN-γ responses to ESAT-6 and WCL were associated with protection from subsequent tuberculosis among HIV-infected subjects with childhood BCG immunization in a region of high tuberculosis prevalence.

Trial registration. ClinicalTrials.gov identifier: NCT00052195.

Tuberculosis is the leading cause of death in people infected with human immunodeficiency virus (HIV) worldwide [1]. Therefore, the development of a new and more effective tuberculosis vaccine is a leading public health priority [2]. Identifying the immune correlates of protection against tuberculosis will accelerate the identification of a novel tuberculosis vaccine.

Multiple lines of evidence indicate that interferon γ (IFN-γ) responses are a critical component of the host immune defense against tuberculosis. Genetic deficiencies in IFN-γ signaling are associated with heightened susceptibility to tuberculosis [3], and deficient IFN-γ responses are thought to contribute to the high vulnerability to tuberculosis of people infected with HIV [4].

In some clinical settings, however, positive IFN-γ responses to tuberculosis-specific antigens are typically interpreted as an indication of tuberculosis risk. Among HIV-negative subjects, positive IFN-γ responses have been associated with presumed latent tuberculosis for those with positive tuberculin skin test (TST) results,

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with an elevated risk of progression to active tuberculosis after exposure to tuberculosis, and with active tuberculosis [5–8]. Among patients infected with HIV, multiple cross-sectional studies have shown positive IFN-γ responses during latent and active tuberculosis [9–11].

To resolve whether IFN-γ responses to mycobacterial antigens are associated with protection from or risk of HIV-associated tuberculosis, we investigated the relationship between IFN-γ and lymphocyte proliferation assay (LPA) responses to both tuberculosis-specific (early secretory antigenic target 6 [ESAT-6]) and nonspecific (antigen 85 [Ag85] and *Mycobacterium tuberculosis* whole cell lysate [WCL]) mycobacterial antigens at baseline and the subsequent risk of tuberculosis among HIV-infected and bacille Calmette-Guérin (BCG)–immunized adults with CD4 cell counts of ⩾200 cells/μL participating in a phase 3 tuberculosis vaccine trial in Dar es Salaam, Tanzania. To our knowledge, this is the first large prospective study to link baseline cellular immune responses to mycobacterial antigens to the risk of subsequent HIV-associated tuberculosis during prospective follow-up.

METHODS

Study subjects. The DarDar trial was a randomized, placebo-controlled, double-blind, phase 3 trial of a prime-boost vaccine strategy for the prevention of HIV-associated tuberculosis among adults in Dar es Salaam, Tanzania [12]. Enrollment occurred from 2001 through 2005, and study follow-up continued through January 2008. Subjects who provided informed consent were eligible for enrollment if they had 2 positive enzyme-linked immunosorbent assay (ELISA) results for HIV, a CD4 cell count of ⩾200 cells/μL, and a BCG scar. At baseline, all subjects were evaluated by means of history, physical examination, single-view chest radiograph, sputum acid-fast bacillus (AFB) smear, or sputum mycobacterial culture. Subjects with active tuberculosis were excluded from enrollment. Subjects were randomized to receive 5 intradermal doses of either heat-inactivated *Mycobacterium vaccae* [13, 14] or matched saline placebo. For the present study, we confined our analyses to subjects who received placebo.

Human research conduct. The human experimentation guidelines of the US Department of Health and Human Services, as well as those of the Committee for the Protection of Human Subjects at Dartmouth College and the Research Ethics Committee of the Muhimbili University of Health and Allied Sciences, were followed in the conduct of this research. This study was registered through the National Institutes of Health (NCT00052195).

Clinical surveillance for tuberculosis. After randomization, we evaluated subjects for active tuberculosis via physical examination and history at months 2, 4, and 6 and every 3 months thereafter. In addition, any subjects who presented with 2 or more weeks of fever, cough, or weight loss underwent evaluation for active tuberculosis via a single-view chest radiograph, 3 sputum collections for AFB smear and mycobacterial culture, phlebotomy for mycobacterial blood culture, and any additional studies as clinically indicated (eg, cultures of other body fluids or tissue biopsies).

Definitions of tuberculosis. A 3-person masked adjudication panel reviewed all episodes of illness evaluated for active tuberculosis and designated subjects to have definite or probable tuberculosis according to rigorous study definitions. Definite tuberculosis was diagnosed if any of the following criteria were met:

1. One or more sputum culture results positive for *M. tuberculosis* with ⩾10 colony-forming units;
2. Two or more sputum culture results with 1–9 colony-forming units of *M. tuberculosis* (indeterminate *M. tuberculosis* culture result);
3. Two or more positive sputum smear results for AFB; or
4. One or more cultures of *M. tuberculosis* from the blood or other sterile body site.

Probable tuberculosis was diagnosed if any of the following criteria were met:

1. Positive chest radiographic result plus either 1 positive sputum AFB smear result or 1 indeterminate *M. tuberculosis* culture result;
2. Clinical symptoms or signs plus either 1 positive sputum AFB smear result or 1 indeterminate *M. tuberculosis* culture result;
3. Clinical symptoms or signs and a positive radiographic result plus a response to antituberculosis therapy;
4. One positive sputum AFB smear result for a sterile site plus clinical symptoms or signs of tuberculosis; or
5. Caseous necrosis on tissue biopsy.

Assays of mycobacterial immune responses. All subjects underwent in vivo and in vitro assessments of immune responses to mycobacteria before vaccination. These assessments included TST, LPA, and an assay of IFN-γ release.

TST. Tuberculin (0.1 mL; RT-23; State Serum Institute, Copenhagen) was injected intradermally on the forearm, and trained personnel measured the size of skin induration at the site after 48–72 h. We considered reactions of ⩾5 mm to be positive and offered isoniazid treatment to all such subjects, 88% of whom completed 6 months of isoniazid therapy [15].

IFN-γ release assays. Freshly isolated and ficolled peripheral blood mononuclear cells (PBMCs) were incubated with study antigens for 5 days in parallel IFN-γ assays and LPAs. At the end of 5 days, centrifuged cell supernatants were frozen and sent to the United States for subsequent measurement of IFN-γ levels by a standard IFN-γ ELISA (R&D Systems). Study

Table 1. Baseline Characteristics of Subjects Who Did or Did Not Develop Definite or Probable Tuberculosis during Prospective Follow-up

Characteristic	No. of subjects	Tuberculosis	No tuberculosis	*P*
Mean age, years	979	34.5	32.9	.066
Male, % (proportion)	979	29.3 (27/92)	23.7 (210/887)	.227
Previous tuberculosis treatment, % (proportion)	979	18.5 (17/92)	7.1 (63/887)	<.001
Mean TST result, mm	961	7.7	4.9	.001
TST result of ⩾5 mm, % (proportion)	961	48.9 (44/90)	30.8 (268/871)	<.001
Antiretroviral therapy at baseline, % (proportion)	979	0.0 (0/92)	3.7 (33/887)	.060
CD4 cell count, cells/μL	977	362.7	481.6	<.001

NOTE. Per protocol, at study entry all subjects were infected with human immunodeficiency virus and had a bacille Calmette-Guérin scar, a CD4 cell count of ⩾200 cells/μL, and no evidence of active tuberculosis (see Methods for details). *P* values were derived using the Mann-Whitney *U* test. TST, tuberculin skin test.

antigens were medium alone (negative control), 1 μg/mL *M. tuberculosis* ESAT-6, 0.5 μg/mL *M. tuberculosis* Ag85, and 0.5 μg/mL WCL (all antigens were acquired through National Institute of Allergy and Infectious Diseases, National Institutes of Health, contract HHSN266200400091C "Tuberculosis Vaccine Testing and Research Materials," awarded to Colorado State University). IFN-γ assays were considered to be valid if the IFN-γ level for the positive control antigen, phytohemagglutinin (PHA) (Sigma; 2.5 μg/mL), was >300 pg/mL. IFN-γ responses to mycobacterial antigens were considered to be positive if the IFN-γ level was ⩾2 standard deviations above the mean of the negative control condition.

LPA. LPAs were conducted on the same PBMCs used in the IFN-γ assay, by a standard 5-day ^{3}H-thymidine incorporation method. After incubation with study antigens, 20 μL of 50 μCi/mL ^{3}H-thymidine was added to wells for 24 h, after which the cells were harvested onto filter paper and sent to the National Public Health Institute in Helsinki, Finland, for data acquisition by means of a scintillation counter. Results were expressed as a proliferation index (PI), derived by dividing the counts per minute for the antigen of interest by the counts per minute for the negative control condition. LPAs were considered to be valid if the PI for the positive control antigen, PHA (2.5 μg/mL), was ⩾3. LPA responses to mycobacterial antigens were considered to be positive if the PI was ⩾5.

Statistical analysis. We compared demographic data and immune responses between subjects who did or did not develop definite or probable tuberculosis during prospective follow-up by a 2-tailed Mann-Whitney *U* test, with a threshold for statistical significance of $P < .05$. We then constructed a multivariate Cox proportional hazards regression model of the hazard of tuberculosis during prospective follow-up, adjusting for age, baseline CD4 cell count, history of previous tuberculosis, and TST status. We confirmed that the proportional hazards assumption was not violated using log-log plots and Schoenfeld residuals. We analyzed the data using Stata software (version 9; StataCorp).

RESULTS

Subject characteristics. All subjects were infected with HIV, had a BCG scar, and had a CD4 cell count of ⩾200 cells/μL at study entry. We diagnosed 92 cases of definite and probable tuberculosis among 979 placebo recipients during a mean follow-up period of 3.2 years (median, 3.3 years; range, 0–6.3 years). Fifty-two cases were designated as definite tuberculosis, and 40 were designated as probable tuberculosis. At baseline, subjects who did not develop tuberculosis during prospective follow-up had higher CD4 cell counts, less frequently had a TST result of ⩾5 mm, and were less likely to have been previously treated for tuberculosis (Table 1).

Relationship between baseline IFN-γ responses and risk of tuberculosis during prospective follow-up. Figure 1*A* shows IFN-γ responses to mycobacterial antigens among subjects who did or did not develop tuberculosis during prospective follow-up. IFN-γ responses to the mycobacterial antigens ESAT-6 and WCL were greater in subjects who did not develop tuberculosis. Subjects with positive IFN-γ responses to WCL were more likely to demonstrate positive IFN-γ responses to the tuberculosis-specific antigen ESAT-6 (42.4% vs 1.8%; $P < .001$). Table 2 shows the incidence and relative risk of tuberculosis among subjects on the basis of the presence of positive IFN-γ responses to mycobacterial antigens. Subjects with positive IFN-γ responses to mycobacterial antigens were less likely to develop tuberculosis during prospective follow-up. In univariate comparisons, there was no relationship between baseline IFN-γ responses to mycobacterial antigens and the risk of developing definite tuberculosis.

Relationship between baseline LPA responses and risk of tuberculosis during prospective follow-up. Figure 1*B* shows LPA responses to mycobacterial antigens among subjects who did or did not develop tuberculosis during prospective follow-up. Subjects who developed tuberculosis had greater LPA responses to the mycobacterial antigen WCL, and there was a trend toward a greater risk of tuberculosis among subjects with

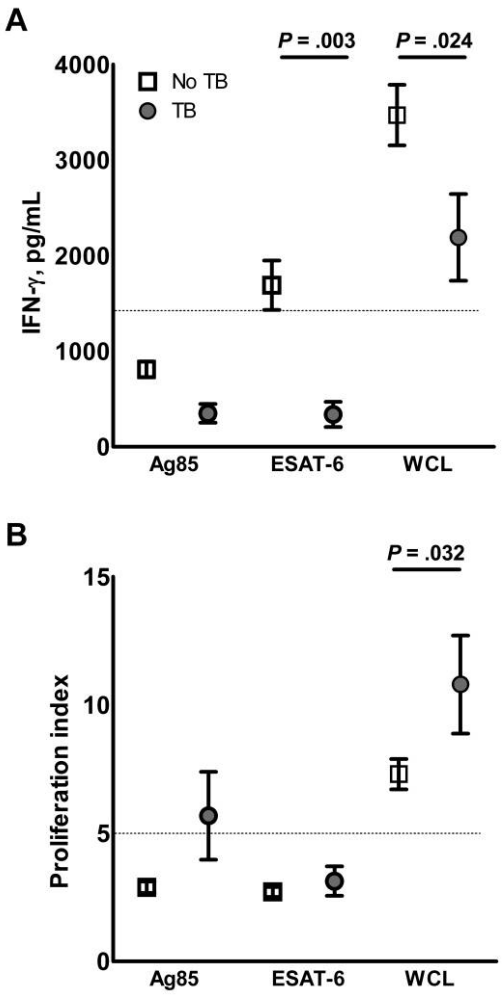

Figure 1. Immune responses to mycobacterial antigens in human immunodeficiency virus–infected adults who did or did not develop tuberculosis during prospective follow-up. Shown are mean interferon γ (IFN-γ) responses (*A*) and mean lymphocyte proliferation assay (LPA) responses (*B*) in subjects. Bars indicate means and standard errors. The dotted gray line indicates the cutoff for a positive response. *P* values were derived using the Mann-Whitney *U* test. For the IFN-γ assay a positive response was defined as an IFN-γ level ⩾2 standard deviations above the mean of the negative control condition, and for the LPA a positive response was defined as a proliferation index of ⩾5 (derived by dividing the counts per minute for the antigen of interest by the counts per minute for the negative control condition). Ag85, antigen 85; ESAT-6, early secretory antigenic target 6; TB, tuberculosis; WCL, *Mycobacterium tuberculosis* whole cell lysate.

detectable baseline LPA responses to WCL (PI, ⩾5) (Table 2). Subjects with positive LPA responses to WCL were more likely to demonstrate positive LPA responses to the tuberculosis-specific antigen ESAT-6 (25.7% vs 3.1%; $P < .001$).

Multivariate model. We assessed the relationship between positive immune responses to mycobacterial antigens and the hazard of developing HIV-associated tuberculosis during prospective follow-up in a multivariate Cox regression model adjusting for age, baseline CD4 cell count, TST status, and history of previous tuberculosis. The hazard of HIV-associated tuberculosis was significantly lower among subjects with positive baseline IFN-γ responses to ESAT-6 and WCL (Table 3). Baseline IFN-γ responses to Ag85 did not significantly correlate with the risk of developing tuberculosis, nor did baseline LPA responses to Ag85, ESAT-6, or WCL (Table 4). If we removed TST status from the multivariate model, IFN-γ responses to ESAT-6 and WCL remained associated with protection from tuberculosis, with a hazard ratio of 0.49 (95% confidence interval [CI], 0.23–1.04; $P = .062$) for ESAT-6 and of 0.55 (95% CI, 0.33–0.91; $P = .021$) for WCL. Among subjects who had a positive TST result at baseline, the hazard of tuberculosis was lower in those who demonstrated positive IFN-γ responses at baseline, with a hazard ratio of 0.38 (95% CI, 0.16–0.91; $P = .030$) for ESAT-6 and of 0.30 (95% CI, 0.13–0.71; $P = .006$) for WCL. Adjustment for receipt of isoniazid did not affect the results of the multivariate analyses. Baseline IFN-γ and LPA responses were not significantly related to the hazard of developing definite tuberculosis.

DISCUSSION

In a prospective study of HIV-infected and BCG-immunized adults in Tanzania, we found that baseline IFN-γ responses to either a tuberculosis-specific antigen (ESAT-6) or a nonspecific mycobacterial antigen (WCL) were associated with protection against subsequent HIV-associated tuberculosis. These data support the concept that IFN-γ responses to mycobacterial antigens are critical to immune protection from tuberculosis during HIV infection.

HIV-infected subjects with positive IFN-γ responses to ESAT-6 and WCL likely have preserved and effective immune responses against tuberculosis. However, having positive IFN-γ responses to ESAT-6 and WCL is more than a marker for intact immunity in general, because not all detectable IFN-γ or LPA responses were associated with protection and controlling for CD4 cell count did not eliminate the relationship between IFN-γ responses to ESAT-6 or WCL and protection from tuberculosis.

The HIV-infected and BCG-immunized subjects in this study live in a region where most adults have evidence of latent tuberculosis, as indicated by a TST result of ⩾5 mm or in vitro assays [11, 16, 17]. Accordingly, the IFN-γ and LPA responses measured here likely stem from a mixture of BCG vaccination, prior tuberculosis, and previous infection with nontuberculous mycobacteria [18]. Each of these mycobacterial infections has been detected by skin test or in vitro studies in African populations [17–25], and all have been shown to protect against tuberculosis [26–31]. Of the 3 antigens tested in the present study, WCL is the most heterogeneous, is the least tuberculosis specific, and had the highest proportion of positives. Among

Table 2. Univariate Analyses of the Incidence and Relative Risk (RR) of Tuberculosis among Subjects with Positive Interferon γ (IFN-γ) or Positive Lymphocyte Proliferation Assay (LPA) Responses

	Incidence					
	Positive		Negative			
Response, antigen	No.	% with tuberculosis	No.	% with tuberculosis	RR point estimate (95% CI)	*P*
IFN-γ response						
Ag85	159	5.1	557	10.2	0.51 (0.26–0.99)	.049
ESAT-6	186	3.8	521	10.9	0.44 (0.23–0.85)	.004
WCL	426	6.3	290	13.2	0.67 (0.49–0.88)	.002
LPA response						
Ag85	70	15.7	465	11.4	1.37 (0.76–2.47)	.300
ESAT-6	65	15.4	470	11.5	1.33 (0.72–2.49)	.365
WCL	214	15.0	321	10.0	1.29 (0.99–1.70)	.082

NOTE. For the IFN-γ assay a positive response was defined as an IFN-γ level ⩾2 standard deviations above the mean of the negative control condition, and for the LPA a positive response was defined as a proliferation index of ⩾5. *P* values were derived using the Mann-Whitney *U* test. The no. of assays for analysis varies based on the no. of valid assays. Ag85, antigen 85; CI, confidence interval; ESAT-6, early secretory antigenic target 6; WCL, *Mycobacterium tuberculosis* whole cell lysate.

subjects with positive IFN-γ responses to WCL, 42% also demonstrated positive IFN-γ responses to ESAT-6. This represents the minimum proportion of subjects in whom antecedent tuberculosis contributed to the observed IFN-γ responses to the heterogeneous WCL antigen.

The IFN-γ responses to mycobacterial antigens detected in this study were associated with protection from either reactivation or reinfection tuberculosis [32–34]. The isoniazid we administered to 312 placebo subjects with a positive TST result would have reduced but not eliminated the risk of reactivation, and such protection wanes with time [35]. Our observation that IFN-γ responses to ESAT-6 and WCL were still associated with protection among subjects who had a positive baseline TST result and received isoniazid underscores the importance of intact IFN-γ responses to mycobacterial antigens in preventing tuberculosis. Large population-based studies from regions of endemicity have found that a majority of culture-confirmed cases of HIV-associated tuberculosis are clustered and thus represent recent infection, but it is unclear whether culture-negative clinical cases—the majority of cases in this study—have a similar pathogenesis [36]. Thus, IFN-γ responses may protect against reactivation tuberculosis, reinfection tuberculosis, or both, or they may be a marker of such protection.

Our prospective data differ from those of previous studies assessing the predictive value of positive IFN-γ responses [5, 8]. Subjects in these studies were typically HIV negative, mycobacteria naive, resident in regions of low tuberculosis prevalence, and were being followed up after an explicit recent exposure to tuberculosis. In that context, a positive IFN-γ response presumably stems from recent infection, which is known to be associated with an elevated risk of active tuberculosis. Aichelburg et al [37] identified 3 of 37 HIV-infected subjects from a region of low prevalence with positive IFN-γ responses who developed tuberculosis during prospective follow-up, but our study of 92 cases of tuberculosis in 979 adults infected with HIV provides a more complete assessment of the relationship between baseline IFN-γ responses to mycobacterial antigens and ongoing protection from tuberculosis. The observation in

Table 3. Multivariate Cox Regression Model of the Relationship between Protection from Human Immunodeficiency Virus (HIV)–Associated Tuberculosis and Baseline Interferon γ (IFN-γ) Responses to Early Secretory Antigenic Target 6 (ESAT-6) and *Mycobacterium tuberculosis* Whole Cell Lysate (WCL)

Antigen, parameter	HR (95% CI)	*P*
ESAT-6		
Age, per 10 years	0.93 (0.66–1.30)	.659
CD4 cell count, per 100 cells/μL	0.68 (0.57–0.81)	<.001
Previous tuberculosis	1.85 (0.98–3.50)	.057
TST result of ⩾5 mm	2.46 (1.47–4.12)	.001
Detectable IFN-γ response to ESAT-6	0.35 (0.16–0.77)	.009
WCL		
Age, per 10 years	0.94 (0.67–1.32)	.735
CD4 cell count, per 100 cells/μL	0.70 (0.59–0.83)	<.001
Previous tuberculosis	1.94 (1.04–3.63)	.038
TST result of ⩾5 mm	3.52 (1.94–6.93)	<.001
Detectable IFN-γ response to WCL	0.30 (0.16–0.56)	<.001

NOTE. The model demonstrated that baseline IFN-γ responses to ESAT-6 and WCL predict protection from HIV-associated tuberculosis. For the IFN-γ assay a positive response was defined as an IFN-γ level ⩾2 standard deviations above the mean of the negative control condition. *P* values were derived using Cox proportional hazards regression. Ag85, antigen 85; CI, confidence interval; HR, hazard ratio; TST, tuberculin skin test.

Table 4. Hazard of Developing Tuberculosis during Prospective Follow-up according to Baseline Interferon γ (IFN-γ) and Lymphocyte Proliferation Assay (LPA) Immune Responses to Mycobacterial Antigens in a Multivariate Cox Regression Model

Response, antigen	No.	HR (95% CI)	*P*
Detectable IFN-γ response to			
Ag85	701	0.54 (0.25–1.19)	.127
ESAT-6	692	0.35 (0.16–0.77)	.009
WCL	701	0.30 (0.16–0.56)	<.001
Detectable LPA response to			
Ag85	524	0.98 (0.49–1.94)	.951
ESAT-6	524	0.95 (0.47–1.92)	.887
WCL	524	1.09 (0.62–1.90)	.766

NOTE. For the IFN-γ assay a positive response was defined as an IFN-γ level ⩾2 standard deviations above the mean of the negative control condition, and for the LPA a positive response was defined as a proliferation index of ⩾5. *P* values were derived using Cox proportional hazards regression. Ag85, antigen 85; CI, confidence interval; ESAT-6, early secretory antigenic target 6; HR, hazard ratio; WCL, *Mycobacterium tuberculosis* whole cell lysate.

our study that positive IFN-γ responses to mycobacterial antigens were associated with decreased risk of subsequent tuberculosis supports the concept that IFN-γ responses reflect intact protective immunity to tuberculosis. It will, however, be important to assess the predictive value of IFN-γ responses to mycobacterial antigens in a large cohort of HIV-infected adults from areas with a low prevalence of tuberculosis, because in such areas it is uncertain whether positive IFN-γ responses will reflect tubercular infection and this risk of tuberculosis or, as in our study, intact protective immunity against tuberculosis.

Our study differs from previous cross-sectional studies conducted in people infected with HIV that have shown a greater frequency of IFN-γ responses among subjects with active or latent tuberculosis than among subjects without active or latent tuberculosis [9–11, 38, 39]. These previous results might suggest that positive IFN-γ responses are always associated with an increased risk of tuberculosis in the future. However, although such cross-sectional studies highlight the importance of IFN-γ in the immune response to HIV-associated tuberculosis [40–42] and demonstrate the diagnostic utility of detecting IFN-γ responses during HIV-associated tuberculosis [43, 44], they do not address the question of how baseline IFN-γ responses affect the risk of subsequent tuberculosis in persons infected with HIV. Our results support the hypothesis that while IFN-γ responses may be detectable during active HIV-associated tuberculosis, they are often weak or absent before the development of tuberculosis.

Our data highlight the complexity of interpreting studies of the relationship between IFN-γ responses and the risk of tuberculosis conducted in different populations with different assay methods. Commercial IFN-γ assays use a 1-day incubation period after antigen stimulation and likely measure effector memory cell responses to mycobacterial antigens, whereas our IFN-γ assay used a 5-day incubation period for antigen stimulation and thus presumably measured both central memory and effector memory responses to mycobacterial antigens [45, 46]. Our results thus raise the possibility that the central memory T cell responses assessed in a 5-day assay are an important component of the long-term immunological control of tuberculosis [47]. Assessing the relative contribution of central and effector memory T cell responses will be a critical step in understanding the protective immune response to novel tuberculosis vaccines [41]. Furthermore, the evolving field of tuberculosis diagnostics may benefit from the identification of assays that separate immune markers of the risk of tuberculosis from immune markers of protection from tuberculosis.

The choice of mycobacterial antigens may also influence the relationship between IFN-γ results and the risk of tuberculosis. Commercial IFN-γ release assays assess IFN-γ responses to the region of difference 1 antigens ESAT-6 and culture filtrate protein 10, while we used ESAT-6, Ag85, and WCL. We found that IFN-γ responses to ESAT-6 and WCL were associated with a reduced risk of developing tuberculosis during prospective follow-up, whereas IFN-γ responses to Ag85 were not. Potential reasons for this finding include the following: (1) IFN-γ responses against ESAT-6 and the heterogeneous antigens in WCL may confer greater protection against tuberculosis than IFN-γ responses to Ag85 and (2) study power to correlate IFN-γ responses to ESAT-6 and WCL with the risk of tuberculosis was greater because IFN-γ responses to ESAT-6 and WCL were more prevalent in our population (12% and 38%, respectively) than responses to Ag85 (6%) [16]. Regardless, we hypothesize that generating IFN-γ responses against a broad array of tuberculosis antigens such as those in a lysate of a whole cell mycobacterium are more likely to result in immunological protection against tuberculosis.

In contrast with our finding that IFN-γ responses to ESAT-6 and WCL were associated with protection from tuberculosis, LPA responses were more closely associated with the risk of tuberculosis in univariate analyses and were not associated with the risk of tuberculosis in multivariate analyses. LPA responses may be analogous to TST responses and reflect a complex mixture of cellular immune responses that indicate tubercular infection but not protection from tuberculosis. This finding highlights the concept that not all cellular immune responses to tuberculosis are protective and has implications for studies of tuberculosis vaccine immunogenicity. Specifically, novel tuberculosis vaccine candidates should elicit immune responses—such as IFN-γ responses to ESAT-6 or WCL in adults infected with HIV—shown to be associated with protection against tuberculosis.

This is the first large study, to our knowledge, to assess the

relationship between baseline immune responses to tuberculosis and the subsequent risk of HIV-associated tuberculosis. Study strengths include its prospective nature, the rigorous exclusion of active or subclinical tuberculosis at baseline [48], the extended and comprehensive follow-up of subjects with suspected tuberculosis cases, and the strict diagnostic criteria used to designate tuberculosis cases. However, our findings may not apply to HIV-infected subjects with lower CD4 cell counts or to those who have not received BCG immunization. Furthermore, we did not find any statistically significant association between IFN-γ responses and the risk of definite tuberculosis alone, a finding that may have been related to the low numbers of definite tuberculosis cases or to the possibility that IFN-γ responses may be more protective against paucibacillary and thus culture-negative tuberculosis.

In summary, we have shown that positive IFN-γ responses to the tuberculosis-specific antigen ESAT-6 and to the multiple and nonspecific mycobacterial antigens in WCL are associated with protection from prospectively detected tuberculosis in HIV-infected subjects with CD4 cell counts of $\geqslant$200 cells/μL and childhood BCG immunization. These novel findings add to our understanding of the protective immune response to tuberculosis in HIV infection and will help guide the development of novel diagnostic and preventive immunization strategies for HIV-associated tuberculosis.

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