Evaluation of the Safety and Immunogenicity of Two Antigen Concentrations of the Mtb72F/AS02A Candidate Tuberculosis Vaccine in Purified Protein Derivative-Negative Adults

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Tuberculosis (TB) remains a major cause of illness and death worldwide, making a new TB vaccine an urgent public health priority. Purified protein derivative (PPD)-negative adults (n = 50) were equally randomized to receive 3 doses at 1-month intervals (at 0, 1, and 2 months) of one of the following vaccines: Mtb72F/AS02A (10 or 40 μg antigen), Mtb72F/saline (10 or 40 μg antigen), or AS02A. Mtb72F/AS02A recipients received an additional dose 1 year after the first dose to evaluate if the elicited immune response could be boosted. Mtb72F/AS02A vaccines were locally reactogenic but clinically well tolerated, with transient adverse events (usually lasting between 1 and 4 days) that resolved without sequelae being observed. No vaccine-related serious adverse events were reported. Vaccination with Mtb72F/AS02A induced a strong Mtb72F-specific humoral response and a robust Mtb72F-specific CD4+ T-cell response, both of which persisted at 9 months after primary immunization and for 1 year after the booster immunization. There was no significant difference between the magnitude of the CD4+ T-cell response induced by the 10- and 40-μg Mtb72F/AS02A vaccines. The Mtb72F-specific CD4+ T cells predominantly expressed CD40L; CD40L and interleukin-2 (IL-2); CD40L and tumor necrosis factor alpha (TNF-α); CD40L, IL-2, and TNF-α; and CD40L, IL-2, TNF-α, and gamma interferon (IFN-γ). Serum IFN-γ, but not TNF-α, was detected 1 day after doses 2 and 3 for the Mtb72F/AS02A vaccine but did not persist. Vaccine-induced CD8+ T-cell responses were not detected, and no immune responses were elicited with AS02A alone. In conclusion, Mtb72F/AS02A is clinically well tolerated and is highly immunogenic in TB-naïve adults. The 10- and 40-μg Mtb72F/AS02A vaccines show comparable safety and immunogenicity profiles.

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MATERIALS AND METHODS

Study design and ethics. This phase I open, randomized, controlled trial (NCT00291889) was conducted between July 2004 and May 2006 at the Center for Vaccinology, Ghent University Hospital, Ghent, Belgium. The protocol was...
approved by the Ethics Committee of the Ghent University Hospital and was undertaken in accordance with the Declaration of Helsinki and good clinical practices. Written informed consent was obtained from all participants before they entered the study.

The participants were equally randomized to one of five treatment groups, according to allocation using a central randomization system on the Internet. The groups were Mtb72F/AS02A (10 or 40 μg antigen, investigational vaccines), Mtb72F/saline (10 or 40 μg antigen, active comparators), and AS02A alone (control). All groups received a primary vaccination course at 0, 1, and 2 months. Participants receiving Mtb72F/AS02A were given a booster dose 9 months after completion of the primary vaccination course, approximately 1 year after dose 1, and were followed for one additional year.

**Study population.** Healthy adults aged 18 to 45 years were enrolled if they were seronegative for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) antibodies and for hepatitis B surface antigen (HBsAg). Participants were excluded if they had a positive PPD skin test, an abnormal chest X-ray, a history of BCG vaccination, documented exposure to M. tuberculosis, or potential contact with TB patients. Female participants who were pregnant or who were planning to become pregnant or to discontinue contraceptive precautions were also excluded.

**Study vaccines.** Mtb72F was manufactured at Corixa Corporation (now GSK Biologicals). Physiological saline and AS02A were manufactured at GSK Biologicals (Belgium). Mtb72F is a proprietary oil-in-water emulsion with two immunostimulants, monophosphoryl lipid A (MPL) and Quillaja saponaria fraction 21 (QS21) (14). The vaccines were reconstituted by adding saline or AS02A to the Mtb72F lyophilizedophil. After gentle shaking of the mixture, a 0.5-ml dose was administered intramuscularly in the deltoid muscle of the nondominant arm. Each 0.5-ml dose of Mtb72F/AS02A and AS02A contained approximately 50 μg MPL and 50 μg QS21.

**Safety and reactogenicity evaluation.** Solicited local and general adverse events (AEs) were recorded on diary cards during a 7-day (including the day of vaccination) follow-up period after each vaccination and were checked by a study physician at scheduled visits. Local AEs recorded were injection-site pain, swelling, and redness, as measured by the longest surface diameter. Solicited general AEs were fatigue, fever (axillary temperature ≥ 37.5°C), gastrointestinal symptoms (nausea, diarrhea, vomiting, abdominal pain), and headache. Unsolicited AEs were recorded after a 30-day follow-up period after each vaccination, and serious AEs (SAEs) were collected throughout the study.

The intensities of the AEs were scored, and grade 3 AEs were defined as AEs preventing normal daily activity, a temperature of ≥39.5°C, and swelling or redness of >50 mm. All local AEs were considered to be related to vaccination. The relationship of all other AEs to vaccination was determined by the investigator to be related to vaccination. The mean age was 28.7 years (standard deviation = 5.9 years), and all participants were white (Caucasian). The overall female-to-male ratio was 1.08 (26 females/24 males).

**Immunogenicity evaluation.** The cell-mediated immune (CMI) response was assessed at specific time points by measuring (i) cytokines in serum by enzyme-linked immunosorbent assay (ELISA) and (ii) immune markers on isolated peripheral blood mononuclear cells (PBMCs) by an intracellular cytokine staining (ICS) assay. Anti-Mtb72F antibody concentrations were measured using ELISA at specific time points.

**Intracellular cytokine staining.** Mtb72F-specific CD4+ and CD8+ T cells were detected using ICS and flow cytometry, based on a previously described method (24, 25, 42). Briefly, whole-blood samples were collected and PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen. After they were thawed, PBMCs were stimulated in vitro using individual Mtb32A and Mtb39A 15-mer peptide pools (1.25 μg/ml) of each peptide, covering the entire sequence of the Mtb72F antigen, in the presence of anti-CD28 and anti-CD49d antibodies (BD Biosciences, Belgium). After 2 h of incubation at 37°C, brefeldin A (BD Biosciences) was added, followed by an overnight incubation. Cells were stained using peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 and allophycocyanin (APC) H7-conjugated anti-CD8 antibodies (BD Biosciences). The CD4+ and CD8+ T-cell responses to a 155-mer peptide pool were measured before 1 week and 1 month after each vaccine dose, 6 months after dose 3, and 1 year after the booster.

**RESULTS**

**Study population.** Figure 1 presents the participant flow. All participants were included in the safety analysis. Nineteen subjects returned for the booster dose. Two individuals in the Mtb72F/AS02A (40-μg) group were eliminated from the ATP analysis for immunogenicity: one withdrew from vaccination after dose 2 and the other was eliminated for noncompliance with the blood sampling schedule after dose 3. One participant from the 10-μg Mtb72F/saline group was not included in the ATP analysis for immunogenicity because he moved away from the study area after dose 2. The demographics were comparable between all groups. The mean age was 28.7 years (standard deviation = 5.9 years), and all participants were white (Caucasian). The overall female-to-male ratio was 1.08 (26 females/24 males).

**Safety and reactogenicity.** The Mtb72F/AS02A vaccine formulations were considered to be clinically well tolerated. Most AEs reported were transient and resolved within 1 to 4 days. Grade 3 AEs resolved or decreased in intensity within 48 h postvaccination, and the incidence of AEs did not increase with subsequent immunizations. All AEs resolved without sequelae, and in addition, no SAEs were judged by the investigator to be related to vaccination.

**Injection-site pain.** The most frequently reported AE was injection-site swelling, reported only in the
Mtb72F/AS02A groups (26.7% and 10.3% for the 10-μg and 40-μg groups, respectively) and the AS02A alone group (46.7%). There was a trend toward a lower frequency of reporting of local grade 3 AEs with the Mtb72F/AS02A (40-μg) vaccine than with the Mtb72F/AS02A (10-μg) vaccine and AS02A. No grade 3 local AEs were reported in the Mtb72F/saline groups. All solicited local AEs resolved within 5 days of vaccination.

The most commonly reported solicited general AEs after primary vaccination were fatigue and headache. The incidence of fatigue was similar in the Mtb72F/AS02A groups (50.0 to 51.7%) and lower in the control/comparator groups (<38%). The incidence of headache was comparable in all groups and ranged from 20.7 to 36.7%. Solicited general grade 3 AEs were infrequent; no cases of grade 3 fever were reported.

The most frequently reported causally related unsolicited AEs after primary vaccination with Mtb72F/AS02A or AS02A were transient mild to moderate influenza-like illness (10.0 to 23.3%) and injection-site warmth (23.3 to 30.0%). Causally related unsolicited AEs were not frequently reported in the Mtb72F/saline groups (<10.3%).

Following booster vaccination with the Mtb72F/AS02A vaccines, comparable frequencies of unsolicited AEs were observed, with transient mild to moderate influenza-like illness (15.0 to 20.0%) and injection-site warmth (26.0 to 30.0%) being the most frequently reported AEs. No increase in the overall frequency of AEs was observed, and the frequencies were comparable in both Mtb72F/AS02A vaccine groups.

No clinically relevant changes in the biochemistry or hematology parameters were noted throughout the study (results not shown).

Two SAEs were reported during the study period. One individual in the Mtb72F/AS02A (40 μg) group reported appendicitis, and one in the Mtb72F/AS02A (10 μg) group suffered a broken ankle. Both SAEs were judged to be not related to vaccination by the investigator.

Immunogenicity. (i) CD4+ and CD8+ T-cell responses by ICS and flow cytometry. In order to evaluate the magnitude of the cell-mediated immune response elicited by the Mtb72F/AS02A vaccines, PBMCs were restimulated in vitro and analyzed by ICS and flow cytometry. A high frequency of Mtb72F-specific CD4+ T cells expressing two or more immune markers among the markers CD40L, IL-2, TNF-α, and IFN-γ was measured after 2 and 3 immunizations for 10-μg Mtb72F/AS02A (median frequencies, 0.19% and 0.23%, respectively) and 40-μg Mtb72F/AS02A (median frequencies, 0.28% and 0.15%, respectively) (Fig. 2). These responses were significantly higher than those for their respective saline comparators (Wilcoxon rank-sum test, *P* < 0.05 at 1 month after doses 2 and 3 for both 10-μg and 40-μg vaccines).

The magnitude of the CD4+ T-cell response at 1 month after dose 2 (day 56) was not significantly different from the response at 1 month after dose 3 (day 86) for either the 10- or 40-μg Mtb72F/AS02A vaccines (Wilcoxon rank-sum test, *P* = 0.93 and *P* = 0.13, respectively).

Boosting by a fourth vaccine dose did not increase the magnitude of the CD4+ T-cell response (at 1 month postbooster, day 338) in either of the Mtb72F/AS02A vaccine groups be-
yond that observed after the second and third immunizations. However, these cellular responses persisted at 1 year postbooster (day 644).

The magnitude of the CD4+ T-cell responses induced by the 10-μg and 40-μg Mtb72F/AS02A vaccines were not significantly different from each other at 1 month after dose 2 (day 56; Wilcoxon rank-sum test, \( P = 0.47 \)), 1 month after dose 3 (day 86; Wilcoxon rank-sum test, \( P = 0.74 \)), 9 months after dose 3 (day 308; Wilcoxon rank-sum test, \( P = 0.64 \)), and 1 year postbooster (day 644; Wilcoxon rank-sum test, \( P = 0.36 \)).

No Mtb72F-specific CD4+ T-cell response was observed in the AS02A group.

No vaccine-induced Mtb72F-specific CD8+ T cells were detected (data not shown).

Functional characterization of the Mtb72F-specific CD4+ T cells showed that both Mtb72F/AS02A vaccines induced polyfunctional CD4+ T cells after vaccination, which persisted (Fig. 3A and B). The predominant phenotypes of the vaccine-induced Mtb72F-specific CD4+ T cells were CD40L+ IL-2+, CD40L+ TNF-α+, polyfunctional CD40L+ IL-2+ TNF-α+, and to a lesser extent, polyfunctional CD40L+ IL-2+ IFN-γ+ TNF-α+. Low frequencies of monofunctional CD4+ T cells expressing IL-2, TNF-α, or IFN-γ were also induced. High frequencies of antigen-specific CD4+ T cells expressing only the activation marker CD40L were elicited by Mtb72F/AS02A.

(ii) Serum IFN-γ and TNF-α responses by ELISA induced by Mtb72F/AS02A vaccines. To evaluate whether the Mtb72F/AS02A vaccine upregulated the expression of Th1 cytokines in the blood, IFN-γ and TNF-α levels were evaluated shortly after vaccine administration. No vaccine-induced serum IFN-γ was detected after dose 1 in the majority of the individuals (Fig. 4). IFN-γ was detected in the serum at 1 day after doses 2 and 3 (days 29 and 57, respectively) but had decreased to baseline levels at 6 days after doses 2 and 3 (days 34 and 62, respectively). At 1 day postbooster (day 309), median serum levels of IFN-γ were comparable to those detected at 1 day after dose 3 (day 57) in the Mtb72F/AS02A groups. Only baseline responses were observed in the Mtb72F/saline and AS02A groups (data not shown). To investigate whether these cytokines were induced early after vaccination, blood samples from additional postbooster time points were evaluated. However, no IFN-γ was detected at 2, 4, and 6 h postbooster. No TNF-α was detected in any of the groups at any time points tested (data not shown).

(iii) Mtb72F-specific antibody responses. Two weeks after the second vaccine dose (day 42), all participants who had received Mtb72F/AS02A were seropositive for anti-Mtb72F IgG antibodies (Fig. 5). During the primary vaccination course, peak levels of anti-Mtb72F antibodies were measured at 2 weeks after dose 3 (day 70). There was a trend toward in-
creased humoral responses with 40 μg Mtb72F/AS02A compared to those with 10 μg Mtb72F/AS02A. All but one vaccinee in the Mtb72F/saline groups became seropositive at 2 weeks after dose 3 (day 70), but the GMCs were significantly lower with no overlapping CIs compared with the values for the Mtb72F/AS02A groups (13.1 and 31.3 EU/ml for the 10- and 40-μg Mtb72F/saline groups, respectively).

Antibody responses after primary vaccination with Mtb72F/AS02A persisted over 9 months. A fourth administration of the Mtb72F/AS02A vaccines boosted the antibody response, and 1 month later (day 338), they were approximately twice those observed after doses 4 and 3, respectively, for the 40-μg antigen dose. All subjects receiving Mtb72F/AS02A remained seropositive at 1 year after the booster dose. No anti-Mtb72F IgG response was observed in the AS02A group.

(iv) PPD skin test reactivity. To assess whether vaccination with Mtb72F/AS02A had an effect on PPD status, participants were evaluated 1 month after the primary vaccination course (i.e., ~day 86 after the first vaccine dose) by intradermal PPD skin test. None of the participants converted from PPD-negative status.

**DISCUSSION**

The safety, reactogenicity, and immunogenicity of Mtb72F/AS02A have recently been evaluated in PPD-negative U.S. adults (42). This randomized, controlled study allowed a better evaluation of safety and was also designed to provide a justification for use of the adjuvant. Therefore, the reactogenicity and immunogenicity induced by the adjuvanted vaccine (Mtb72F/AS02A) were compared to those of the antigen in saline (Mtb72F/Saline) and the adjuvant alone (AS02A). A head-to-head comparison was also needed to examine whether a higher concentration of antigen (40 μg) was well tolerated and was able to elicit better immune responses compared to those elicited with the 10-μg concentration used previously. Here we also assessed if a fourth vaccine administration could boost the persistent immune responses. Finally, the quality of the T-cell response was extensively analyzed in order to achieve a full characterization of the vaccine-elicited mono- and polyfunctional T-cell phenotypes. This study demonstrates that up to four immunizations with the Mtb72F/AS02A vaccine (10 and 40 μg) are clinically well tolerated in PPD-negative healthy adults. No vaccine-related SAEs were reported. The local reactogenicity profile of Mtb72F/AS02A was comparable to that observed with the AS02A adjuvant.
Fewer AEs were observed in the Mtb72F/saline groups, suggesting that AS02 A rather than the Mtb72F antigen contributes to the observed injection-site reactions. The solicited general AEs fatigue and fever were reported more frequently in the Mtb72F/AS02A groups than in the Mtb72F/saline or AS02 A groups, suggesting that this may be the expected consequence of the vigorous antigen-specific immune response observed after Mtb72F/AS02A administration. All AEs reported were transient, and most resolved within 1 to 4 days. The pattern of AEs observed has previously been reported with other vaccines containing AS02 A (14, 16, 37, 40–42). No clinically significant changes in laboratory test (biochemistry and hematology) values related to vaccination were observed. In a study described in a previous publication (42), eosinophilia and decreased hemoglobin levels were frequently reported when the vaccine was given to healthy PPD-

FIG. 3. Polyfunctional profiles of Mtb72F-specific CD4+ T cells expressing any combination of immune markers among IFN-γ, IL-2, TNF-α, and CD40L. PBMCs from subjects vaccinated with 10 μg Mtb72F/AS02A (A) and 40 μg Mtb72F/AS02A (B) were obtained at prevaccination (day 0), 2 weeks after dose 2 (day 42), 2 weeks after dose 3 (day 70), prebooster (day 308), 1 month postbooster (day 338), and 1 year postbooster (day 644). PBMCs were evaluated using ICS and flow cytometry after short-term in vitro stimulation with 15-mer peptide pools spanning the Mtb32A and Mtb39A antigens. Data are represented in box-and-whiskers plots as the number of Mtb72F-specific CD4+ T cells expressing single markers and any combination of IFN-γ, IL-2, TNF-α, and/or CD40L per 10^6 CD4+ T cells.
negative adults, although these were reported to be mainly mild and not clinically significant. In this study, which was controlled, in contrast to the previous study, which had no comparators, there were infrequent and comparable reports of mild eosinophilia and a decrease in hemoglobin levels in all vaccine and control groups (maximum of 1 to 3 individuals per group). Most of these mild fluctuations occurred either at prevaccination or screening, and no vaccination-related pattern emerged. These reports were also not considered to be clinically significant, in line with what was previously reported.

It is interesting to note that there appeared to be a trend toward a lower frequency of reporting of AEs in the 40-μg Mtb72F/AS02A group than in the 10-μg Mtb72F/AS02A and AS02A groups. However, given the small sample size and overlapping CIs, this observation appears not to be clinically significant. The Mtb72F/AS02A vaccines induced a strong Mtb72F-specific CD4+ T-cell response compared to that induced by Mtb72F/saline, showing that the AS02A adjuvant system enhanced the immunogenicity of the Mtb72F antigen.

No Mtb72F-specific immune responses were observed in the group receiving AS02A alone. A robust CD4+ T-cell response was induced after two doses of Mtb72F/AS02A, with no increase in the magnitude of the response occurring after a third and a fourth vaccine dose. The Mtb72F-specific CD4+ T-cell response was persistent in the Mtb72F/AS02A vaccine groups at 9 months after the primary vaccination course. A booster dose increased Mtb72F-specific CD4+ T-cell responses to magnitudes comparable to those observed after primary vaccination and persisted at 1 year postbooster, suggesting that memory CD4+ T cells may have been induced. There was no significant difference between the magnitude of the CD4+ T-cell response induced by the 10-μg and 40-μg Mtb72F/AS02A vaccines. The magnitude of the response after 1 month after dose 3 was comparable to that seen previously with the 10-μg Mtb72F/AS02A vaccine when it was tested in PPD-negative U.S. adults (10).

Functional characterization showed that Mtb72F-specific polyfunctional CD4+ T cells were induced by both Mtb72F/AS02A vaccines and persisted 1 year after boosting. The predominant CD4+ T-cell phenotypes were CD40L+, CD40L+ IL-2+, CD40L+ TNF-α+, and CD40L+ IL-2+ TNF-α+. Mtb72F/AS02A also induced Mtb72F-specific CD40L+ IL-2+ TNF-α+ IFN-γ+ CD4+ T cells, albeit at a lower frequency. Remarkably, a higher frequency of single positive CD40L+ CD4+ T cells was also observed, indicating that a distinct population of antigen-specific activated CD4+ T cells was elicited by the Mtb72F/AS02A vaccine. This is the first study which demonstrates the polyfunctionality of vaccine-induced CD4+ T cells in PPD-negative subjects. Other studies of TB vaccine
candidates have demonstrated polyfunctional CD4+ T-cell responses in PPD-positive adults (2, 5, 18, 31). Earlier work showed that the MVA85a TB vaccine candidate was immunogenic in PPD-negative British adults and that the magnitude of the IFN-γ enzyme-linked immunospot assay response was positively influenced by BCG priming (26).

Our results are promising, since studies in mouse models showed that induction of polyfunctional T cells correlated with protection against tuberculosis (1, 19) and that assessment in humans of T-cell polyfunctionality might allow more accurate identification of a protective T-cell population (9). Moreover, it is generally accepted that the induction of cellular Th1 immune responses is associated with protection against TB (36). It is generally accepted that the induction of cellular Th1 immunity is associated with protection against tuberculosis (36).

In conclusion, the Mtb72F/AS02A candidate vaccine is clinically well tolerated and highly immunogenic in PPD-negative British adults and that the magnitude of the IFN-γ enzyme-linked immunospot assay response was positively influenced by BCG priming (26).

We demonstrated that vaccination with Mtb72F/AS02A induced IFN-γ secretion in the serum at 1 day after vaccination. No TNF-α was detected. The transient presence of IFN-γ in the serum was clearly linked to Mtb72F/AS02A vaccination, as no such response was observed in the other study groups.

Robust and persistent antibody responses were induced by the Mtb72F/AS02A vaccines, with 100% seropositivity occurring after the second dose. Each vaccine administration increased the humoral immune response during the primary vaccination course, and the antibody responses induced by the Mtb72F/AS02A vaccines were significantly higher than those induced by their Mtb72F/saline counterparts. Anti-Mtb72F titers tended to be higher in the Mtb72F/AS02A (40-μg) group than in the Mtb72F/AS02A (10-μg) group in the primary vaccination course. The antibody response could be boosted to the same level for both Mtb72F/AS02A vaccines by a fourth dose 9 months later. All subjects remained seropositive 1 year postbooster.

The contribution of humoral responses in protection against TB is somewhat controversial, but it has been suggested that antibody-mediated immunity can alter the course of M. tuberculosis infection in certain situations (15).

In conclusion, the Mtb72F/AS02A candidate vaccine is clinically well tolerated and highly immunogenic in PPD-negative adults. A favorable safety profile was observed with 4 injections of the two Mtb72F/AS02A vaccines (10 μg and 40 μg) tested in this study. The vaccine induced a robust Mtb72F-specific humoral and polyfunctional CD4+ T-cell response.

The next steps in the clinical development of this vaccine will be to evaluate Mtb72F/AS02A in adults who are PPD positive, either from natural exposure to M. tuberculosis or from prior vaccination with BCG, and to initiate its evaluation in individuals living in regions where TB is endemic.

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