

Greater Preexisting Interferon γ Responses to Mycobacterial Antigens and Lower Bacillary Load During HIV-Associated Tuberculosis

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The role of preexisting interferon (IFN) γ responses in controlling bacillary burden in human immunodeficiency virus (HIV)-associated tuberculosis is not known. Among BCG-immunized HIV-infected adults who developed tuberculosis in a phase III trial of an investigational tuberculosis vaccine, greater baseline IFN- γ responses to early secretory antigenic target 6 and *Mycobacterium tuberculosis* whole-cell lysate were associated with reduced bacillary burden on sputum smear grade, days to culture positivity on agar, and sputum culture grade during subsequent tuberculosis. This association was most consistent among recipients of the investigational vaccine. When HIV-associated tuberculosis develops, greater preexisting IFN- γ responses to mycobacterial antigens are associated with reduced tuberculosis bacillary burden.

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Heritable interferon (IFN) γ signaling deficiencies are associated with heightened susceptibility to tuberculosis [1] and deficient IFN- γ responses probably contribute to the high vulnerability to tuberculosis of persons with human immunodeficiency virus (HIV) infection [2]. Among HIV-infected and

BCG-immunized adults, we have shown that detectable IFN- γ responses to single and multiple mycobacterial antigens are associated with protection from HIV-associated tuberculosis [3, 4]. To our knowledge, however, there have been no prospective studies evaluating whether IFN- γ responses reduce the tuberculosis bacillary load when active tuberculosis occurs. To address this question, we correlated baseline IFN- γ responses to mycobacterial antigens with the bacillary burden of tuberculosis disease among HIV-infected adults who subsequently developed culture-confirmed tuberculosis in the DarDar phase III tuberculosis vaccine trial in Tanzania.

METHODS

Human Research Conduct

We followed human experimentation guidelines of the US Department of Health and Human Services in a research protocol approved by the research ethics committees of Dartmouth College and the Muhimbili University of Health and Allied Sciences. All subjects provided written informed consent.

Study Subjects

The DarDar trial was a phase III randomized placebo-controlled double-blind trial of an inactivated whole-cell nontuberculous mycobacterial booster vaccine (SRL 172) for the prevention of HIV-associated tuberculosis among BCG-immunized adults in Dar es Salaam, Tanzania [5]. Enrollment occurred in 2001–2005; study follow-up continued through January 2008. Subjects were eligible for enrollment if they had 2 positive enzyme-linked immunosorbent assay results for HIV, a CD4 T-cell count $\geq 200/\text{mm}^3$, and a BCG vaccine scar. At baseline, all subjects were evaluated with history, physical examination, single-view chest radiography, sputum acid-fast bacillus (AFB) smear and mycobacterial culture, and blood mycobacterial culture. Subjects with active tuberculosis at baseline were excluded from enrollment. After randomization, subjects received 5 intradermal doses of either SRL 172 or matched borate-buffered saline placebo. Subjects who received vaccine exhibited protection against definite tuberculosis [5].

Sputum Sample Collection, Processing, and Smears

Subjects submitted the first expectorated spot sputum sample on enrollment and 2 additional samples collected first thing in the morning at home. We requested repeated samples if a sample was inadequate, and we used a jet nebulizer to induce sputum with 3% hypertonic saline if all expectorated samples were inadequate. Samples were stored in an air-tight leak-proof container at 4°C–8°C and processed within 1 week of collection. Sputum specimens were diluted in 2% sodium hydroxide

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and centrifuged at 4000 rpm for 15 minutes before sterile water resuspension and repeated centrifugation at 3500 rpm. Sputum samples were stained for AFB with auramine O for initial reading; results were confirmed with Ziehl-Neelsen staining. Smear staining was categorized by grade (grade 0, no AFB per 100 fields; positive, 1–9 AFB per 100 fields; grade 1+, 10–99 AFB per 100 fields; grade 2+, 1–10 AFB per field; and grade 3+, >10 AFB per field), and mean smear grades were calculated for each subject.

Sputum Culture

We used a sterile technique to inoculate the sputum centrifuge pellet onto glycerol and pyruvate Lowenstein-Jensen slopes supplemented with trypan blue before incubation in an air incubator at 35°–36°C with caps loose for ≥ 1 week. Cultures were examined 72 hours after inoculation, caps tightened, and incubated for 10 weeks. Visual inspection for culture growth was conducted at least weekly. Contaminated slants or slants with nonviable dark-green Lowenstein-Jensen medium were discarded. Sputum culture grade was categorized as follows: grade 0, <20 colonies; grade 1, 20–100 colonies; grade 2, discrete innumerable colonies; and grade 3, confluent growth of colonies. Among subjects with ≥ 1 positive sputum culture, we included in analyses the sputum culture with the greatest growth.

Tuberculin Skin Tests

The same day as phlebotomy for IFN- γ responses, all subjects underwent tuberculin skin tests (TSTs) performed by forearm intradermal injection of purified protein derivative, (0.1 mL; RT-23; State Serum Institute). Trained personnel measured injection site skin induration after 48–72 hours, and reactions of ≥ 5 mm were deemed positive. We offered isoniazid treatment to all subjects with positive reactions [6].

IFN- γ Release Assay

Freshly isolated Ficoll-treated peripheral blood mononuclear cells (2×10^5 cells at 1×10^6 cells/mL per well) collected at

baseline were incubated in triplicate in Corning Costar 96-well plates with study antigens for 5 days. Centrifuged cell supernatants were frozen and sent to the United States for later IFN- γ level measurement using a standard IFN- γ enzyme-linked immunosorbent assay (R&D Systems). Study antigens were medium alone (negative control), 1 μ g/mL *M. tuberculosis* early secretory antigenic target 6 (ESAT-6), 0.5 μ g/mL *M. tuberculosis* antigen 85 (Ag85), or 0.5 μ g/mL *M. tuberculosis* whole-cell lysate (WCL), with all antigens acquired through a contract awarded to Colorado State University from the National Institute of Allergy and Infectious Disease, National Institutes of Health (NIH). Owing to logistical difficulties, 58 of the 74 subjects with positive sputum cultures had data available regarding IFN- γ responses to WCL and Ag85, and 57 had data regarding IFN- γ responses to ESAT-6. All assay values were included in these analyses even if they were below the lower limit of detection of 156 pg/mL.

Clinical Surveillance for Tuberculosis Disease

After randomization, we evaluated subjects for active tuberculosis disease by interim history and physical examination at months 2, 4, and 6, and every 3 months thereafter. In addition, whenever subjects presented with ≥ 2 weeks of fever, cough, or weight loss, they underwent evaluation for active tuberculosis with single-view chest radiography, 3 sputum collections for AFB smear and mycobacterial culture, phlebotomy for mycobacterial blood culture, and additional studies as clinically indicated. Subjects with ≥ 1 culture positive for *Mycobacterium tuberculosis* from published DarDar Trial diagnostic categories of definite and probable tuberculosis [5] were included in this study.

Statistical Analysis

Using Stata 9 software (StataCorp), we correlated IFN- γ responses with categorized sputum culture grade (Cuzick test for trend) and with the continuous variables of mean sputum smear grade and days to sputum culture positivity (Spearman rank correlation coefficient).

RESULTS

Subject Characteristics

Among 2013 enrolled adult HIV-infected subjects, 58 subjects (25 vaccine recipients and 33 placebo recipients) with available IFN- γ response data developed tuberculosis with positive sputum cultures during prospective follow-up (median, 965 days; interquartile range [IQR], 598–1282 days). At baseline the mean subject age was 35 years, 20% were male, the TST result was positive in 56%, 14% reported prior treatment for active tuberculosis before enrollment, the mean baseline CD4 T-cell count was 396 cells/ μ L, and no subjects were receiving antiretroviral therapy. The mean baseline HIV load among the 39 subjects with available measurements was 54 134 copies/mL.

Table 1. IFN- γ Responses to Mycobacterial Antigens Among 58 Adults With Culture-Confirmed HIV-Associated Tuberculosis in a Phase III Tuberculosis Vaccine Trial

Antigen	Subjects, No		IFN- γ Response, pg/mL		
	With Available Data	With IFN- γ Responses Above Lower Limit of Detection	Minimum	Median	Maximum
ESAT-6	57	27	45	138	16 600
WCL	58	39	45	590	54 350
Ag85	58	21	50	125	2294

Abbreviations: Ag85, antigen 85; ESAT-6, early secreted antigenic target 6; IFN, interferon; WCL, whole-cell lysate.

Tuberculosis Bacillary Burden

The median sputum smear grade was 0.167 of 3 (IQR, 0–1), and the median time to sputum culture positivity was 35 days (IQR, 26–48 days). The sputum culture grade distribution was as follows: grade 0 in 19 subjects, grade 1 in 21, grade 2 in 6, and grade 3 in 12. There was no correlation between baseline CD4 T-cell count and median sputum smear grade (Spearman ρ , -0.078 ; $P = .52$), days to sputum culture positivity (Spearman ρ , 0.028 ; $P = .81$), or sputum culture grade (Spearman ρ , -0.124 ; $P = .29$).

IFN- γ Responses to Mycobacterial Antigens

Table 1 depicts IFN- γ responses to the mycobacterial antigens ESAT-6, WCL, and Ag85 among 58 study subjects in whom HIV-associated tuberculosis subsequently developed during study follow-up.

Association Between Baseline IFN- γ Responses and Tuberculosis Bacillary Burden

Table 2 depicts the correlation between baseline IFN- γ responses to mycobacterial antigens with 3 markers of tuberculosis burden: mean sputum smear grade, sputum culture grade, and days to sputum culture positivity.

Mean Sputum Smear Grade

Greater baseline IFN- γ responses to the mycobacterial antigen ESAT-6 were associated with lower subsequent mean sputum smear grade at the time of tuberculosis diagnosis. In stratified analyses, lower subsequent mean sputum smear grade was significantly associated with greater IFN- γ responses to ESAT-6 among vaccine recipients, subjects without prior active tuberculosis, subjects with a positive TST results, and subjects who received isoniazid. Baseline IFN- γ responses to WCL did not correlate significantly with mean sputum smear grade among all subjects, but in stratified analyses they were correlated among vaccine recipients, subjects without prior active tuberculosis, and those who did not receive isoniazid. There were no overall differences between vaccine and placebo recipients in mean sputum culture grade (0.681 vs 0.896; $P = .27$) and there was no correlation between IFN- γ responses to the mycobacterial antigen Ag85 with mean sputum smear grade.

Sputum Culture Grade

Greater baseline IFN- γ responses to ESAT-6 were significantly correlated with lower subsequent sputum culture grade among multiple subject strata. Greater baseline IFN- γ responses to WCL were correlated with lower sputum culture grade only among vaccine recipients and subjects without prior active tuberculosis. There were no overall differences between vaccine and placebo recipients in sputum culture grade (1.0 vs 1.33, $P = .20$), and there was no correlation between IFN- γ responses

to the mycobacterial antigen Ag85 and subsequent sputum culture grade.

Days to Sputum Culture Positivity

There was a trend toward a correlation between greater baseline IFN- γ responses to the mycobacterial antigen ESAT-6 and longer time to sputum culture positivity, but the correlation was significant only among vaccine recipients. Greater baseline IFN- γ responses to WCL were associated with more days to sputum positivity among all subjects as well as among multiple subgroups. There were no overall differences between vaccine and placebo recipients in days to sputum culture positivity (36.9 vs 35.9 days; $P = .86$) and there was no consistent correlation between IFN- γ responses to Ag85 and days to sputum culture positivity.

DISCUSSION

IFN- γ responses protect HIV-negative humans from tuberculosis in genetic analyses [1], and HIV-infected adults with preexisting IFN- γ responses to mycobacterial antigens have a reduced risk of subsequent tuberculosis [3, 7]. Our new data support the hypothesis that when preexisting IFN- γ responses to mycobacterial antigens fail to prevent tuberculosis they still modify tuberculosis bacillary burden, strongly suggesting that IFN- γ responses are involved in immunological containment of disease even when tuberculosis does develop.

Greater baseline IFN- γ responses were correlated with smaller tuberculosis bacillary burden, particularly among subjects who received SRL 172 vaccine but not placebo. We hypothesize that immunization with SRL 172, shown in a phase III trial to protect against HIV-associated tuberculosis [5], boosts baseline IFN- γ responses in a way that led to improved immune containment of tuberculosis [8]. This novel observation has important implications for tuberculosis vaccine development. Findings of a recent study indicate that generation of culture-positive cough aerosols is the best predictor tuberculosis transmission [9], but high-magnitude smear positivity remains an important predictor of transmission risk, and both are reasonable secondary end points in new tuberculosis vaccine trials.

The correlations between IFN- γ response and markers of tuberculosis disease burden were not seen uniformly across all antigens for all markers of tuberculosis burden. One explanation for these findings is the possibility that only IFN- γ responses targeting specific antigens are associated consistently with protection from HIV-associated tuberculosis; borderline study power is another possible explanation. The observed correlations between IFN- γ responses and markers of tuberculosis disease burden could result from chance, but this is unlikely

Table 2. Correlation of 3 Markers of Tuberculosis Bacillary Burden With Interferon γ Responses to the Mycobacterial Antigens ESAT-6, WCL, and Ag85 Among 58 Adults With Culture-Confirmed HIV-Associated Tuberculosis in a Phase III Tuberculosis Vaccine Trial

Subjects by Stratified Analyses	Mean Sputum Smear Grade						Sputum Culture Grade						Days to Sputum Culture Positivity					
	ESAT-6 (n = 55)		WCL (n = 56)		Ag85 (n = 56)		ESAT-6 (n = 57)		WCL (n = 58)		Ag85 (n = 58)		ESAT-6 (N = 55)		WCL (n = 56)		Ag85 (n = 56)	
	Spearman Rank Correlation Coefficient		Spearman Rank Correlation Coefficient		Spearman Rank Correlation Coefficient		Cuzick Test for Trend		Cuzick Test for Trend		Cuzick Test for Trend		Spearman Rank Correlation Coefficient		Spearman Rank Correlation Coefficient		Spearman Rank Correlation Coefficient	
	ρ	PValue	ρ	PValue	ρ	PValue	z	PValue	z	PValue	z	PValue	ρ	PValue	ρ	PValue	ρ	PValue
All subjects	-0.336	.01	-0.156	.25	0.033	.81	-2.49	.013	-1.13	.26	-0.38	.70	0.255	.06	0.263	.047	0.173	.20
Baseline CD4 T-cell count, cells/ μ L																		
<350	-0.283	.14	-0.035	.86	0.049	.81	-0.64	.52	0.37	.71	0.83	.41	0.199	.30	0.189	.32	0.065	.74
\geq 350	-0.285	.15	-0.111	.58	0.023	.91	-2.25	.02	-1.69	.09	-1.26	.21	0.289	.14	0.393	.04	0.262	.17
Vaccine receipt																		
SRL 172 vaccine	-0.625	.001	-0.457	.03	-0.219	.30	-3.19	.001	-2.32	.02	-1.67	.10	0.517	.008	0.445	.03	0.328	.11
Placebo	-0.050	.79	0.043	.81	0.122	.51	0.00	>.99	0.41	.68	0.36	.72	0.129	.48	0.181	.31	0.078	.67
Prior active tuberculosis																		
Yes	0.205	.63	0.675	.07	0.321	.44	1.03	.30	1.42	.16	0.52	.60	0.359	.38	0.755	.03	0.769	.03
No	-0.503	<.001	-0.348	.02	-0.083	.58	-3.07	.002	-1.98	.048	-0.79	.43	0.255	.08	0.210	.14	0.088	.54
TST reaction, mm																		
<5	-0.244	.24	-0.121	.57	-0.108	.62	-2.25	.02	-1.56	.12	-1.24	.21	0.126	.56	-0.019	.93	-0.088	.67
\geq 5	-0.432	.02	-0.199	.29	0.253	.18	-1.53	.13	-0.13	.90	1.23	.22	0.171	.36	0.250	.18	0.003	.98
Isoniazid receipt																		
No	-0.209	.25	0.011	.95	-0.012	.95	-1.54	.12	-0.36	.72	-0.82	.41	0.292	.10	0.187	.29	0.223	.20
Yes	-0.535	.009	-0.414	.049	0.136	.54	-1.97	.049	-1.36	.18	0.74	.46	0.094	.66	0.275	.19	0.014	.95

Abbreviations: Ag85, antigen 85; ESAT-6, early secreted antigenic target 6; TST, tuberculin skin test; WCL, whole-cell lysate; z, Cuzick z statistic.

because all correlations share the same biologically plausible directionality and there were more significant correlations than expected from chance alone.

Two groups of investigators have previously evaluated the correlation of IFN- γ responses to mycobacterial antigens with concurrent sputum tuberculosis bacillary load among HIV-infected adults [10, 11]. Unlike in our study, in which baseline IFN- γ responses were related prospectively to subsequently acquired sputum results, IFN- γ responses in those studies were measured simultaneously with the assessment of tuberculosis bacillary burden. Furthermore, we used a 5-day assay that, unlike the overnight assays used by the other authors, may also have assessed central memory responses and T-cell proliferative potential [12, 13]. Our study is thus the first to assess the relation of baseline IFN- γ responses with tuberculosis bacillary burden during subsequent rather than concurrent disease.

Our study was prospective, conducted in a phase III trial in a resource-limited setting with high tuberculosis prevalence, and subjects with active tuberculosis were excluded at enrollment. Our small sample size precluded multivariate analyses, and thus confirmation of our results will be important. We hypothesize that greater-magnitude IFN- γ responses contribute to lower tuberculosis burden among HIV-negative individuals too, but we could not address this hypothesis in the current study.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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