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Ag85B–ESAT-6 adjuvanted with IC31® promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection

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ABSTRACT

New TB vaccines are urgently needed because of the apparent lack of effect of the BCG vaccine on rates of adult contagious pulmonary tuberculosis and the risk of disseminated BCG disease in immunocompromised individuals. Since BCG appears to protect children, the primary target for vaccine development is a booster vaccine for adults but such vaccines ideally need to be able to efficiently prime mycobacterially naïve individuals as well as boost individuals previously vaccinated with BCG and those latently infected with TB. Protective immunity against *Mycobacterium tuberculosis* depends mainly on the generation of a Th1-type cellular immune response characterized by interferon-gamma (IFN-γ) production. In the present study, we monitored safety and IFN-γ responses in healthy BCG-vaccinated and prior or latently TB-infected individuals receiving a novel vaccine composed of the fusion protein Ag85B–ESAT-6 combined with the adjuvant IC31®, administered at 0 and 2 months. Vaccination caused few local or systemic adverse effects besides transient soreness at the injection site, but it elicited strong antigen-specific T cell responses against Ag85B–ESAT-6 and both the Ag85B and ESAT-6 components, that could be augmented by second vaccination. The strong responses persisted through 32 weeks of follow-up, indicating the induction of a persistent memory response in the vaccine recipients.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, an intracellular microorganism that is still considered to be among the world's most devastating pathogens. An estimated one third of the world's population is infected with TB, and 8–10 million cases of disease and 2–3 million deaths annually are caused by this pathogen [1]. Currently, the only widely available vaccine against TB is BCG, the live attenuated vaccine derived from *Mycobacterium bovis* by Calmette and Guérin early last century. BCG has shown to protect against some childhood forms of TB but the protective efficacy with regard to adult contagious pulmonary tuberculosis varies considerably, from 0 to 85% [2]. Adult pulmonary TB is the most infectious form of the disease and as a consequence, BCG has had little impact on the global TB epidemic. Improved second generation TB vaccines are therefore urgently needed [3].

Multiple new TB vaccines are being developed – which include recombinant BCG, attenuated *M. tuberculosis*, and DNA-based vaccines as well as recombinant proteins [4,5] and many of these have reached clinical trials. We recently published the first human phase I clinical trial data using a molecularly well-defined TB subunit vaccine, consisting of a hybrid protein of Early Secretory Antigenic Target (ESAT-6) and Antigen 85 (Ag85B) [6]. These antigens are strongly recognized by T cells from TB patients, are strongly conserved in clinical strains [7] and they have demonstrated protective efficacy in animal models [8–10]. However, because the protein component of the subunit vaccine is poorly immunogenic, the hybrid protein vaccine was adjuvanted with IC31®, a novel, two-component adjuvant system composed of the cationic polyaminoacid KLK and the oligodeoxynucleotide ODN1a [11–13]. This adjuvant was chosen on the basis of its ability to induce strong

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protective immunity in animal models of *M. tuberculosis* infection [11], by delivering a TLR9 ligand into the endosomal pathway [14] after comparison to the results of a substantial earlier adjuvant screening program [15]. The immunogenicity findings of the first trial showed that the adjuvanted Ag85B–ESAT-6 vaccine succeeded in inducing strong and long lasting Th1 cellular immune responses (>2 years after vaccination in a low endemic region) in volunteers without detectable prior recall responses to mycobacterial antigens [6]. This is encouraging, given earlier findings that protective immunity against mycobacteria depends on the generation of a Th1 cellular immune response characterized by the secretion of IFN-γ, and that the levels of IFN-γ appear to correlate with disease and recovery in TB patients [16,17]. Thus, even though IFN-γ by itself is clearly not the only factor required for immunity to *M. tuberculosis*, it is universally used as a marker of "vaccine take" [18].

For a TB vaccine that is to be employed in developing countries, showing safety and efficacy in mycobacterially naïve individuals is not enough, as globally most individuals have been sensitized by either prior BCG, by exposure to environmental mycobacteria or by latent or manifest TB infection. However, as the IC31®/H1 vaccine, containing both a new synthetic adjuvant and a novel recombinant protein had no prior data on safety of either component in humans, we adopted a cautious approach to testing. Therefore, only after the first clinical trial with the TB subunit vaccine in mycobacterially naïve individuals was shown to be safe and immunogenic, the vaccine was subsequently given to two groups of PPD (purified protein derivative) positive subjects. The first group included PPD positive BCG-vaccinated individuals with no known risk of *M. tuberculosis* infection and the second group consisted of PPD positive subjects with prior documented manifest or presumed latent TB infection. The primary objective of the present phase I trial was to evaluate the safety of the vaccine and the secondary objective was to evaluate the immunogenicity in PPD positive individuals.

2. Materials and methods

2.1. Ethics statement

All subjects gave informed consent for blood sampling, X-rays and tuberculin skin testing after verbal and written information was provided. The study protocol (EUDRACT No.: 2006-006366-42, ClinicalTrials.gov Identifier: NCT00929396, LUMC protocol no.: P07.132), the Investigator's Brochure and the Investigational Medicinal Product Dossier were approved by the accredited Ethical Review Board of LUMC and the relevant national authorities.

2.2. The investigational products

Ag85B–ESAT-6 is the recombinant fusion protein of Ag85B and ESAT-6, developed and manufactured by Statens Serum Institute (Copenhagen, Denmark). IC31® is a two-component adjuvant system developed by Intercell AG (Vienna, Austria), composed of the cationic polyaminoacid KLK and the oligodeoxynucleotide ODN1a in specific molar ratio of 25:1 KLK to ODN1a. KLK is composed of the amino acids lysine (K) and leucine (L). ODN1a is a single-stranded oligodeoxynucleotide based on alternating sequences of the nucleic acids inosine and cytidine. The final products were manufactured by Statens Serum Institute, in an accredited GMP facility and supplied to the study site as a sterile suspension for injection with a pH of 7.4. The injected volume was 0.5 ml. The vaccine was analyzed and released according to documented specifications before shipment to the clinical site. A GLP compliant repeated dose toxicity study in rabbits was conducted on Ag85B–ESAT-6 + IC31® (LAB Scantox, study no. 55926) in accordance with the CPMP Note for Guidance on preclinical pharmacological and toxicological testing of vaccines for human use (CPMP/SWP/465/95) providing guidance in the EU as of June 1998.

2.3. Study design and objectives

The study was an open label, single-centre, non-randomized phase I exploratory trial in PPD positive subjects. Two vaccinations of 50 μg Ag85B–ESAT-6 + IC31® (500 nmol KLK + 20 nmol ODN1a) were administered intramuscularly in the deltoid muscle, the first at time 0 of the study and the second at 2 months. The primary objective was to evaluate safety. The primary endpoints comprised sequential monitoring of a standard set of laboratory safety parameters (such as differential white blood cells, platelets, alanine aminotransferase, and creatinine), active solicitation for local and systemic adverse events following vaccine injections, and passive surveillance for solicited and unsolicited local and systemic adverse events by scheduled telephone interviews and self-reporting in a diary for up to 8 months after the first vaccination.

The secondary objective was to evaluate the immunogenicity of the vaccine. Cell-mediated responses were measured through detection of IFN-γ spot-forming cells by ELISPOT and of IFN-γ production in supernatants of peripheral blood mononuclear cells (PBMC) by ELISA, after stimulation with Ag85B–ESAT-6 protein, or with overlapping peptides of Ag85B and ESAT-6 (representing the individual components of Ag85B–ESAT-6). Reactivity was also monitored through detection of IFN-γ and TNF-α levels in plasma before and 7 days after the first and second vaccination. Finally, humoral responses were measured by ELISA for total IgG to the vaccine (Ag85B–ESAT-6).

2.4. Study subjects

The trial population consisted of 20 PPD positive volunteers divided into two groups: the 'BCG group' (BCG) and the 'TB infection group' (TBI). The BCG group consisted of 10 subjects (3 female, 7 male) who had been vaccinated with BCG >2 years before, whose tuberculin-skin-test (TST) was positive (range 6–15 mm or any documented value between 6 and 15 mm on medical file in the past), who did not have active, chronic or past TB disease as confirmed by chest X-ray at screening, a negative QuantiFERON®-TB Gold In Tube test and a negative 6-day lymphocyte stimulation test (LST: as described in [6]). The TB infection group consisted of 10 subjects (4 female, 6 male) who were either known to have been previously diagnosed with latent TB and whose TST was positive (≥10 mm, or documented ≥10 mm positive on medical file in the past), or of subjects with past documented (i.e., clinically manifest) TB infection but with no currently (>2 years) active disease as confirmed by chest X-ray at screening. According to the inclusion criteria, subjects with a past or latent TB infection should not have received treatment/chemoprophylaxis for TB within the preceding 2 years. They should also have evidence of prior *M. tuberculosis* infection as assessed by specific antigen recall responses. In the screening, many persons with documented past or latent TB infection tested QuantiFERON®-TB Gold negative. Therefore, besides 6 subjects with positive QuantiFERON®-TB Gold test, we selected 4 subjects with documented previous TB infection who did not have a positive QuantiFERON®-TB Gold test at screening, but who had positive antigen-specific responses to ESAT-6 or CFP10 as evidences by a response in the 6-day LST [6].

The general health of all participants was assessed by reviewing their recorded medical history, and performing a physical examination, chest radiography, and standard blood (including hepatitis B, hepatitis C and HIV testing) and urine tests. Exclusion criteria were having clinically relevant laboratory parameters outside of normal range, presence of a granulomatous disease (as evidenced by laboratory screening and chest radiography), being vaccinated with a

live vaccine within three months before the first vaccination, use of immune modulating drugs (e.g., glucocorticosteroids, immunosuppressive drugs or immunoglobulins) within the three months before the first vaccination, hypersensitivity to vaccine components, HBV, HCV or HIV sero-positive, pregnant women/planned pregnancy and/or breastfeeding within the trial period, or participation in another clinical trial. For the BCG group, 13 volunteers were screened and 3 were excluded: one because of an intention to become pregnant, one withdrew consent and one because of a positive TST > 15 mm. For the TB infected group, 16 volunteers were screened and 6 were excluded: one because of an intention to become pregnant, and 5 because of a negative QuantiFERON®-TB Gold in association with a negative LST. The median age was 49 (IQR: 24–54) years in the BCG group and 26 (IQR: 21–34) years, in the TB infected group, respectively. Volunteers were financially compensated as approved by the Institutional Review Board for the number and amount of blood and urine samples, inconvenience with respect to the intramuscular administration and for the time spent on trial visits and transportation to the study site.

The expected trial duration for each subject was 224 days (32 weeks or 8 months) following the first vaccination (day 0), which was preceded by a pre-vaccination screening period of approximately 10–14 days. The second vaccine dose was administered 2 months after the first. Each subject was expected to attend a total of 11 visits during the trial, two of which preceded the first vaccination. The final visit was planned eight months after the first and 6 months after the second (and final) vaccination.

2.5. Safety parameters

Volunteers remained under medical observation for 3 h after each intramuscular vaccination, to monitor possible immediate reactions. The volunteers were asked to record any symptoms and their body temperature in a diary. During the first week after each vaccination, symptoms and temperature (armpit) were recorded on a daily basis, thereafter on a weekly basis (the temperature being recorded in the evening). A medical examination of local reactions and temperature was performed on days 0, 1 and 7 and 42 after each vaccination. Extended physical examinations comprising ear, nose and throat, cardiovascular, pulmonary, neurological, gastrointestinal, urogenital and dermatological systems were performed at screening, just before the second vaccination and 98 and 224 days after the first vaccination. Adverse event recording took place from day 0 to day 224. On days 14 and 70 a standardized telephone interview was carried out.

Urine samples and blood sampling for hematologic and biochemistry clinical safety tests were taken before the vaccinations, 1, 7 and 42 days after the first and second vaccination, and 24 weeks after the last vaccination. All routine laboratory assays were performed by the certified central laboratories at LUMC according to standard procedures.

Adverse events were described in the Case Record Forms with onset date, duration, intensity of symptoms (grade I, II or III; i.e., mild, moderate, severe), outcome of the event and relationship (not related, possible, probable or certain related) of the event to the vaccine. Abnormal laboratory findings were scored for severity into severity grades 1–4 (based on "Toxicity grading scale for healthy adults and adolescent volunteers enrolled in preventive vaccine clinical trials" – FDA 2007 guidelines).

2.6. Quantiferon-TB Gold assay

Blood samples for testing with the QuantiFERON®-TB Gold in-tube assay were drawn before the first vaccination and at the last visit and performed according to the manufacturer's instructions with the result categorized as positive when the result was ≥0.35 IU/mL.

2.7. PBMC isolation and immunogenicity parameters

Baseline blood samples were collected two weeks before and on the same day as (i.e., immediately prior to) the first vaccination, and at 6 weeks of follow-up (i.e., 2 weeks before the second vaccination), and at weeks 14 and 32. Briefly, 40 ml of heparinized blood was centrifuged (5 min/322 g), the plasma removed for storage at −70 °C and cells resuspended and transferred to Leucosep filter tubes (Greiner-bio-one) containing 15 ml Ficoll (LUMC pharmacy #902861). After centrifugation, the fluid phase containing the PBMCs was removed and washed three times with 45 ml of sterile PBS (LUMC pharmacy). The PBMCs were aliquoted and stored in liquid nitrogen in medium (IMDM, Cambrax #BE112-722F) containing 20% fetal calf serum (FCS, PAA Laboratories #A15-043)/10% DMSO (Sigma #41650) until assaying. Assays were batch-processed with frozen cells to reduce variation. One ml aliquots of PBMC were thawed in a water bath at 37 °C, supplemented with 2 ml of 50% FCS/IMDM and centrifuged. PBMCs were pelleted, washed with 10 ml of serum-free culture medium (AIMV, Invitrogen #12055-91), resuspended in 1 ml medium, and counted. A minimum viability of 80% was considered acceptable for assay purposes. Assays using PBMC samples from each individual were performed in a single experiment or on the same day in case of different assays, to minimize temporal and inter-assay variation.

2.8. Quantifying IFN-γ spot forming units (SFU) after PBMC stimulation

To enable the determination of all longitudinal samples of single volunteers on a single day, limiting as far as possible inter-assay variability within subjects, it was necessary to start with frozen samples. In this protocol, cells were thawed and pre-stimulated for 16–18 h, followed by 24 h in the ELISpot plate. Of note, the pre-incubation step is done in a different plate, and samples are transferred to the ELISpot plate to reduce the background in the ELISpot and enhance the sensitivity of the assay when proteins or large peptides are used [19]. Briefly, 1×10^6 thawed cells/well were stimulated in 24 well plates with the same antigens and concentrations as described below for ELISA. All samples were assayed in triplicate. Incubation was done overnight in a fully humidified incubator at 37 °C, 5% CO_2. Subsequently, cells were resuspended and divided over 3 wells (250,000 cells/well, for antigen-stimulated cells, 100,000/well for PHA-stimulated cells) of a mixed cellulose ester-backed 96 well plate (MAHAS45, Millipore) which had been pre-coated with anti-IFN-γ-antibody (mAb 1-D1K, Mabtech, Sweden) and blocked with AIMV medium. Plates were incubated overnight in a fully humidified incubator at 37 °C, 5% CO_2. The next day biotinylated detector antibody (mAb 7-B6-1, Mabtech) was added and spots colored with alkaline phosphatase-conjugated streptavidin (Mabtech, Sweden) and Fast™NBT/BCIP (Sigma–Aldrich, The Netherlands), all according to the manufacturer's instructions. Substrate incubation was done at room temperature for 10 min and stopped by rinsing the plates with tap water. Plates were dried and spots were counted in the Bioreader 3000 pro (BioSys, Germany) using calibrated parameters.

2.9. Quantifying IFN-γ production (ELISA) after PBMC stimulation

After thawing, 150,000 cells/well were stimulated in triplicate in 96 well flat bottom plates (Greiner) with medium (AIMV, Invitrogen), 4 μg/ml PHA (Remel Europe), 5 μg/ml PPD (SSI, RT48 for *in vitro* use), 10 μg/ml Ag85B-ESAT-6 (SSI, GMP grade), an ESAT-6 peptide pool (9 peptides; 5 μg/ml of each), or an Ag85B peptide pool

(28 peptides; 5 μg/ml of each). The peptide pools consisted of 20-mer peptides, with an overlap of 10 amino acids. Every peptide was elongated at the N terminus with two lysine residues (K) to improve solubility. Cells were cultured for 6 days in a fully humidified incubator at 37 °C, 5% CO_2. Supernatants were harvested and stored at −20 °C until performing the IFN-γ ELISA. For the IFN-γ ELISA, Maxisorp™ plates (Nunc) were coated with mAb MD-2 (U-CyTech, The Netherlands) followed by blocking with 0.2% (w/v) milk powder (Fluka) in PBS (LUMC pharmacy). An IFN-γ standard (U-CyTech, The Netherlands) ranging from 5 to 5000 pg/ml was used, and to control for data consistency an external IFN-γ reference standard was included in all assays and demonstrated consistency over the assays (NIBSC, UK). Supernatants were diluted two-fold in 1% bovine serum albumin (BSA) in PBS. Following standard and sample incubation, biotinylated detector pAb (U-CyTech) was added. The ELISA was developed with streptavidin–horseradish peroxidase conjugate (CLB, The Netherlands) and tetramethylbenzidine substrate (BioSource, The Netherlands). Substrate incubation was done for 20 min at 37 °C. The reaction was stopped with H_2SO_4 and the OD was read at 450 nm.

2.10. Quantifying anti-Ag85B–ESAT-6 specific IgG antibodies in plasma by ELISA

Polysorb™ plates were coated with 4 μg/ml H1 or human serum albumin (Octalbine, Octapharma) as control for aspecific binding, followed by blocking with 1% (w/v) BSA (Sigma–Aldrich, UK)/1% (v/v) tween-20 (Sigma–Aldrich, UK) in PBS. A plasma dilution range was added to the wells, starting with a 1:12 dilution, followed by 2-fold dilution steps to 1:24,576 (12 dilutions in total). As a positive control, plasma from a treated TB patient was used; as negative control plasma from a healthy individual was used. After overnight incubation at 4 °C, HRP conjugated detector pAb (Dako, Denmark) was added. Development was done with TMB substrate (BioSource, Denmark) for 15 min at room temperature. The reaction was stopped with H_2SO_4, and the OD was read at 450 nm. Graphs with dilution factor versus OD450 yielded an S-curve. The antibody titre was defined as the dilution factor corresponding with the midpoint of the linear portion of this curve.

2.11. Quantifying IFN-γ and TNF-α levels in plasma by ELISA

Plasma was harvested after centrifugation of blood, and stored at −70 °C until performing the IFN-γ and TNF-α ELISA (Sanquin, Amsterdam). ELISAs were performed according to the instructions of the manufacturer.

2.12. Statistical analysis

The statistical analysis of the THYB-02 data was performed by JGConsult, an independent Contract Research Organisation in accordance with GCP and ICH-Guidelines.

Primary and secondary laboratory endpoints were evaluated within vaccination groups with respect to change from baseline to last visit using Wilcoxon signed rank test and between vaccination groups with respect to differences in change from baseline to end of trial using Wilcoxon rank sum test. Pairwise comparisons were conducted using Mann–Whitney. Adverse events were evaluated descriptively.

No formal sample size calculation was performed in this trial. A test significance level of 5% was used throughout the study. The statistical analysis was performed using SAS software (SAS®, Cary, NC 27513, USA, version 9.2 TS Level 1M0) on Platform Windows XP PRO Version 5.1.2600. Further subanalyses at SSI to produce the figures were conducted using Prism 4.0b MacOSX 10.5.8.

Table 1
Adverse events (AE), possibly vaccination-related, all classified as grade I, in BCG vaccinated and previously or latently TB infected subjects given two intramuscular injections with the Ag85B–ESAT-6 hybrid protein with IC31® adjuvant, relative to group size (n/N) or episode relative to persons affected.

Vaccination	Subjects			
	BCG vaccinated		TB infected	
	I	II	I	II
Any local adverse event (n/N)	5/10[a]	6/10	7/10	6/10
Stiffness	2	3	4	1
Soreness and/or pain at injection site	4	4	5	3
Erythema and/or swelling	1	1	1	5
Nodule at injection site	–	2	–	3
Any systemic adverse event (n/N)	2/10	1/10	0/10	0/10
Fever	1	1[b]	–	–
Tired and/or malaise	2	1[b]	–	–

[a] Number of subjects affected in each group/number of subjects in group.
[b] Reactions in same individual, temperature once elevated.

3. Results

3.1. Safety

All 20 subjects completed both vaccinations and the follow-up period. All subjects with at least one vaccination were included with all data in the safety analysis. No serious adverse events occurred in either of the two vaccination groups. In all, 52 local, potentially vaccine-related adverse events and 9 systemic, potentially vaccine-related adverse events were reported. Local vaccine-related adverse reactions included stiffness (defined as injection site movement impairment, $n = 6$ at first vaccination) and soreness and/or pain at injection site one day after vaccination ($n = 9$ at first vaccination). After the first vaccination such reactions were noted in about half of individuals in the BCG group and in the TB infection group (Table 1). After the second vaccination these reactions were more frequent. Most local adverse reactions were classified as mild (grades I–II) which means the event did not interfere with daily activities. In the BCG group however, one "injection site pain" reaction and one "injection site movement impairment" was classified as grade II, i.e., moderate, and one "injection site pain" reaction as severe (grade III) i.e., temporarily interfering with activities. All subjects with local reactions experienced full recovery within a maximum of 4 days. None of the subjects required analgesics. A small, cold nodule at the injection site was noted in 2 subjects in the BCG group and 3 in the TB infection group. This was without signs of attendant inflammation: local vesiculation, axillary lymphadenitis or fistula did not occur, and in all cases, the nodule had disappeared within one week. The systemic, potentially vaccine-related adverse events included fatigue ($n = 1$), malaise and feeling cold ($n = 2$) and were mild except for one individual where fatigue and malaise was classified as moderate.

Extensive follow-up of blood and urine parameters did not reveal any obvious trends within or differences between the two vaccination groups, or laboratory abnormalities with respect to change from baseline that could be related to the vaccinations.

3.2. Immunogenicity: IFN-γ ELISPOT and ELISA

Immune responses were evaluated by ELISA and ELISPOT. Responses were monitored to the Ag85B–ESAT-6 fusion protein, and also to the two individual components (ESAT-6 and Ag85B). The data in all cases are shown and analyzed without background subtraction.

For ELISA, there were no significant differences between any of the groups with regard to the negative (culture medium only: median value 8 pg/ml, Interquartile range 1 pg/ml) control or the

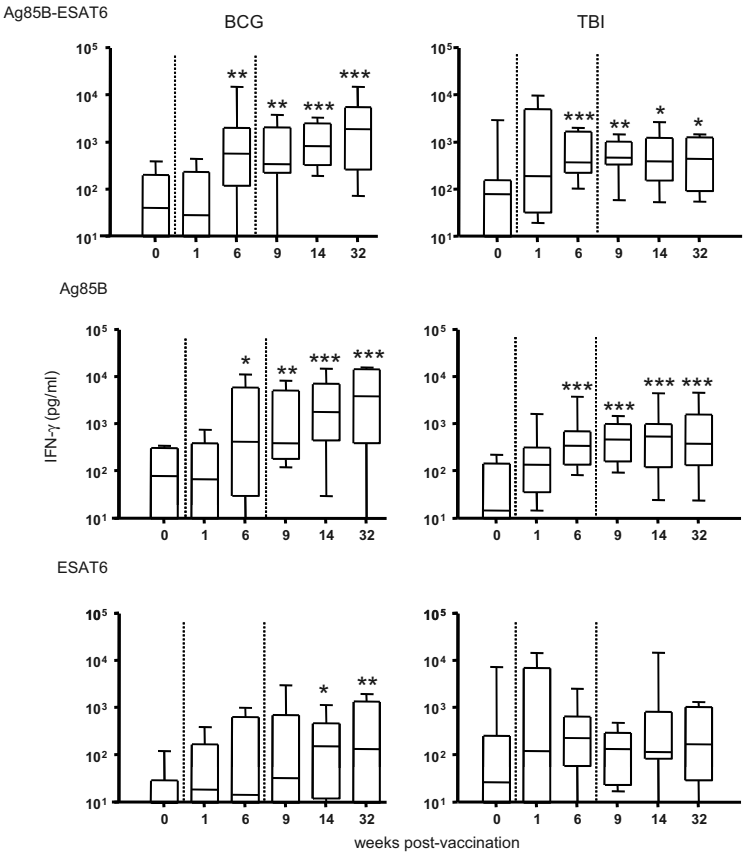

Fig. 1. IFN-γ production by PBMC as assayed by ELISA (expressed as pg/ml) after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations (TBI). Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians and quartiles of assays performed in duplicate, with medians significantly different from those of the pre-vaccination bleeds marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

positive control (PHA: median value 15,000 pg/ml, interquartile range 226 pg/ml) (data not shown). Unlike in the previous study [6] – but as expected – these mycobacterially exposed individuals had detectable immune responses prior to vaccination (Fig. 1, time point 0). The magnitude of these responses were not significantly different between the groups for Ag85B, or the fusion molecule Ag85B–ESAT-6 (both had $p > 0.1$), but BCG vaccinees had a significantly lower response to ESAT-6 pre-vaccination than the TB infection group (Mann–Whitney, $p = 0.002$). By 6 weeks post-vaccination, however, there was no difference between the groups with regard to their ESAT-6 response ($p = 0.7$) and in both groups, it was higher after the booster vaccination than at baseline ($p = 0.05$).

Although baseline immune responses were higher as compared to the previous study in mycobacterially naïve individuals, for both Ag85B and Ag85B–ESAT-6 prior to vaccination, both groups showed significant increases in IFN-γ production to these antigens from 6 weeks post-vaccination ($p < 0.001$ for Ag85B–ESAT-6, $p = 0.007$ for Ag85B), and this elevated response was maintained without any apparent diminution up to 32 weeks after vaccination (Fig. 1).

The same overall pattern was observed when the response was assessed by ELISpot (Fig. 2): a detectable immune response to Ag85B and Ag85B–ESAT-6 was found prior to vaccination in both BCG-vaccinated and TB infected groups, which was efficiently boosted by the vaccination ($p < 0.0001$ for Ag85B–ESAT-6, $p < 0.001$ for Ag85B) in both groups. These p values were calculated using the 32-week endpoint, but significant increases were also seen at much earlier time points. As would be expected, the BCG vaccinees did not have a significant positive response to ESAT-6 prior to vaccination (Median response 0 SFU/million, interquartile range 22 SFU) while the TB infected individuals had a substantial response (median response 198 SFU/million, interquartile range 427 SFU, $p < 0.0001$ compared to BCG group). At 32 weeks, post vaccination, the response to ESAT-6 was significantly ($p < 0.0001$) higher than at baseline in the BCG group. The same is true in the TB group,

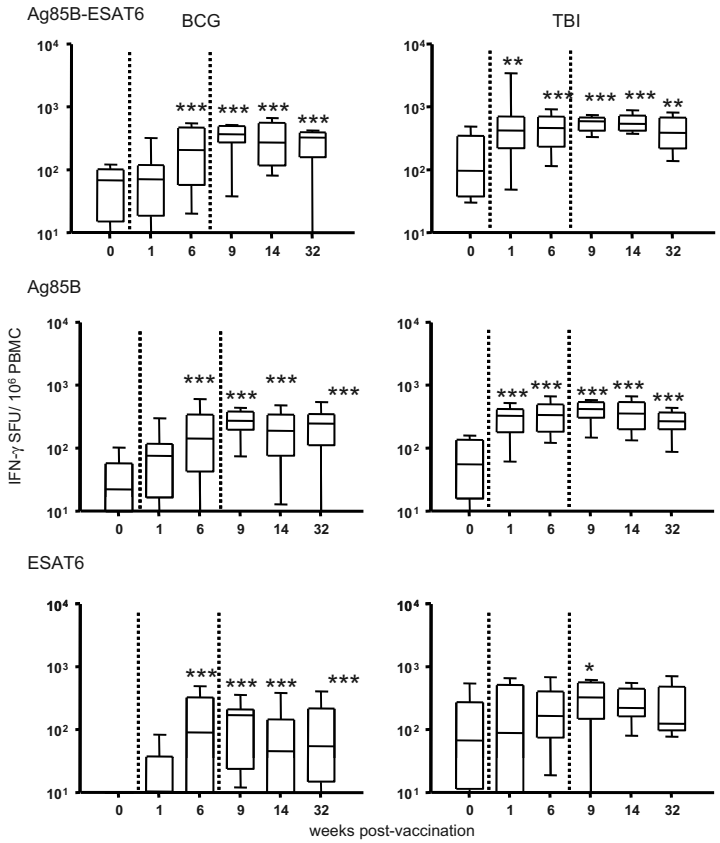

Fig. 2. IFN-γ production by PBMC as assayed by ELISPOT (expressed as spot-forming units per million peripheral blood mononuclear cells) after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations (TBI). Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken and just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians and quartiles of assays performed in triplicate, with medians significantly different from those of the pre-vaccination bleeds marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

though the difference was less pronounced due to higher baseline responses ($p < 0.03$).

We also tested the linker region between Ag85B and ESAT-6 to assess if any neo-epitopes were generated and were unable to detect any responses above background, whether humoral or cell mediated (data not shown). Thus, for both groups, in both assays, vaccination with Ag85B–ESAT in IC31® drove either the emergence of, or – in case of a preexisting response, an increase in – antigen-specific T cell responses that persisted up to the end of 32 weeks follow-up.

3.3. Kinetics of the response

While both groups increased their antigen-specific responses significantly after vaccination (Figs. 1 and 2), it also appeared that the kinetics of the response were somewhat different. This was particularly noticeable in the magnitude of the T cell responses against the hybrid protein at different times between the BCG-vaccinated (but TB naïve) group and the TB infection group (i.e., subjects either with past manifest TB or a clinical history indicative of latent TB infection). Prior to vaccination, the groups did not respond differently to the Ag85B–ESAT-6 fusion protein or to Ag85B. However, after vaccination, a relatively stronger T cell response to Ag85B–ESAT and Ag85B emerged in the TB infection group, compared to the BCG group, at the earliest time points, as assessed by ELISPOT ($p < 0.001$) (Fig. 3). This difference in antigen-specific ELISpot reactivity between BCG-vaccinated and the TB infection group remained significant following the second vaccination, until the numbers of IFN-γ positive cells in the ELISPOT converged by week 32. If the results from the previous clinical trial, THYB-01, where the same vaccine was given to individuals with no prior mycobacterial sensitization ($n = 11$) [6] are compared with the results from this study, it appears that the ELISpot response to Ag85B–ESAT and Ag85B in mycobacterially naïve indi-

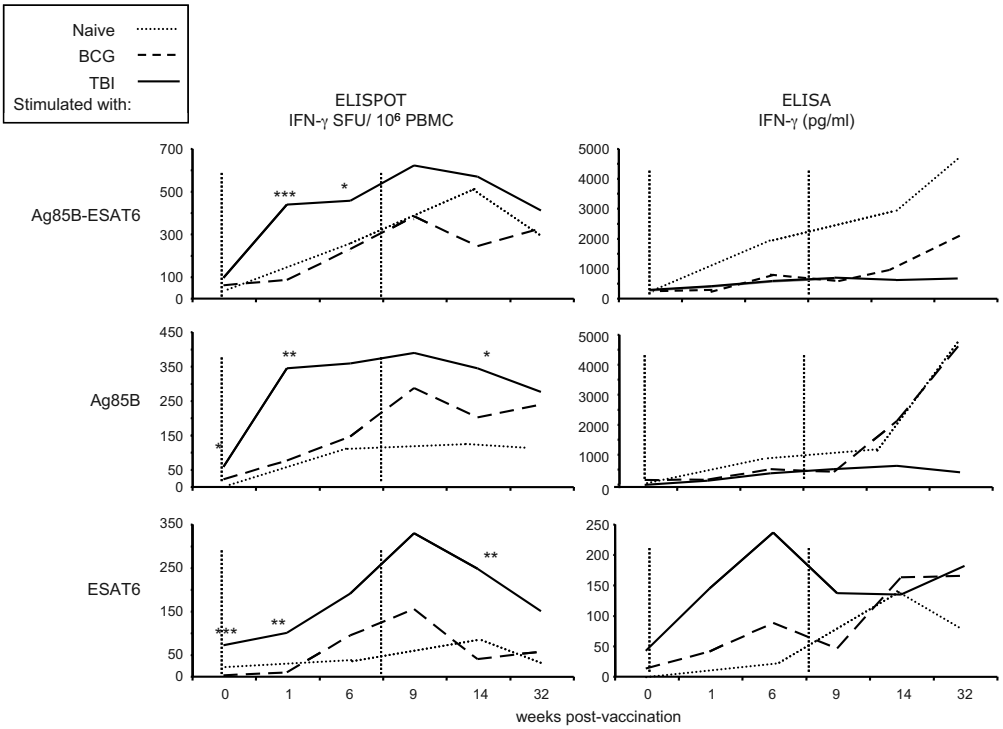

Fig. 3. Comparison of median IFN-γ responses over time in mycobacterially naïve (dotted line: data drawn from [6]) previously BCG-vaccinated individuals (dashed line) or in individuals previously been diagnosed with an *M. tuberculosis* infection >2 years prior to the vaccinations (solid line). IFN-γ production by PBMC was assayed after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations. Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians of the assays performed in duplicate (ELISA: expressed as pg/ml) or triplicate (ELISpot, expressed as SFU per 10^6 PBMC), with medians significantly different between groups at the same time point marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

viduals most resembled that of the BCG group in the present study.

IFN-γ responses as assessed by ELISA, however, show a different trend, though the differences were not significant through the course of the study. The TB infection group responded more rapidly to Ag85B–ESAT and Ag85B in terms of the numbers of cells producing IFN-γ (Fig. 3), but not necessarily in the amount of IFN-γ produced. This difference was even more marked for the mycobacterially naïve recipients (Fig. 3 and [6]).

For ESAT-6, the pattern was slightly different, with the TBI group having a significantly higher baseline response ($p < 0.001$) prior to vaccination than the two groups with *M. tuberculosis* infection as assessed by ELISpot, but this difference diminished post-vaccination and was not significantly different thereafter. Again, despite higher levels of ESAT-6-induced IFN-γ in the ELISA, in the TBI group, the results were not significantly different between the three groups (Fig. 3).

These results indicate that after vaccination, all of the groups respond to vaccination by significantly increasing both the numbers of cells producing IFN-γ and the overall amount of IFN-γ produced, but that the kinetics of the response are different – not only between the groups but also between the different assays. This is interesting, given the trend (also seen in the prior study in mycobacterially naïve vaccinees [6]) for the magnitude of the ELISpot response to decline, even as the magnitude of the ELISA response increased, suggesting that the nature of the responding cells may be changing after vaccination, perhaps as a consequence of the development of long-term immune memory.

3.4. Humoral response to vaccination

With respect to a humoral response to the vaccinations no increase in anti-Ag85B–ESAT-6 IgG levels was observed after the first vaccination ($p > 0.15$) in the BCG group, but a small increase was seen in the TB group. However, after the booster vaccination, IgG antibodies reactive against Ag85B–ESAT-6 hybrid protein were significantly elevated above base-line by sampling at 14 weeks, in both groups. Thereafter, antibody levels decreased significantly again ($p < 0.05$) both in the BCG-vaccinated and TBI individuals (Fig. 4) and although in the TBI group, the over all response was greater and IgG levels remained above baseline at the final visit (32 weeks), the trend was clearly identical for both groups. There was no clear correlation at the individual level between the magnitude of IFN-γ and IgG responses.

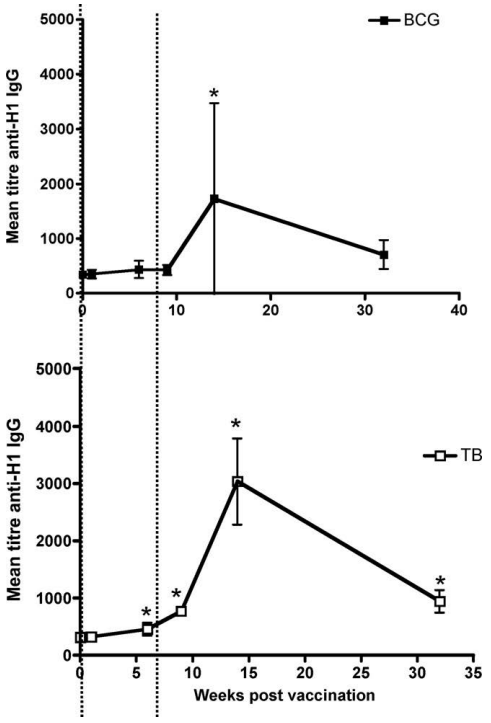

Fig. 4. IgG antibody production against the Ag85B–ESAT-6 hybrid protein in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Samples for antibody measurement were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Vaccination time points are marked by dotted vertical lines. Results shown are geometric means and SD of the titre, from assays performed in triplicate, with medians significantly different from those of the pre-vaccination bleeds marked ($^*p < 0.05$).

3.5. Plasma levels of IFN-γ and TNF-α

Before the first vaccination and the week after each of the two vaccinations, blood was sampled for measurement of cytokine levels. In neither of the two groups was TNF-α above the limit of detection (i.e., <5 pg/mL). The same was largely true of IFN-γ, although in one instance in one individual in the BCG group, the level of IFN-γ detected was just above the limit of detection one week after first vaccination (data not shown). It can be concluded therefore, that plasma levels of TNF-α and IFN-γ are weakly – if at all – increased by vaccination. Consistent with this, plasma cytokine concentrations were not associated with occurrence or intensity of adverse effects.

3.6. Infrequent QuantiFERON conversion after vaccination with H1/IC31®

In the developed world, the QuantiFERON TB Gold test is now broadly used as a diagnostic assay for detection of TB infection. To assess the risk of conversion to a positive QuantiFERON TB Gold after intramuscular administration of the adjuvanted Ag85B–ESAT-6 vaccine to BCG-vaccinated individuals, the QuantiFERON TB Gold assay was done before vaccination and 24 weeks after the last vaccination. Test results of all subjects were negative before vaccination (as per the inclusion criterion in this group); one of the 10 had a positive response at the end of follow-up indicating that two injections of the adjuvanted Ag85B–ESAT-6 vaccine can induce a positive *in vitro* T cell response to ESAT-6 in a small percentage of BCG-vaccinated subjects. This is consistent with earlier results in mycobacterially naïve individuals [6]. In the TB-infected subjects, 6 out of 10 cases were QuantiFERON TB Gold positive before vaccination; of these, one subject tested negative after the last vaccination, whereas of 4 with a negative QuantiFERON TB Gold one subject reacted positively at the end of follow-up, indicative of boosting of the response by the vaccination. There was no obvious difference in the magnitude or kinetics of the immune response to any of the antigens between the QuantiFERON TB Gold converters and the rest of the group, though the numbers were too small to draw a definitive conclusion.

4. Discussion

This is the first study of a molecularly well-defined TB subunit vaccine adjuvanted with IC31®, a new adjuvant aimed at boosting the Th1-type immune reactivity, in BCG-vaccinated individuals and individuals with prior *M. tuberculosis* infection. The findings indicate that intramuscular injection of the SSI-produced Ag85B–ESAT-6 hybrid protein with adjuvant was safe and well tolerated. In TB-infected individuals, a single vaccine dose induced a strong cellular immune response to the hybrid protein; the strong reaction to the first vaccination is suggestive of a booster effect from prior *M. tuberculosis* exposure. In individuals who had received prior BCG vaccination, the ELISpot responses after the first vaccination lagged significantly behind those observed in the TB infected group, though they did eventually converge. In both groups, T cell responses were strongest against the Ag85B–ESAT-6 hybrid protein and Ag85B component, and following a second administration were sustained or even increased during the 32-week follow-up. The adjuvanted vaccine thus induced a strong and lasting Th1-type cellular immune response with a significant but perhaps less durable antibody response against the hybrid protein or its components.

The present study extends previous findings in mycobacterially naïve individuals that intramuscular injection of Ag85B–ESAT-6 hybrid protein with adjuvant IC31® is safe and well tolerated. In this study, as in the prior one, no serious adverse events occurred and systemic adverse events reported were all considered to be unrelated to the administration of the vaccine. In the two cases in which such a relation could not be excluded totally, the adverse events were mild or moderate and disappeared within one day. The only significant trial-related response that was observed in about half of the subjects consisted of a feeling of soreness and pain at the site of the injection, the day after administration of the vaccine. In most of those affected, the pain at injection site was generally mild, i.e., grade I, and disappeared within one to two days, did not hinder function of the extremity nor require analgesics, and was not accompanied by local lymphadenitis, febrile responses or systemic symptoms. To further evaluate the safety of vaccination, extensive hematological and biochemical assessments were conducted before starting, at the time of each of the two vaccinations, and after completing the long-term follow-up. The vaccine did not noticeably affect any hematological or biochemical measurement. Thus, the results of this clinical study demonstrate that there are no safety concerns associated with the administration of the subunit vaccine to PPD positive individuals – obviously within the limitations of any exploratory phase I study, which can exclude with certainty only very frequent adverse reactions. On

the other hand, the lack of any significant reactogenicity despite impressive immunogenicity of the adjuvanted vaccine can only be considered a breakthrough, given the current paucity of effective human adjuvants for the induction of cell-mediated immune responses [20]. Based on these results, a phase I trial to evaluate safety and immunogenicity in PPD negative volunteers, BCG vaccinees and volunteers with previous disease or latent TB infection is currently ongoing in Ethiopia: the first-ever vaccine phase I trial in Ethiopia.

The vaccination induced specific and strong Th1-type immune reactivity to the vaccine antigen that persisted and in many instances even increased up to 32 weeks following first vaccination. In *M. tuberculosis* infected individuals, assessment by ELISpot indicated that a single dose of antigen adjuvanted with IC31® induced a significant cellular immune response – in general, stronger than that observed previously in mycobacterially naïve subjects [6] or in BCG vaccinated subjects, and the response to H1 was further amplified by booster vaccination. In general, T cell responses were strongest against the Ag85B–ESAT-6 hybrid protein and secondarily against the Ag85B component and were sustained during a follow-up, suggesting induction of potent immunological memory. However, the differences in the kinetics of the response as assessed by ELISpot and ELISA observed here suggest that the nature of the immune response is slightly – but perhaps importantly – different between the groups, with those assumed to have a high level of prior exposure to mycobacterial antigens – the TBI group – responding to vaccination with a rapid increase in the number of cells making IFN-γ, but a much smaller increase in the amount of IFN-γ produced. In our previous study we found that individuals with no prior markers of mycobacterial sensitization responded by more slowly inducing antigen-specific cells (as assessed by numbers) but reaching a peak – in terms of IFN-γ concentration – higher than that seen in the TBI group [6]. The BCG group, who presumably fall in between the other two groups in terms of mycobacterial exposure also fell between them in terms of the difference in the number of IFN-γ producing cells compared to total amount of IFN-γ produced.

There are no defined surrogate biomarkers of protective immunity in TB. It appears clear that host defense against TB involves a number of factors, but that it crucially depends on establishment of a Th1-type immune response [21,22]. IFN-γ in particular appears to be essential, and the IFN-γ response is rapid and robust. Therefore, the number of IFN-γ-releasing *M. tuberculosis* antigen-specific T-cells and the amount of IFN-γ released – even though imperfect markers – remains widely used as a surrogate marker of potential vaccine efficacy in TB vaccines [18]. Although the mechanism controlling the composition of immune responses to *M. tuberculosis*-derived antigens is unclear, it is not unexpected that individuals with different immunological histories will respond differently to re-exposure to antigen. The data presented in Fig. 3 thus underline the importance of looking at vaccine induced immunity using different approaches and using well defined cohorts that can be differentiated with regard to their history of mycobacterial exposure.

Consistent with earlier findings in mycobacterially naïve individuals, the adjuvanted vaccine induced a strong and persistent Th1 cell immune response, with a relatively modest antibody response against the Ag85B–ESAT-6 protein, increasing after the second dose. The IgG response, though significant, showed a tendency to wane that was not observed for IFN-γ production. This suggests an initial response heavily biased towards the cell-mediated arm of the immune response. While this is unusual, it matches results obtained with this vaccine in mice, where relatively weak humoral responses were generated alongside high levels of antigen specific IFN-γ production [11], across a range of dosing regimens, possibly due to the apparent targeting of dendritic cells [12]. It is not clear if this response is specific for the *M. tuberculosis* antigens used, but if these characteristics of IC31® are confirmed for other types of vaccines, it may establish this adjuvant as one of the first adjuvants that may specifically induce a cellular immune response in humans. This is different from currently approved adjuvants like aluminum salts and MF59, both of which primarily promote a Th2 or humoral immune response [20,23–25], and also from the report from von Eschen and colleagues who recently reported that the mtb72F/AS02A adjuvanted protein vaccine gave a mixture of both Th1 T cell activity and a humoral response [26].

ESAT-6 is included in several widely used commercial diagnostic tests (the QuantiFERON and T.Spot.TB tests) and a positive response to ESAT-6 due to vaccination may interfere with follow up of potentially *M. tuberculosis* sensitized/infected patients. In the present study, vaccination was associated with conversion of the QuantiFERON assay in only one out of ten BCG-vaccinated volunteers. Even if subsequent trials gave higher levels of ESAT-6 responses, this is not anticipated to be a major problem: it should be realized that the employment of *in vitro* diagnostics for tuberculosis and vaccination against *M. tuberculosis* are quite distinct: the former is mostly used in western, low TB-incidence countries whereas the latter is aimed primarily towards TB endemic regions. Nonetheless, the interaction between diagnostic tests based on *in vitro* IFN-γ-production and the possible impact of the vaccine on the usefulness of the QuantiFERON assay is being studied in ongoing trials in Ethiopia.

Sustained responses seen here compare well with that induced by BCG vaccination in adolescents, where waning of the overall response is already be observed after 12 months [27]. Given the previous report of long lasting and persistent T cell responses in mycobacterially naïve individuals, and the present sustained T cell responses in PPD positive subjects, the Ag85B–ESAT-6 subunit vaccine may well be able to overcome what has previously been a stumbling block in vaccination against TB – the requirement for a long-lasting memory response [28,29]. Prolonged maintenance of immune memory elicited by an adjuvanted subunit vaccine is in good agreement with recent observations from a mouse study of Ag85B–ESAT-6 in an adjuvant consisting of cationic liposomes, and suggest that the adjuvants, perhaps through establishment of an antigen depot and subsequent slow release, or targeting of dendritic cells [12], may have unique abilities to maintain strong immune memory [30].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.12.135.

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