Prevention of tuberculosis in Bacille Calmette–Guérin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine

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Objective: To determine whether a multiple-dose series of an inactivated whole cell mycobacterial vaccine, *Mycobacterium vaccae*, can prevent HIV-associated tuberculosis.

Design and methods: The DarDar trial was a randomized, placebo-controlled, double-blind trial. The study was carried in an outpatient facility in Dar es Salaam, Tanzania. HIV-infected patients with CD4 cell counts of at least 200 cells/μl and a Bacille Calmette–Guérin scar were chosen for the study. The intervention was carried out by random 1:1 assignment to five intradermal doses of *M. vaccae* or placebo. Tuberculin skin tests were performed, and patients with reactions of at least 5 mm were administered isoniazid for 6 months. The main outcome measures were disseminated (primary endpoint), definite, and probable tuberculosis (secondary endpoints).

Results: Two thousand thirteen individuals were randomized (1006 to *M. vaccae*, 1007 to placebo) and followed every 3 months for a median of 3.3 years. The trial was terminated early because of slow accrual of cases of disseminated tuberculosis and significant protection against definite tuberculosis. Hazard ratios were disseminated tuberculosis 0.52 (95% confidence interval 0.21–1.34; seven cases in *M. vaccae*, 13 cases in placebo; log-rank $P = 0.16$), definite tuberculosis 0.61 (95% confidence interval 0.39–0.96; 33 cases in *M. vaccae*, 52 cases in placebo; $P = 0.03$), and probable tuberculosis 1.17 (95% confidence interval 0.76–1.80; 48 cases in *M. vaccae*, 40 cases in placebo; $P = 0.46$). Immunization was well tolerated, with no adverse effect on CD4 cell count or HIV viral load, and no increase in the rate of serious adverse events.

Conclusion: Administration of a multiple-dose series of *M. vaccae* to HIV-infected adults with childhood Bacille Calmette–Guérin immunization is safe and is associated with significant protection against definite tuberculosis. These results provide evidence that immunization with a whole cell mycobacterial vaccine is a viable strategy for the prevention of HIV-associated tuberculosis.

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Introduction

The global tuberculosis (TB) epidemic continues to grow with an estimated 9.3 million new cases and 1.7 million deaths in 2007 [1]. HIV infection is a major contributor to the epidemic, and neither isoniazid (INH) preventive therapy (IPT) nor antiretroviral therapy (ART) is completely effective in reducing this risk [2−5]. Consequently, TB remains the major cause of death in most regions where TB and HIV coexist [4−7]. Disease due to multiple-drug resistant (MDR) TB and extensively-drug resistant (XDR) TB has also fueled the global epidemic [8−10]. Despite the wider application of ART and the development of newer and more potent antimicrobials, the current multifaceted TB epidemic continues to grow at a rate of 1% per year [7].

An effective preventive TB vaccine would have the potential to impact each of the components of the global epidemic [11]. The existing TB vaccine, *Mycobacterium bovis*, Bacille Calmette−Guérin (BCG), is more than 80% effective in childhood when given to mycobacteria-naive newborns but is generally considered to offer minimal or no protective effect when administered to previously unvaccinated adults [12,13]. BCG is also ineffective for boosting immunity in those who received the vaccine in childhood [14−16].

Before the widespread acceptance of BCG, inactivated whole-cell mycobacterial vaccines, derived from both *Mycobacterium tuberculosis* and nontuberculous mycobacteria, had been shown to be effective in preventing TB in experimental models and in clinical trials but were not widely adopted, presumably because of the need for multiple-dose immunization [17−19]. We considered that an inactivated whole-cell mycobacterial vaccine could be used in a prime-boost immunization strategy after childhood BCG and would be expected to be well tolerated in HIV-infected persons. Our international epidemiologic studies [20−24] of disseminated mycobacterial infections in AIDS suggested that although African AIDS patients were exposed to organisms of the *Mycobacterium avium* complex (MAC), they were generally protected against disseminated MAC, presumably from immunity induced by prior TB and/or prior BCG immunization. This conclusion is consistent with extensive epidemiologic and experimental studies demonstrating that infection with one species of mycobacteria provides cross-protection against other (heterologous) species (e.g., infection with nontuberculous mycobacteria protects against TB) [25−27]. Further, recent evidence suggests that antigens of the DosR regulon, present in most nontuberculous mycobacteria, but not in BCG, are associated with the control of latent TB [28].

Our hypothesis was that in patients with HIV infection, the mycobacterial immunity that had already been primed by childhood BCG immunization might be boosted to provide protection against TB. We posited that a successful prime-boost strategy against TB in HIV infection would need to meet the following several requirements. First, it would need to be given early in HIV infection for an optimal immune response. Second, it would need to present multiple antigens because of the reduced T-cell diversity in HIV infection. Third, to be well tolerated in HIV, it could not include live mycobacteria. An inactivated whole-cell mycobacterial reagent would satisfy all these requirements.

Such a vaccine was developed by Drs John Stanford and Graham Rook using a strain of *Mycobacterium vaccae* [29]. Single-dose studies [30,31] of *M. vaccae* for the treatment of drug-sensitive TB were unsuccessful. We realized that an inactivated vaccine would need to be administered in a multiple-dose series to induce the immune response we sought for the prevention of TB. We, therefore, conducted phase I studies [32−35] to demonstrate the safety of a multiple-dose series of *M. vaccae* in healthy adults and in HIV-infected adults and children. A phase II study [36] in Zambia indicated that five doses of *M. vaccae* could induce T-cell responses against mycobacteria in HIV-infected patients, and that responses were maximal in recipients primed with childhood BCG immunization. A subsequent, randomized, controlled, phase II trial [37] in Finland demonstrated that five doses of *M. vaccae* were well tolerated in HIV-infected patients and boosted T-cell mycobacterial responses in recipients primed with BCG. The present phase III trial was designed to test the efficacy of a five-dose series of *M. vaccae* in the prevention of HIV-associated TB among BCG-primed recipients in a TB-endemic country.

Methods

Participants

Eligible participants were ambulatory HIV-positive residents of Dar es Salaam, Tanzania, with age at least 18 years with CD4 cell count of at least 200 cells/μl, a visible BCG scar from childhood immunization (sensitivity >90% [38]), a negative pregnancy test, and no evidence of active TB. Participants were recruited from HIV testing centers. At baseline, all patients were interviewed, examined, and had the following tests performed: HIV ELISA, CD4 cell count, tuberculin skin test (TST; RT-23; State Serum Institute, Copenhagen, Denmark), single posterior−anterior chest radiograph, and collection of three sputum samples for acid fast bacillus (AFB) stain and mycobacterial culture. Patients with a TST result of at least 5 mm and no history of prior treatment for TB were given 6 months of IPT, 300 mg daily for 6 months [39].

Immunization protocol

A 1 : 1 randomization list was generated at Dartmouth and assigned sequential participants in Dar es Salaam to one of

the 20 numbered but otherwise unidentified boxes of *M. vaccae* or placebo (10 each) for their entire treatment course. Treatment assignments were generated in randomly permuted blocks of eight, 10, 12, 14, and 16 participants. Thus, all patients and study personnel, including the technical nurse who administered the vaccine, were blinded to treatment assignment; no evaluations for vaccine site reactions or suspect TB were performed by the technical nurse. Patients received a five-dose series of 0.1 ml intradermal *M. vaccae* (*M. vaccae*, SRL 172, ~1 mg, 10^9 colony-forming units; Immodulon, London, UK) or placebo (borate buffered isotonic saline, same appearance, identical vial) over the deltoid at 0, 2, 4, 6, and 12 months.

Follow-up

All patients were seen routinely every 3 months and were instructed to return to the study site whenever they experienced new symptoms or clinical events and to report any hospitalizations. CD4 cell counts and HIV viral loads were performed annually on all patients through 22 August 2003. Patients with CD4 cell count of less than 200 cells/μl, new active TB, or who met other Ministry of Health (MOH) guidelines for ART were referred to an MOH clinic for HIV treatment. Evaluation for active TB was conducted by study staff and included a physical examination, chest radiograph, three expectorated sputum samples for AFB stain and culture (one spot sample and two first morning samples; standard

techniques including culture on Lowenstein Jensen agar), and an automated mycobacterial blood culture (a single 5-ml sample until March 2004, then two 5-ml samples; MB BacT; bioMérieux, Durham, North Carolina, USA). Patients with suspect TB were referred to the MOH for TB treatment. Patients with suspect or treated TB were subsequently categorized as disseminated, definite, or probable TB based on the consensus of three blinded experts who reviewed all clinical and laboratory data, interpreted all chest radiographs, and applied definitions for study endpoints (Table 1).

Statistical analysis

Study endpoints defined prospectively by protocol included disseminated, definite, and probable TB (Table 1). The primary endpoint was the time to disseminated TB (defined by a positive blood culture) in the intention-to-treat (ITT) population. The three secondary endpoints were time to first episode of definite, probable, or definite along with probable TB ('all tuberculosis'). For patients with more than one endpoint, only the first endpoint was included in the analysis.

The ITT population was defined as all randomized patients. The per-protocol population was defined as all patients who received at least three doses of vaccine. Time until TB outcome was calculated on the basis of the study population and the outcome. The starting

Table 1. Tuberculosis endpoints.

Endpoint	Subcategory	*n*
Definite	A1 – ≥1 positive blood culture for *M. tuberculosis* (disseminated)	20
	A2 – ≥1 positive sputum culture(s) with ≥10 CFU	51
	A3 – ≥2 positive sputum cultures with 1–9 CFU	6
	A4 – ≥2 sputum smears with ≥2 AFB/100 oil immersion fields	7
	A5 – ≥1 positive culture, or positive AFB smear and caseous necrosis from a sterile site (other than blood)	1
Probable	B1 – caseous necrosis on a biopsy	2
	Pulmonary	
	B2 – a positive chest radiograph[a] along with either one sputum AFB smear with ≥2 AFB/100 oil immersion fields or one sputum culture with 1–9 CFU	5
	B3 – clinical symptoms/signs[b] along with either one positive sputum AFB smear, ≥2 AFB/100 oil immersion fields or one sputum culture with 1–9 CFU	11
	B4 – clinical symptoms/signs and a positive radiograph and a response to antituberculous therapy[c]	62
	Extrapulmonary	
	B5 – clinical symptoms/signs and a positive imaging study and a response to antituberculous therapy	5
	B6 – one positive AFB smear from a sterile site along with clinical symptoms/signs of tuberculosis	1

AFB, acid fast bacillus; CFU, colony-forming units; *M. tuberculosis, Mycobacterium tuberculosis*.
[a]A positive chest radiograph, compared with a baseline film, is defined as exhibiting any of the following new findings: pulmonary infiltrates (with or without cavitation), pleural effusion, pulmonary fibrosis, or intrathoracic lymphadenopathy. These findings would be expected to persist after 10 days of treatment for routine community-acquired pneumonia.
[b]Symptoms are defined as any of the following: cough for at least 2 weeks, fever for at least 2 weeks, and weight loss of at least 5 kg/year. Signs include any of the following findings on physical examination: rales, dullness to percussion, egophony or bronchial breath sounds (consistent with consolidation), hepatosplenomegaly, lymphadenopathy of at least 1 cm at at least two sites, pericardial friction rub, and focal bone tenderness.
[c]Response to therapy is defined as at least two of the following: gain of more than 3 kg since start of treatment, clearing of fever, reduction of at least 50% in cough frequency, reduction of at least 50% in pleural effusions, infiltrates or intrathoracic or extrathoracic adenopathy, reduction of at least 50% by imaging of other disease due to TB (e.g., pericardial effusion, osteomyelitis, and so on), conversion of AFB smear from positive to negative thrice, conversion of sputum culture from at least 1 CFU to negative thrice.

point for ITT population analyses was the date of randomization, and for the per-protocol population, it was the date of the third dose of vaccine. Each episode of TB was assigned a diagnosis date. For cases with a positive smear or culture, this was the date when the first positive specimen was obtained; otherwise, it was the date when treatment was started. Participants with no TB found during follow-up were censored at the date of the last examination.

The planned sample size was selected to have 80% power to detect a 50% reduction in disseminated TB hazard at the 0.05 (two-sided) level of significance. The required enrollment was a total 2274 patients expected to experience 71 cases of disseminated TB (1.60% per person-year) over a median follow-up of 48 months. One interim efficacy analysis, planned to occur at the midpoint of follow-up, was based on an O'Brien–Fleming stopping boundary [40]. Baseline characteristics of the randomized participants were compared using descriptive statistics, Student's t-test, and Fisher's exact test as appropriate. The probability of TB over time was estimated using the Kaplan–Meier product limit method; treatment arms were compared using the log-rank test, and hazard ratios were calculated using proportional hazards regression (*M. vaccae* relative to placebo). To evaluate the effect of *M. vaccae* in the presence of ART, we performed a proportional hazards regression analysis, which included a time-dependent covariate reflecting ART status. Prespecified subgroup analyses evaluated the vaccine effect according to age, sex, baseline TST, and baseline CD4 cell count. Effect modification by these factors was assessed using proportional hazards regression with appropriate inter-action terms. The time-to-event was measured in days; repeat analyses using interval-censored follow-up design with 3-month intervals yielded indistinguishable results. Efron's method of handling ties was used. Serious adverse events were compared using Fisher's exact test. All P values are two-sided, and a P value of less than 0.05 was considered statistically significant.

Study design
The study protocol was approved by the Dartmouth Committee for the Protection of Human Subjects, by the Muhimbili University of Health and Allied Sciences (MUHAS) Research Ethics Committee, and by the Division of AIDS Clinical Science Review Committee, National Institutes of Health (NIH). All study participants gave written informed consent. Regulatory oversight of the trial was conducted by the Tanzanian Food and Drug Authority. The study results (including blinded safety and efficacy data) were reviewed every 6 months by an independent Data Safety and Monitoring Board (DSMB) comprising six members from the United States and Tanzania, with expertise in vaccines, HIV, TB, and statistics. The DSMB could request unblinded data and

make nonbinding recommendations regarding the completion of enrollment and continuation of the study.

Results

The first patient was enrolled in September 2001 and the last patient in September 2005. A total of 4973 patients were screened to identify 2013 eligible, randomized patients before expiration of the vaccine lot. The study was terminated early on 31 January 2008 based on a recommendation from a closed session of the DSMB after their independent consultation with the sponsor [National Institute of Allergy and Infectious Diseases (NIAID), Division of AIDS, NIH]. The DSMB cited two reasons for termination. First, cases of the primary endpoint, disseminated TB, although showing a trend toward protection, were not accruing at a sufficient rate to provide adequate power for detecting a difference between vaccine and placebo arms. Second, the rate of definite TB indicated a significant level of protection by the vaccine.

Figure 1 shows the recruitment and follow-up of all patients. Of the 2013 patients randomized, 1006 were allocated to *M. vaccae* and 1007 were allocated to placebo and form the ITT population. Baseline characteristics of the two treatment groups in the ITT population were similar (Table 2). The per-protocol population who received at least three doses was composed of 1853 (92%) patients. The median duration of follow-up from enrollment, with interquartile values, was 3.3 years for *M. vaccae* (2.5–4.2) and 3.3 years for placebo(2.4–4.3). Loss to follow-up occurred in 184 (18.3%) of *M. vaccae* recipients and 182 (18.1%) of placebo recipients.

Table 3 summarizes cases of TB endpoints and hazard ratios. The number of disseminated TB endpoints was insufficient to determine whether *M. vaccae* was effective against the primary endpoint of disseminated TB; protection was significant for the secondary endpoint of definite TB but not for probable TB (or the
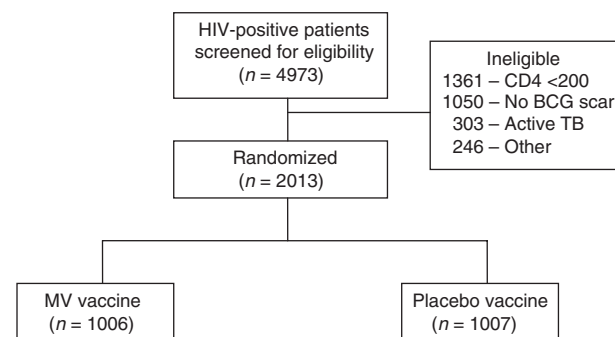

Fig. 1. Consort diagram. BCG, Bacille Calmette–Guérin; MV, *Mycobacterium vaccae*; TB, tuberculosis.

Table 2. Baseline characteristics of study participants.

Characteristic	MV ($n = 1006$)	Placebo ($n = 1007$)	P
Age (years), mean (SD)	33.2 (8.0)	33.0 (7.8)	0.57
Female sex	774 (77%)	766 (76%)	0.68
BMI (kg/m²), median (range)	23.7 (15.3–50.8)	23.6 (16.1–47.4)	0.45
CD4 cell count (cells/μl), median (minimum, 25th–75th percentile, maximum)	428 (200, 317–573, 1645)	404 (200, 298–580, 2000)	0.50
Log HIV viral load (copies/ml), median (range)	4.12 (1.70–5.70)	4.08 (1.70–5.70)	0.26
HIV antiviral therapy	26 (2.6%)	34 (3.4%)	0.36
Prior treatment for TB	88 (9%)	82 (8%)	0.68
Tuberculin skin test ≥5 mm[a]	315 (31%)	324 (32%)	0.71

MV, *Mycobacterium vaccae*; TB, tuberculosis.
[a]Two hundred forty-six MV recipients and 247 placebo recipients completed isoniazid preventive therapy.

combination of definite and probable, Appendix). Prespecified subgroup analyses showed no significant modification of vaccine efficacy for definite TB by age, sex, baseline TST, INH treatment, or baseline CD4 cell count. For patients with a history of prior TB ($n = 169$), the hazard ratio for definite TB was 0.31 [95% confidence interval (CI) 0.10–0.98] compared with 0.70 (95% CI 0.43–1.13) among recipients without such a history (interaction $P = 0.18$).

The time-to-event analyses for the ITT population are shown in Fig. 2 (a and b). We examined the effect of ART, although this was not a prespecified analysis, as ART use was uncommon at the start of the study. Overall, 566 (28%) patients received ART at some point during the study (292 vs. 274 at *M. vaccae* and placebo, respectively). There were 857 person-years of follow-up of patients on ART and 5639 person-years of follow-up of patients not on ART. Using a proportional hazards regression model with ART status as a time-dependent covariate, there was no significant interaction between vaccine efficacy and ART (interaction $P = 0.37$). However, the estimated hazard ratio for definite TB in the absence of ART (0.56; 95% CI 0.34–0.93) was numerically lower than in the presence of ART (0.94; 95% CI 0.14–6.17).

A total of 4616 doses of *M. vaccae* and 4603 of placebo were administered. For the first 165 patients, the vaccine site was assessed at days 7 and 28 after each of the five doses. Among *M. vaccae* recipients, the median vaccine site induration after each dose was 5–6 mm (range 0–30 mm) at both 7 and 28 days; among placebo recipients, all median values were 0 mm (range 0–8 mm). At 7 days after each dose, skin desquamation was observed in 35–61% of *M. vaccae* recipients (0–3% placebo) and local drainage in 9–35% (0–3% placebo). At 28 days, these changes were seen in 18–23% and 7–19% of *M. vaccae* recipients (0–1% and 0% placebo). Local reactions in *M. vaccae* recipients were maximal after third dose and typically resolved within several weeks. The proportion of *M. vaccae* and placebo recipients who reported systemic symptoms including fever, malaise, or headache, ranged from 4 to 23% across all assessments and was similar for both treatment groups (Table A in Appendix).

Immunization was discontinued by the on-site investigator for 12 (1.2%) *M. vaccae* recipients (and one placebo recipient) because of adverse reactions considered related to vaccine. These events included three vaccine site abscesses, five other local reactions, and four generalized rashes. Vaccine site abscesses occurred after the first dose in one patient and after the third dose in two patients (both with a history of prior TB); all abscesses drained spontaneously and resolved with routine wound care and oral antibiotic therapy. An additional 65 patients (35 *M. vaccae*, 30 placebo) withdrew themselves from the study before completing immunization, typically citing a switch to alternative medicine, perceived vaccine side effects, or inconvenience.

There were 94 deaths in each treatment group (hazard ratio 0.97, 95% CI 0.73–1.30, $P = 0.86$). The causes of death as reported by relatives or confidantes did not differ

Table 3. Study endpoints and protection against tuberculosis.

Endpoint	Intention-to-treat ($n = 2013$)				Per protocol[a] ($n = 1853$)			
	No. of endpoints MV	Placebo	Hazard ratio (95% CI)	P	No. of endpoints MV	Placebo	Hazard ratio (95% CI)	P
Disseminated tuberculosis	7	13	0.52 (0.21–1.34)	0.16	7	13	0.54 (0.21–1.37)	0.17
Definite tuberculosis	33	52	0.61 (0.39–0.96)	0.03	33	51	0.64 (0.41–1.00)	0.04
Probable tuberculosis	48	40	1.17 (0.76–1.80)	0.46	47	39	1.20 (0.78–1.85)	0.39

CI, confidence interval; MV, *Mycobacterium vaccae*.
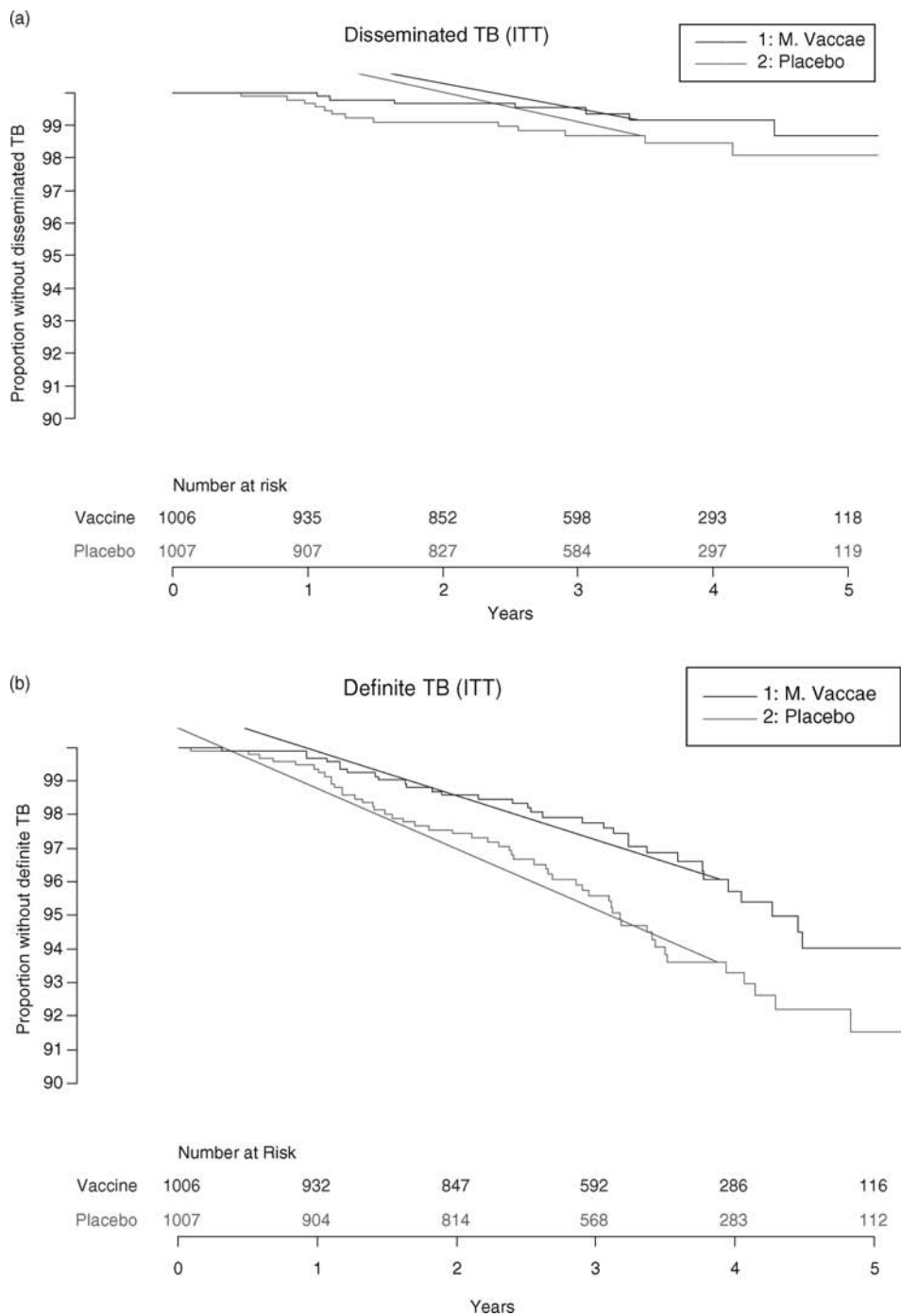[a]Per protocol, at least three doses of MV or placebo.

Fig. 2. Tuberculosis study endpoints (Kaplan–Meier, intention-to-treat). (a) Disseminated TB. (b) Definite TB. ITT, intention-to-treat; M. vaccae, Mycobacterium vaccae; TB, tuberculosis.

between the two treatment groups, including deaths attributed to TB, AIDS, and other febrile illnesses. The proportion of patients with one or more serious adverse events was similar in both groups (Table 4); events attributed to malaria (including some clinical diagnoses and some false–positive smears, thus some 'malaria' cases were possibly due to other causes of fever such as TB) were significantly lower in M. vaccae recipients. No

adverse effects were noted on CD4 cell count or HIV viral load (data not shown). Repeat TST data on 215 patients showed that among those with a TST of less than 10 mm at baseline, the frequency of a TST of at least 10 mm following the fifth dose was similar in both treatment groups [8/81 (9.9%) M. vaccae vs. 8/71 (11.3%) placebo; $P = 0.92$]. No patients were observed to have a Koch reaction.

Table 4. Serious adverse events in the intention-to-treat population.

Category	MV	Placebo	P
Hospitalization, tuberculosis	2	6	0.29
Hospitalization, malaria	76	103	0.04
Hospitalization, other	128	159	0.06
Infection, other	41	44	0.82
Obstetric	8	6	0.61
All other	57	40	0.08
Total patients with ≥1 SAE	209	232	0.24

MV, *Mycobacterium vaccae*; SAE, serious adverse event.

Discussion

The present study demonstrates that a multiple-dose series of inactivated *M. vaccae* given to BCG-primed recipients with HIV infection and baseline CD4 cell count of at least 200 cells/μl is well tolerated and provides significant protection against definite TB with rigorous microbiologic confirmation. It was underpowered to determine the efficacy of *M. vaccae* in preventing the terminal HIV complication of disseminated TB. To our knowledge, this is the first placebo-controlled trial to demonstrate effective vaccine-based prevention of an opportunistic infection in HIV-infected adults. Vaccine-based prevention of pneumococcal disease has been demonstrated in a placebo-controlled trial in HIV-infected children [41].

We chose disseminated or bacteremic TB as the primary study endpoint based on our prior studies [24,42–44], indicating that this form of disease is common in advanced HIV infection (CD4 cell count <75 cells/μl). Further, it is strictly defined, more readily classified than pulmonary TB, and might represent the most sensitive indicator of a biologic effect of immunization (e.g., BCG is most effective in preventing miliary and meningeal TB, which are likely mediated via bacteremia [13]). At the time of termination, only 20 cases of disseminated TB had been detected compared with the 71 expected cases. Factors that may have directly reduced the rate of disseminated TB include prevention of dissemination of TB by aggressive surveillance for and treatment of the earlier complication of pulmonary TB, the increasing use of ART over time, administration of INH to patients with positive TSTs, and the return of patients with advanced AIDS to their home villages, making follow-up less complete for this endpoint. The observed reduction in disseminated TB cases (seven *M. vaccae* vs. 13 placebo) paralleled the statistically significant reduction in definite TB cases.

Definite TB, defined here as a secondary endpoint, is the usual primary endpoint in trials for the prevention of TB [3,45], and protection was significant against this rigorously defined and clinically meaningful endpoint. The lack of *M. vaccae*-induced protection against the other secondary endpoint, clinically defined or 'probable' TB, is

parallel to the finding in the recent update by Aronson *et al.* [46] of the 3287 persons BCG trial conducted in the western United States in the 1930s. BCG was found to offer significant protection against culture-confirmed TB for as long as 50–60 years but no protection against clinical/probable TB (nine probable TB cases in BCG recipients and three probable cases in the placebo group) [46]. In the one, other successful BCG trial from the meta-analysis by Colditz *et al.* [12] of prospective trials, which included definitions for both culture-confirmed definite TB and clinically categorized probable TB, vaccine efficacy was lower for probable than definite TB [47]. There are two possible explanations for this repeated observation in TB vaccine trials: case misclassification or a fundamental and as yet unidentified characteristic of 'clinical' or probable TB. In either case, future trials of new vaccines against TB should carefully distinguish definite vs. probable cases of TB.

Local and systemic reactions to *M. vaccae* were milder and less frequent than generally observed with BCG [13]. We observed three vaccine site abscesses among the 1006 *M. vaccae* recipients (0.3%); these resolved with simple treatment. Overall, 94% of enrolled patients had a preexisting humoral or cellular immune response to one or more of the five mycobacterial antigens [48] and 8% had a history of prior TB. Nevertheless, we did not observe any Koch reactions or other pathologic local or systemic responses to repeated doses of *M. vaccae* [49]. Immunization with *M. vaccae* was not associated with 'conversion' of the TST and would not be expected to affect newer interferon gamma release assays, as the *M. tuberculosis* RD1 antigens used in these tests are absent from *M. vaccae* [50].

These data conclusively establish the safety of this inactivated whole-cell mycobacterial vaccine in persons with HIV and are consistent with those reported in other trials of *M. vaccae* in persons without HIV [30,31]. However, the major differences in both the regimens and objectives for our *M. vaccae* trial compared with previous ones are important to note. We examined the ability of multiple-dose *M. vaccae* to boost immunity and prevent TB in persons primed by BCG. Previous trials studied single-dose *M. vaccae* as an adjunct to treatment of drug-susceptible TB [30,31]. Inactivated whole-cell vaccines must be given in a multiple-dose schedule to obtain an optimal preventive immune response [51].

Although morbidity due to TB was reduced by immunization, we did not observe an effect on mortality. In our research setting, all patients were followed closely, had TB diagnosed promptly, sometimes before the onset of clinical symptoms or radiologic abnormalities [52,53], and were referred for ART. It is possible that a mortality benefit might accrue among patients with more limited access to medical care, with delayed diagnosis and treatment of TB. Only a limited number of patients in this

trial were receiving ART. Although there was a trend toward lower vaccine efficacy in those receiving ART, the difference was not statistically significant.

Notable strengths of the trial include that it was randomized, placebo-controlled, double-blinded, and well executed. We were able to achieve high rates of compliance with a five-dose schedule, a regimen that might encourage closer follow-up of patients with HIV infection. However, we have shown that a three-dose schedule is immunogenic [37], and further study of this more practical schedule should also be considered.

Visible acute vaccine site reactions in *M. vaccae* recipients, such as those in the early phases of the immunization schedule, could have led to unblinding by on-site staff, an issue also identified as a potential problem in BCG trials. However, as the trial proceeded, only small scars remained in *M. vaccae* recipients, all of whom also had BCG scars and some of whom had smallpox scars. Further, decisions to obtain sputum samples and chest radiographs or refer patients for treatment were never made by the technical nurse who administered the immunizations. If a referral bias existed for TB treatment, it might have favored patients with no local reactions who may have been felt to be at a greater risk of TB. Finally, all study endpoints were adjudicated by a blinded panel that did not have access to information regarding vaccine site reactions. Enrollment in this study was restricted to patients with a CD4 cell count of at least 200 cells/μl, a BCG scar, and no active TB; consequently, the results are only applicable to this population.

Conclusion

This study indicates that boosting childhood BCG with a five-dose series of *M. vaccae* provides 39% protection against definite TB in HIV-infected adults with a CD4 cell count of at least 200 cells/μl in Tanzania. The reduction in risk observed in this study supports further development of a prime-boost immunization strategy using BCG and a whole-cell inactivated mycobacterial vaccine for preventing the most important opportunistic infection affecting HIV-infected persons in the developing world. As the risk of TB in HIV begins before the current CD4 cell threshold for initiation of ART, booster immunization could be considered at the time of initial diagnosis of HIV in areas where TB is endemic.

Acknowledgements

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Appendix

Participants

HIV viral load was performed at baseline until August 2003. Blood was obtained at baseline and after dose 5 for immunologic assays including lymphocyte proliferation assays and interferon gamma release assays in response to four mycobacterial antigens (these studies are the subject of a separate report). Persons with tuberculin skin test reactions (TST) ≥ 5 mm at baseline and no history of prior treatment for tuberculosis were given isoniazid 300 mg daily for 6 months and followed monthly during this interval [54]. TSTs were repeated 2 months after dose 5.

Subjects were randomized 1 : 1 in blocks of 6–14 to receive a 5-dose series of 0.1 mL intradermal *Mycobacterium vaccae* (MV; SRL 172; batch MV 017 until July 31, 2004 then batch S00631; Silence Therapeutics, London) or placebo (P; borate buffered saline in an identical vial) on the forearm at 0, 2, 4, 6, and 12 months.

Substudies

In Substudy A (safety), the first 165 subjects were scheduled to return at one and four weeks after each dose of vaccine for detailed examination of vaccine site reactions and for interviews on local and systemic side effects (Table A). Subjects also had HIV viral load and CD4 count determined 2 months after each dose of vaccine. In Substudy B (immunogenicity), the next 150 subjects had vaccine immunology performed 2 months after dose 3 and 5, and annually. Subjects in both Substudy A and Substudy B had repeat tuberculin skin testing performed 2 months after dose 3 and 2 months after dose 5.

Follow-up

Patients with suspect tuberculosis were referred to the National Tuberculosis and Leprosy Control Program (NTLP) with results of these evaluations for decisions about treatment. Standard tuberculosis treatment at the inception of the study was directly observed isoniazid, rifampin, ethambutol and pyrazinamide for 2 months followed by isoniazid and ethambutol for another 6 months.

On February 1, 2006 NTLP treatment guidelines were changed to directly observed isoniazid, rifampin, ethambutol and pyrazinamide for 2 months followed by isoniazid and rifampin for another 4 months. Follow up data on treatment outcome were obtained by interview and examination of patients at 2, 5 and 8 months of treatment (and after the change in treatment policy in 2006 at 2, 4 and 6 months).

All subjects were treated for intercurrent infections at the study site or by referral to district hospitals for inpatient care. At each visit, subjects were evaluated to determine if they met current national criteria for initiation of antiretroviral therapy (ART). ART-eligible subjects were counseled and referred to a Tanzanian Ministry of Health Care and Treatment Center for ART. Dates and putative causes of death were obtained from interviews with a relative or confidante named at the time of study entry, and, less commonly, from treating medical facilities and personnel.

Laboratory studies

Sputum AFB stains were performed using the Ziehl-Neelsen method. Mycobacterial sputum cultures were performed at the study laboratory in Tanzania using standard methods: 1% NaOH decontamination followed by culture on Lowenstein-Jensen agar slants with 10 weeks of air incubation. Blood cultures were processed by inoculation of 5 mL blood into an automated broth system with a 6 week incubation period (MB/BacT, bio-Merieux, Lyon, France) with back-up use of the Isolator mycobacterial blood system (Inverness Medical Professional Diagnostics, Princeton, NJ) at times when the automated system was not operational [55]. Beginning November 19, 2004 a 6 week terminal subculture to Lowenstein-Jensen medium was performed on all MB/BacT negative blood cultures. AFB positive isolates were shipped to Dartmouth Medical School and tested for the *M. tuberculosis* complex (MTBC) using DNA probes (Accuprobe, Gen-Probe, San Diego, CA).

IS*6110* typing was performed on study isolates and concurrent community isolates obtained from the NTLP laboratory by Dr. Barry Kreiswirth using standard methods [56]. Isolates with IS*6110* patterns that were identical or differed by 1 band were examined for date of processing to determine possible cross contamination [57]. The date processed (or date collected if former was unavailable) was reviewed for matching patterns to identify clusters that may have represented false positive results due to either laboratory cross contamination or mislabeling. Date parameters used to define clusters were ±4 days from date processed and ±10 days from date of specimen collection.

CD4 counts were performed by flow-cytometry (FACS-Count, Becton Dickinson, Franklin Lakes, New Jersey). Viral loads were performed using Versant HIV-1 RNA 3.0––bDNA (Bayer Diagnostics, Emeryville, CA) or Versant 340 bDNA (Siemens Diagnostics, Norwood, MA).

Table A. Systemic reactions 7 days after immunization.

Reaction	MV n = 367	Placebo n = 380	P value
Measured temperature ≥38°C	2/367 (1%)	5/380 (1%)	0.45
After Dose A	0	3/82 (4%)	
Dose B	2/81 (2%)	1/79 (1%)	
Dose C	0	1/76 (1%)	
Dose D	0	0	
Dose E	0	0	
Self-reported fever	40/367 (11%)	31/380 (8%)	0.21
After Dose A	9/81 (11%)	9/82 (11%)	
Dose B	11/77 (14%)	8/79 (10%)	
Dose C	10/77 (13%)	7/76 (9%)	
Dose D	5/72 (7 %)	3/72 (4%)	
Dose E	5/60 (8%)	4/71 (6%)	
Headache	50/367 (14%)	37/380 (10%)	0.11
After Dose A	14/81 (17%)	11/82 (13%)	
Dose B	14/77 (18%)	10/79 (13%)	
Dose C	4/77 (5%)	8/76 (11%)	
Dose D	12/72 (17%)	3/72 (4%)	
Dose E	6/60 (10%)	5/71 (7%)	
Malaise	64/367 (17%)	53/380 (14%)	0.19
After Dose A	19/81 (23%)	15/82 (18%)	
Dose B	18/77 (23%)	10/79 (13%)	
Dose C	8/77 (10%)	9/76 (12%)	
Dose D	11/72 (15%)	11/72 (15%)	
Dose E	8/60 (13%)	8/71 (11%)	

Statistical analysis

The trial was designed to detect with 80% power a 50% decrease in the risk of disseminated tuberculosis at the 0.05 (2-sided) statistical significance level. An annual disseminated tuberculosis rate of 1.60% and an annual death rate of 9.16% were expected in the placebo group. Follow-up loss was expected to be 27% over the entire study duration. Based on these assumptions, a total of 71 events was needed and was expected to be observed among 2274 participants followed over a median of four years.

One interim efficacy analysis for the primary endpoint of disseminated tuberculosis was conducted in November 2005 when 9 cases had been observed. This resulted in an information time of 0.13 with a corresponding critical value using an O-Brien Fleming boundary of 6.206. The DSMB reviewed the log rank tests and compared their P values to the nominal P value at the interim analysis, which did not indicate a significant difference warranting stopping (or considering to stop) the trial.

Results

Among 246 ineligibles whose reason was 'other' (consort diagram) subcategories were: failure to return − 79, HIV negative − 43, no CD4 count − 52, serious underlying disease − 26, refused to continue − 18, moved − 8, screening incomplete when enrollment stopped − 20. Subjects who were lost to follow-up compared to subjects who remained in the study were less often female (69% vs. 77%, respectively, $P < 0.0001$), less likely to have had prior tuberculosis (4.4% vs. 9.3%, $P < 0.001$), and less likely to have a positive TST (27% vs. 33%, $P = 0.02$); age, sex, and baseline CD4 counts did not differ between the two groups.

The following number of subjects received the following minimum number of doses: one dose 1975 (988 MV, 987 P); two doses 1902 (950,952); three doses 1853 (928, 925); four doses 1804 (903, 901); five doses 1687 (848, 839).

The HR for definite plus probable tuberculosis in the ITT analysis was 0.83 (95% CI: 0.61−1.13; 79 MV, 92 P; $P = 0.22$) and in the PP analysis was 0.86 (95% CI: 0.63−1.17; 78 MV, 90 P, $P = 0.31$). Two MV subjects had episodes of both definite and probable tuberculosis; only one episode was included for each of these subjects in the definite plus probable calculation.

M. tuberculosis was isolated from 81 subjects; 71 study isolates and 168 concurrent community control isolates were available for IS*6110* typing. Five clusters (four with identical IS*6110* results and one with similar IS*6110* results) were identified and reviewed by the expert panel. One cluster was determined to represent a false positive result and resulted in the change of one tuberculosis case classification from definite to unlikely.