Safety, reactogenicity, and immunogenicity of MTBVAC in infants: a phase 2a randomised, double-blind, dose-defining trial in a TB endemic setting

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Summary

Background Safer and more effective tuberculosis (TB) vaccines than Bacille Calmette Guérin (BCG) are needed. We evaluated the safety, reactogenicity, and immunogenicity of three dose levels of the live-attenuated *Mycobacterium tuberculosis* (*Mtb*) vaccine, MTBVAC, compared to BCG, in South African infants.

Methods Healthy, HIV-unexposed, BCG-naïve infants were randomised to receive a single intradermal dose of BCG (2.5×10^5 CFU, n = 24) or MTBVAC (2.5×10^4, 2.5×10^5, or 2.5×10^6 CFU, each n = 25). Safety endpoints were solicited systemic, solicited injection site, and unsolicited adverse events (AE), and serious AE (SAE). Immunogenicity was measured using interferon-γ release assay (IGRA) and whole blood intracellular cytokine staining assay. Follow-up was 12 months post-vaccination.

Findings Ninety-nine infants were enrolled between 18 February 2019 and 08 March 2021. Seventy-eight infants experienced reactogenicity AE (all mild except one grade 2 erythema). Induration, swelling, and erythema were more frequent as MTBVAC dose increased. All reactogenicity events were less frequent in infants receiving MTBVAC 2.5×10^5 CFU compared with BCG. Twelve infants (three BCG and nine MTBVAC recipients) experienced 14 vaccine-unrelated SAE, including one death due to bronchopneumonia (MTBVAC recipient). Eight infants were treated for unconfirmed pulmonary TB (four BCG and four MTBVAC 2.5×10^4 CFU recipients); one BCG recipient was treated for unconfirmed TB meningitis. MTBVAC was immunogenic at all 3 doses, inducing predominantly Th1-cytokine-expressing CD4 T cells, which peaked at Day 56. The 2.5×10^5 and 2.5×10^6 CFU MTBVAC doses induced similar response magnitudes and were more immunogenic than BCG. Day 56 IGRA conversion was observed in 61 (87.4%) infants receiving any MTBVAC dose, but only 28 (42.4%) remained positive by Day 365.

Interpretation MTBVAC appeared safe, well-tolerated, and immunogenic at doses between 2.5×10^4 and 2.5×10^6 CFU in South African infants. The 2.5×10^5 CFU MTBVAC dose, being less reactogenic and more immunogenic than BCG, was selected for a multi-centre, phase 3 trial.

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Keywords: Tuberculosis; Vaccine; *Mycobacterium tuberculosis*; BCG; Infant

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Research in context

Evidence before this study

We searched PubMed for original research articles and systematic reviews published in any language up to 30 Apr 2024. We combined terms for "live"," *Mycobacterium tuberculosis*", "Human" and "vaccine". The only published clinical trials of live whole cell *M. tuberculosis* were that of Spertini et al. of MTBVAC in BCG-naïve Swiss adults and Tameris et al. of MTBVAC in adults and neonates in an endemic region.

Added value of this study

This dose-finding trial follows on that of Tameris et al. by enrolling 99 neonates for vaccination with either BCG or one of three escalating dose strengths of MTBVAC.

Implications of all the available evidence

MTBVAC is safe and well tolerated at all three dose levels used in the study. MTBVAC 2.5 × 10^5 CFU is less reactogenic and more immunogenic that the licensed BCG an thus this dose was selected for the Phase 3 efficacy trial currently recruiting in Sub-Saharan Africa.

Introduction

The World Health Organisation (WHO) Global TB Report 2023 estimated that 12% of the 10.6 million tuberculosis (TB) cases in 2022 were children (aged 0–14 years)[1]; and most of these child TB cases occurred in countries with universal infant Bacille Calmette Guérin (BCG) coverage as part of the Expanded Programme on Immunisation. BCG vaccination protects against the most severe forms of miliary and meningitic TB,[2] but offers partial, variable protection against pulmonary TB in children,[3,4] which rarely lasts into adolescence.[5] BCG is a live, attenuated *Mycobacterium bovis* vaccine, which may be associated with local, regional, and disseminated disease in infants with untreated HIV infection and other congenital immunodeficiencies.[6]

The WHO has identified development of a new infant TB vaccine with improved safety and efficacy compared to BCG as a major strategic goal.[7] The WHO Preferred Product Characteristics (PPC) for such a vaccine include: improved safety compared to BCG, including among immuno-compromised infants; improved efficacy compared to BCG, including against all forms of TB; and protection lasting at least 10 years.[7] Modelling suggests that by 2050 an infant vaccine with these characteristics could avert up to 18 million TB cases, 2.6 million TB deaths, and would be cost-effective in almost all high TB burden countries.[8]

MTBVAC is a live-attenuated clinical strain of *Mycobacterium tuberculosis* (*Mtb*) (lineage 4) that has been genetically engineered to contain two independent, stable deletions of the virulence-associated gene *phoP*, which regulates secretion of ESAT-6; and *fadD26*, which is required for synthesis of the cell-wall lipid phthiocerol dimycocerosate (PDIM).[9] MTBVAC expresses a broader collection of potentially protective mycobacterial antigens than BCG, since MTBVAC contains *Mtb* genes absent from *M. bovis,* in addition to the RD1 genes lost from *M. bovis* during sub-cultivation to derive BCG. The RD1 region includes ESAT-6 and CFP-10,[9] which were protective against *Mtb* challenge in mice,[10] and may be important for protection against TB in humans.

A similar safety profile to BCG was observed for MTBVAC in several preclinical models, including mice, immunocompromised SCID mice, guinea pigs, and non-human primates; as well as superior immunogenicity and efficacy against *Mtb* challenge.[11–13] MTBVAC is being developed in parallel for both adult and infant populations. Previously it has appeared safe and well-tolerated in BCG-naïve and BCG-vaccinated adults[14,15]; showed a safety profile similar to BCG in infants; and showed evidence of MTBVAC dose-related conversion of the interferon-γ release assay (IGRA).[15]

This phase 2a, randomised, double-blind, BCG-controlled, dose-escalation trial of MTBVAC was conducted to select the optimum safe and immunogenic dose for a subsequent licensure efficacy trial (NCT04975178), designed to demonstrate conformity of MTBVAC with WHO PPC requirements for a new TB vaccine with improved protection against pulmonary TB, which could potentially replace BCG vaccine in infants.[7]

Methods

Design and study population

The trial was conducted in the Cape Winelands District of the Western Cape Province, South Africa, where TB burden is approximately 5478 new cases per year and infant *Mtb* exposure is high.[16] HIV-negative pregnant women in their third trimester were recruited at antenatal clinics. Eligible women had no history of TB and no identified household TB contact. Proof of maternal HIV status needed to be within 30 days of delivery. In South Africa pregnant women are tested for HIV up to 3 times during pregnancy and routinely at delivery; HIV unexposed infants are routinely tested for HIV at 18 months. Eligible infants were BCG-naïve, generally healthy, aged 96 h or younger, weighed at least 2450 g at birth, with at least 37 weeks' gestation, and a 5-min Apgar score of at least 7.

Randomisation and blinding

We aimed to enrol 99 eligible infants to randomly (~1:3) receive either the standard infant dose of BCG Vaccine SSI 2.5×10^5 CFU, or MTBVAC in three sequential cohorts of increasing dose (2.5×10^4 CFU, 2.5×10^5 CFU, or 2.5×10^6 CFU in 0.05 mL). Each cohort was designed to comprise 25 MTBVAC and 8 BCG recipients. The principal investigator reviewed cohort 1 Day 28 safety data before enrolling cohort 2; the Data and Safety Monitoring Board (DSMB) reviewed Day 28 safety data for both cohort 1 and 2 before enrolling cohort 3.

Randomisation was by a pre-prepared schedule with blocks of 33, linked to treatment number, with sequential allocation by the pharmacist, who prepared and masked the vaccine syringe using semi-transparent tape. All clinical staff remained blind to group allocation until all infants in a cohort completed 365 days of follow-up. Immunology and microbiology laboratory staff remained blinded until analyses were completed.

Procedures

Infants were administered either BCG (2.5×10^5 CFU Danish strain 1331 in 0.05 mL diluent) or MTBVAC (2.5×10^4, 2.5×10^5, or 2.5×10^6 CFU in 0.05 mL) intradermally on study Day 0 in the left deltoid region.

Follow-up visits occurred on study Days 7, 28, 56, 182, and 365. Additional visits were conducted on study Days 120 and 270 to screen for TB signs and symptoms if the infant tested IGRA positive by QuantiFERON-TB Gold Plus (QFT) test at Day 56.

Venous blood was collected for safety outcomes (serum chemistry, including liver and renal function, and haematology, including full blood, differential white cell, and platelet counts) on Day 7; and for immunogenicity outcomes on Days 28, 56, 182, and 365.

Unsolicited adverse events (AE) and serious adverse events (SAE) were collected from vaccination until end of study; solicited systemic AE until Day 28; and solicited injection site AE until Day 56. Adverse events were graded using a toxicity table based on DAIDs Table for Grading the Severity of Adult and Pediatric Adverse Events, Corrected Version 21 July 2017[17]

Infants were screened for symptoms compatible with TB or history of a new household TB contact at all visits. IGRA was performed on Days 56, 182, and 365. A positive IGRA at Day 56 post-vaccination, which might have been vaccine-induced, did not trigger further action. A positive IGRA at Day 182 or 365, presence of TB symptoms, or a reported household contact triggered TB investigations, including gastric aspirate and induced sputum on two consecutive days, chest radiograph, blood for IGRA (if not the trigger) and HIV testing, and referral to the local public health clinic, for either isoniazid preventive therapy (IPT) or TB treatment.

After all infants in a dose cohort completed study Day 365, the cohort was unblinded and MTBVAC recipients were offered BCG vaccination. If IGRA conversion occurred at Day 365, the infant was BCG vaccinated after completion of IPT, or if the infant was on TB treatment, after treatment completion.

Outcomes

The primary study aim was to evaluate the safety, reactogenicity, and immunogenicity of escalating dose levels of MTBVAC compared to BCG. Safety and reactogenicity outcomes were frequencies of solicited systemic and local injection site AE, unsolicited AE, and SAE.

Immunogenicity endpoints were frequencies of antigen-specific IFN-γ, TNF, IL-2, IL-17, and/or IL-22 expressing CD4 and CD8 T cells, measured by 12-h whole blood intracellular cytokine staining (WB-ICS) at Days 28,56,182, and 365.

A secondary aim was to evaluate IGRA responses by measuring quantitative and qualitative IGRA results at the manufacturer's QFT threshold on Days 56, 182, and 365.

Immunogenicity evaluations

WB-ICS assay was performed as previously described.[18] Because only a very small blood volume could be safely drawn at days 28 and 56, 2 mL was drawn at these visits, while 4 mL could be drawn at the later timepoints. Whole blood collected in sodium heparin tubes was stimulated with MTBVAC (10^6 CFU/mL), a pool of 122 peptides of immunodominant *Mtb* epitopes (Megapool, Genscript, Piscataway, NJ, USA), phytohemagglutinin (PHA, 5 μg/mL, Thermo Fisher), or left unstimulated (UNS) in the presence of co-stimulatory antibodies (anti-CD28 and CD49d, at 0.25 μg/mL each, BD) for 12 h at 37 °C. After 7 h of stimulation, Brefeldin A (10 μg/mL, Sigma Aldrich) was added, and the blood was incubated for another 5 h. Erythrocytes were lysed and leukocytes fixed using FACSLysing solution (BD). For flow cytometry, leukocytes were stained with the antibody panel in Table 1 directed against the following targets: γδTCR (BD, #562560, AB_2737655), CD8 (BD, #563919, AB_2722546), CD16 (BD, #563172, AB_2744297), IL-17 A (BD, #563746, AB_2738402), CD56 (Biolegend, #318336, AB_2562417), CD4 (BD, #563877, AB_2738462), IL-2 (BD, #340448, AB_400424), HLA-DR (Biolegend, #307629, AB_893575), IL-22 (eBiosciences, #12-7229-42, AB_1834463), CD26 (BD, #565158, AB_2739085), CD161 (BD, #551138, AB_394068), TNF (eBiosciences, #25-7349-82, AB_469686), CD153 (R&D, #FAB1028A, AB_416825), IFN-γ (BD, #557995, AB_396977), CD3 (BD, #641406, AB_1645730). All antibodies were commercially available validated reagents. Titrations, panel optimisation and dummy runs were performed before study samples were acquired. Acquisition on a

Lineage/function	Specificity	Fluorochrome	Clone	Manufacturer	Cat #	Titer[a]	RRID
gd T cells	gd TCR	BV421	B1	BD	562560	2	AB_2737655
T cells	CD8	BV510	SK1	BD	563919	0.5	AB_2722546
NK cells	CD16	BV605	3G8	BD	563172	1.2	AB_2744297
Th17 cytokine	IL-17 A	BV650	N49-563	BD	563746	0.1	AB_2738402
NK cells	CD56	BV711	HCD56	Biolegend	318336	0.6	AB_2562417
T cells	CD4	BV786	SK3	BD	563877	0.3	AB_2738462
Th1 cytokine	IL-2	FITC	5344.111	BD	340448	2.5	AB_400424
Activation	HLA-DR	PerCP-Cy5.5	L243	Biolegend	307629	0.3	AB_893575
Th22 cytokine	IL-22	PE	22URTI	eBiosciences	12-7229-42	0.15	AB_1834463
MAIT cells	CD26	PE-CF594	M-A261	BD	565158	0.3	AB_2739085
MAIT cells	CD161	PE-Cy5	DX12	BD	551138	2.5	AB_394068
Th1 cytokine	TNF	PE-Cy7	MAb11	eBiosciences	25-7349-82	0.015	AB_469686
Host resistance to Mtb	CD153	APC	116614	R&D	FAB1028A	1	AB_416825
Th1 cytokine	IFN-g	AF700	B27	BD	557995	1	AB_396977
T cells	CD3	APC-H7	SK7	BD	641406	0.3	AB_1645730

[a]µL/50 µL of staining volume.

Table 1: **Flow cytometry antibody panel for the whole blood ICS assay.**

BD Fortessa flow cytometer was performed in batches that included samples collected from the same participant (all time points and conditions) to minimise variability. The QuantiFERON-TB Gold Plus assay (Qiagen) was performed per the manufacturers' protocol.

Statistics

The sample size was selected as adequate for a preliminary assessment of the safety profile of MTBVAC in infants, with a sample size similar to that of other Phase 1 trials, and was not powered to detect specific differences in outcomes. The safety analysis set included all participants who received study vaccine. The immunogenicity set included all participants who received study vaccine and had at least one evaluable immunogenicity result. Summary measures minimum, maximum, medians are reported for continuous data while frequency and proportions/percentages are used for binary and categorical data. Point estimates and corresponding 95% confidence intervals for risk-ratios are presented comparing MTBVAC dose arm to BCG as well for comparing MTBVAC doses. Forest plots are used to present these point and interval estimates. For immunogenicity frequencies of cytokine producing T cells were analysed by linear mixed effect models, fit to log-transformed data and comparisons between MTBVAC dose arms were evaluated. P-values were obtained using a linear mixed model, and adjusted using Holm's multiplicity method. Adjustments were made within each visit.

Ethics

The study was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (UCT HREC 475/2021) and the South African Health Products Regulatory Authority (SAHPRA) and was registered on ClinicalTrials.gov (#NCT03536117). All mothers gave written informed consent for their infants' participation before the onset of labour.

Role of funders

This trial was funded by the European and Developing Countries Clinical Trials Partnership (EDCTP). The sponsor, Biofabri, had a role in study design, data interpretation, review of the clinical study report, which was compiled by FHI Clinical, and review of the final manuscript. All authors had full access to the data.

Results

Between 11 February 2019 and 26 February 2021, 228 pregnant women consented, and 99 infants were enrolled (Fig. 1). Reasons for exclusion included: closure to enrolment in a cohort pending safety review; COVID-19 pandemic measures; withdrawal of consent; receipt of BCG at birth; gestational age <37 weeks; weight less than 2450 g; family relocation plans; age older than 96 h; participation in previous vaccine trial; and infant being unwell. Cohort 1 had completed follow up by the start of the SARS CoV2 pandemic in South Africa and Cohort 2 participants had all completed the day 180 clinic visit. Enrolment into Cohort 3 was delayed until researchers could return to the public health clinics in October 2020.

Median age of enrolled infants was 2 days; median Apgar score at 5 min was 10; median birth weight was 3140 g; and 46.5% were male (Table 2).

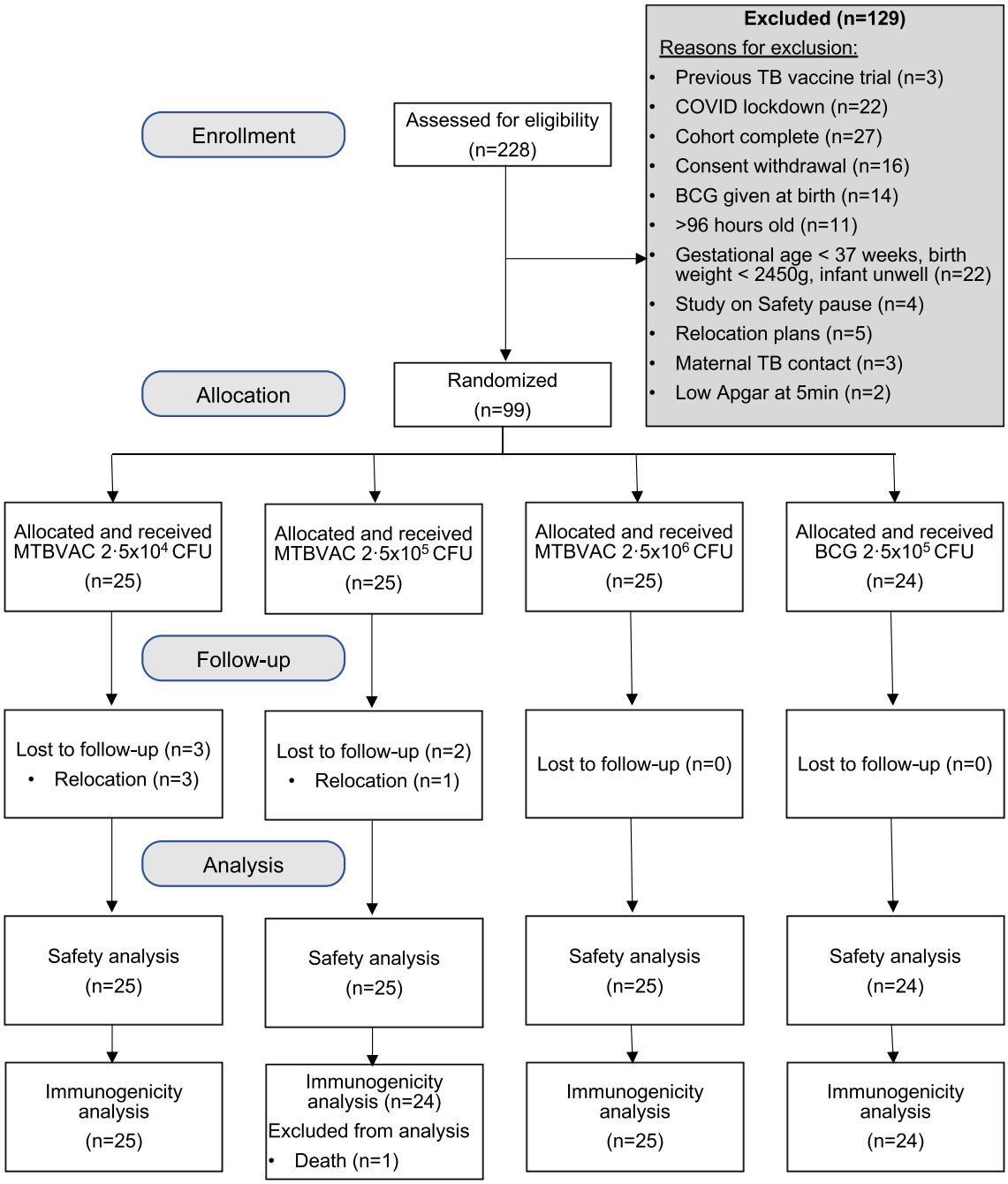

***Fig. 1:* Consort diagram**. CFU, colony forming units.

Four of 99 participants (4%) were lost to follow-up due to relocation. One infant died after the Day 182 visit (see below).

Sixty-three of 99 infants across all three cohorts had 86 solicited AE events through Day 56, all of which were rated mild, except one grade 2 erythema in an MTBVAC 2.5×10^5 CFU recipient (Tables 3 and 4). Induration, swelling, and erythema were more frequent with increasing MTBVAC dose (Fig. 2). Redness, swelling, pain, ulceration, and discharge were less frequent in infants receiving MTBVAC 2.5×10^5 CFU compared to BCG, but induration and swelling were more frequent in infants receiving MTBVAC 2.5×10^6 CFU than those receiving BCG. Scarring was recorded as unsolicited AE

Description	BCG 2.5×10^5 CFU (n = 24)	MTBVAC 2.5×10^4 CFU (n = 25)	MTBVAC 2.5×10^5 CFU (n = 25)	MTBVAC 2.5×10^6 CFU (n = 25)	Total (n = 99)
Age (days)					
Median (Q1-Q3)	2 (1–3)	3 (2–3)	2 (1–3)	2 (1–2)	2 (1–3)
Min—Max	1–4	1–4	1–4	1–4	1–4
Apgar score at 5 min					
Media (Q1-Q3)	10 (9–10)	10 (9–10)	10 (9–10)	9 (9–10)	10 (9–10)
Min—Max	9–10	9–10	8–10	7–10	7–10
Birth weight (g)					
Median (Q1-Q3)	3170 (2998–3650)	3130 (2885–3330)	3350 (2885–3330)	3090 (2890–3340)	3140 (2920–3465)
Min—Max	2680–4490	2465–3860	2480–5950	2550–3845	2465–5950
Sex, n (%)					
Male	13 (54)	10 (40)	12 (48.0)	11 (44)	46 (46)
Female	11 (45)	15 (60)	13 (52.0)	14 (56)	53 (54)
Race, n (%)					
Mixed cape ancestry	16 (67)	16 (64)	15 (60)	15 (60)	62 (63)
Black	8 (33)	9 (36)	10 (40)	10 (40)	37 (37)

BCG: Bacillus Calmette-Guérin. CFU: colony forming units. Max: maximum. Min: minimum. n: number of participants. %: percentage of participants.

Table 2: **Infants demographics.**

(Supplementary Table S1) and was seen in 16/24 (67%) BCG recipients, 1/25 (4%) MTBVAC 2.5×10^4, 16/25 (64%) 2.5×10^5 and 22/25 (88%) 2.5×10^6 CFU recipients. Solicited and unsolicited systemic AE reflected common early childhood ailments and were evenly distributed between study vaccines across groups.

Twelve of 99 infants (three of 24 BCG and nine of 75 MTBVAC recipients, including six in the 2.5×10^4 CFU, two in the 2.5×10^5 CFU, and one in the 2.5×10^6 CFU groups, experienced 14 vaccine-unrelated SAE. These SAE included the death of one MTBVAC 2.5×10^5 CFU recipient, one month after the day 182 visit, due to bronchopneumonia confirmed at autopsy. The day 182 QFT had tested negative on this infant. Six hospitalisations occurred for respiratory tract infection (5 of 75 MTBVAC recipients, 1 of 24 BCG recipients); three for gastroenteritis (2 in MTBVAC recipients, 1 in BCG recipients); two for neonatal jaundice; and one each for breast abscess, pertussis, and possible TB meningitis.

In total, nine infants (9%) were treated for TB. Four of 24 BCG recipients (17%) and four of 75 MTBVAC recipients (5%), all of whom received 2.5×10^4 CFU MTBVAC, were treated for unconfirmed pulmonary TB. One infant in each of the MTBVAC 2.5×10^4 CFU and BCG groups had a single trace positive Xpert MTB/RIF Ultra result on induced sputum, which was classified as unconfirmed TB. No infants in the MTBVAC 2.5×10^5 or 2.5×10^6 CFU cohorts were treated for TB (Supplementary Table S2).

One BCG recipient (4%) was diagnosed with unconfirmed TB meningitis nine days post-vaccination, following a generalised neonatal convulsion. Cerebrospinal fluid (CSF) examination showed a Xpert MTB/RIF Ultra trace result which could not be replicated;

Description	MTBVAC 2.5×10^4 CFU (n = 25)	MTBVAC 2.5×10^5 CFU (n = 25)	MTBVAC 2.5×10^6 CFU (n = 25)	BCG 2.5×10^5 CFU (n = 24)	Total (n = 99)
Participants with at least one related AE, n (%)	18 (72)	20 (80)	25 (100)	22 (92)	85 (86)
Participants with at least one related unsolicited AE, n (%)	2 (8)	20 (80)	24 (96)	21 (88)	67 (68)
Participants with at least one related solicited AE, n (%)	17 (68)	7 (28)	22 (88)	17 (71)	86 (90)
Participants with at least one related injection site AE n (%)	13 (52)	20 (80)	24 (96)	20 (83)	77 (78)
Injection site ulcer, n (%)	0	1 (4)	1 (4)	3 (13)	5 (5)
Injection site pain, n (%)	5 (20)	1 (4)	1 (4)	3 (13)	10 (10)
Injection site discharge, n (%)	0	1 (4)	7 (28.0)	6 (25)	14 (14)
Injection site erythema, n (%)	3 (12)	4 (16)	17 (68)	9 (38)	33 (33)
Injection site swelling, n (%)	4 (16)	6 (24)	15 (60)	7 (29)	32 (32)

Table 3: **Solicited injection site reaction adverse events.**

Adverse Events	MTBVAC 2.5×10^4 CFU (n = 25)	MTBVAC 2.5×10^5 CFU (n = 25)	MTBVAC 2.5×10^6 CFU (n = 25)	BCG 2.5×10^5 CFU (n = 24)	Total (n = 99)
	n (%)	n (%)	n (%)	n (%)	n (%)
Participants with at least one related solicited (local and systemic) AE					
Mild	17 (68)	7 (28)	22 (88)	17 (71)	63 (64)
Moderate	0	1 (4)	0	0	1 (1)
Severe	0	0	0	0	0
Injection site redness					
Mild	3 (12)	3 (12)	17 (68)	9 (38)	32 (32)
Moderate	0	1 (4)	0	0	1 (1)
Severe	0	0	0	0	0
Injection site swelling					
Mild	4 (16)	5 (20)	15 (60)	7 (29)	31 (31)
Moderate	0	0	0	0	0
Severe	0	0	0	0	0
Injection site pain					
Mild	5 (20)	1 (4)	1 (4)	3 (13)	10 (10)
Moderate	0	0	0	0	0
Severe	0	0	0	0	0
Injection site discharge					
Mild	0	1 (4)	7 (28)	4 (17)	12 (12)
Moderate	0	0	0	0	0
Severe	0	0	0	0	0
Injection site ulceration					
Mild	0	1 (4)	0	3 (13)	4 (4)
Moderate	0	0	0	0	0
Severe	0	0	0	0	0

Table 4: **Severity of solicited related adverse events and injection site reactions induced by MTBVAC doses and BCG administration.**

CSF culture for *Mtb* was not done despite repeating the lumbar puncture. The infant completed an 8-month course of TB treatment, at no point displaying any neurological deficit, and was generally healthy, well grown, with normal developmental milestones at end of study. The infant's mother tested negative for TB and HIV and congenital TB was not suspected.

Vaccination with MTBVAC and BCG induced CD4 T cells predominately expressing IFN-γ, IL-2, or TNF, which were readily detected 28 days after vaccination, but peaked at D56 in most infants (Fig. 3). At all time-points (Days 28, 56, 182, and 365), frequencies of MTBVAC-specific CD4 T cells expressing any combination of IFN-γ, IL-2, TNF, IL-17, and/or IL-22 (any cytokine-expressing cells) were higher in the MTBVAC 2.5×10^5 and 2.5×10^6 CFU groups than the MTBVAC 2.5×10^4 CFU group (Fig. 3b and c). When compared to BCG, frequencies of any cytokine-expressing MTBVAC-specific CD4 T cells were higher in the MTBVAC 2.5×10^5 and 2.5×10^6 CFU groups at Day 28 (Fig. 3c). Very similar patterns were observed for polyfunctional Th1-cytokine expressing CD4 T cells, co-expressing IFN-γ, TNF, and IL-2 (Fig. 3c). IL-17-expressing and IL-22-expressing CD4 T cells were very low and no dose-associated response patterns were observed (Fig. 3d).

We also analysed CD8 T cell responses; frequencies of MTBVAC-specific CD8 T cells expressing IFN-γ, IL-2, TNF, IL-17, and/or IL-22 were generally low (Fig. 3d), with the MTBVAC 2.5×10^6 CFU group displaying significantly higher responses than MTBVAC 2.5×10^4 CFU and BCG vaccinated infants at Day 182 and Day 28, respectively (Supplementary Figure S1). Similar CD4 and CD8 T cell functional patterns, but of lower magnitude, were observed in the different cohorts when cells were re-stimulated with a Megapool of Mtb-specific peptides (Supplementary Figure S2).

IGRA conversion and reversion were also measured (Fig. 4), as quantitative IFN-γ levels or as qualitative outcome, in the TB1-Nil or TB2-Nil QFT assay tubes, whichever provided the higher value. QFT conversion was observed at day 56 in 20/23 (87%), 20/24 (83%), and 21/23 (91%) infants who received the 2.5×10^4 CFU, 2.5×10^5 CFU and 2.5×10^6 CFU MTBVAC doses, respectively. Responses in all MTBVAC dose groups waned over time, such that by day 182, 17/24 (71%), 16/24 (67%) and 15/23 (65%) MTBVAC-vaccinated infants had a QFT-positive response. Similarly, by day 365, 10/21 (48%), 8/21 (38.1%) and 10/24 (42%) MTBVAC-vaccinated infants had a QFT-positive response. The quantitative levels of IFN-γ also waned over time, as was

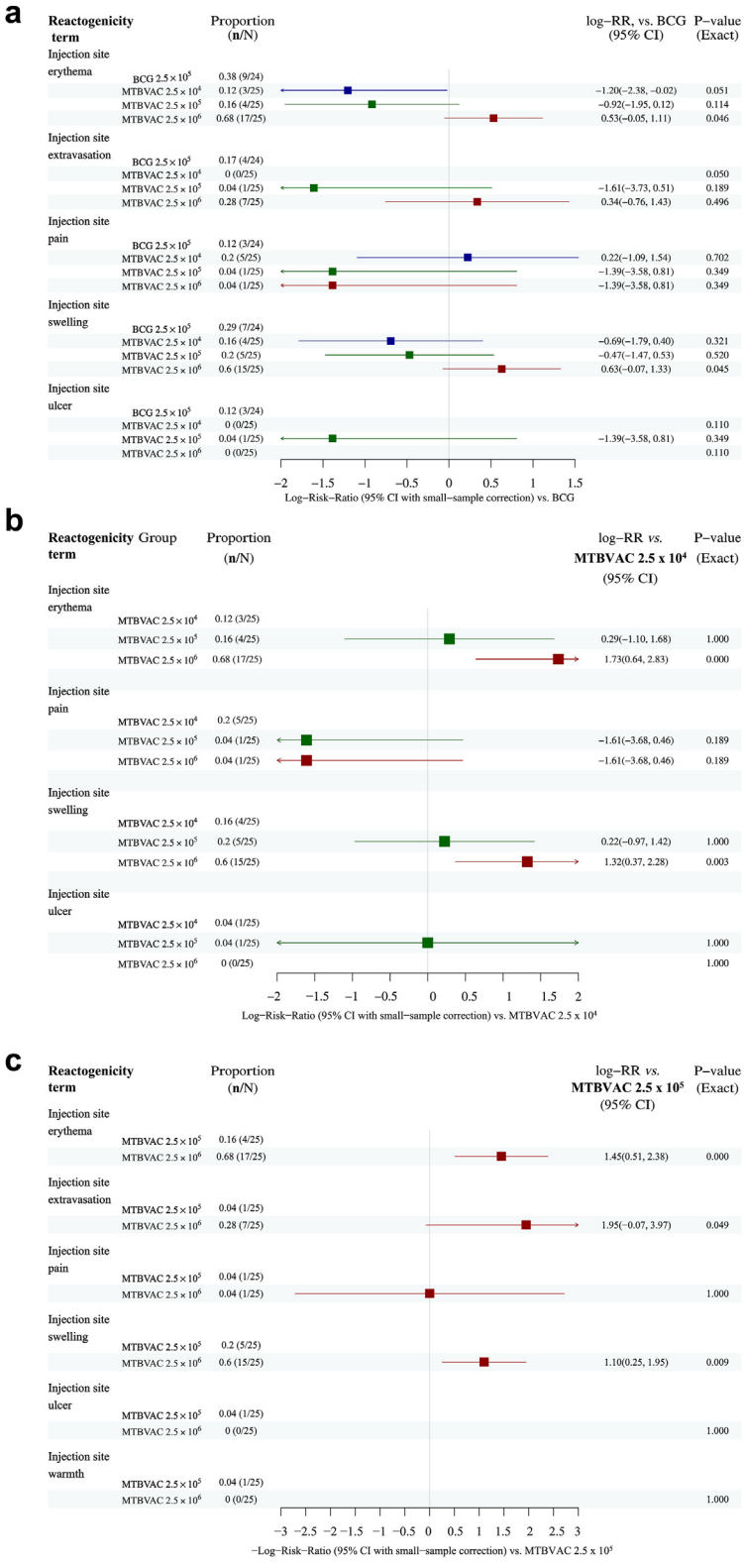

***Fig. 2:* Comparison of reactogenicity events at the injection site between cohorts and vaccination regimens.** Graphs depict risk ratios with 95% confidence intervals, and P-values (Exact's test) which represent the compared proportions of infants who presented with the listed local adverse events between (a) BCG and the different MTBVAC doses, (b) the lowest MTBVAC dose (2.5×10^4 CFU) and the other MTBVAC doses, and (c) the mid MTBVAC dose (2.5×10^5 CFU) and the highest MTBVAC dose (2.5×10^6 CFU). Shifts to the right indicate a greater risk of adverse reactions in infants who received the test vaccine dose (below each comparator) compared to infants who received the comparator vaccine (in bold, above). Number of infants per group are presented in Tables 3 and 4.

observed for the MTBVAC-specific CD4 T cell response (Fig. 3). By comparison, 1/24 (4.%) infants who received BCG had a positive QFT at day 56; 2/24 (8%) by day 182; and 4/24 (17%) by day 365. BCG vaccination cannot lead to IGRA conversion since it does not encode ESAT-6 and CFP-10, the antigens used in the test. These conversions are likely the result of Mtb exposure of these infants.

In summary, MTBVAC was immunogenic at all three doses, inducing predominantly Th1-cytokine-expressing CD4 T cells, which peaked at Day 56. The 2.5×10^5 and 2.5×10^6 CFU MTBVAC doses induced similar response magnitudes and were both more immunogenic than BCG. Vaccination with any MTBVAC dose resulted in IGRA conversion in 61 (87%) infants at Day 56, but these responses waned and reverted so that 38 (57%) infants were IGRA-negative by Day 365.

Discussion

We have shown that MTBVAC, a live-attenuated *Mtb* candidate vaccine developed to provide more effective protection against childhood TB by inducing a broader immune response than BCG, appeared safe and well tolerated in South African infants receiving escalating MTBVAC doses ranging from 2.5×10^4 to 2.5×10^6 CFU. Injection site reactogenicity to MTBVAC was dose-dependent, but it is notable that the 2.5×10^5 CFU MTBVAC dose was associated with fewer solicited adverse events, including fewer injection site reactogenicity events, than BCG The vaccine was also immunogenic, inducing predominantly Th1-cytokine-expressing CD4 T cells, which peaked at Day 56. The 2.5×10^5 and 2.5×10^6 CFU MTBVAC doses induced similar response magnitudes, and both were more immunogenic than BCG.

This study was designed to identify the optimal dose of MTBVAC for a subsequent multicentre, multi-country infant efficacy trial of MTBVAC, compared to BCG, for protection against childhood TB. This phase 3 trial (NCT04975178) will enrol 7000 infants across 6 African sites. The WHO Preferred Product Characteristics for New Tuberculosis Vaccines for neonates and infants stipulate a new vaccine should demonstrate

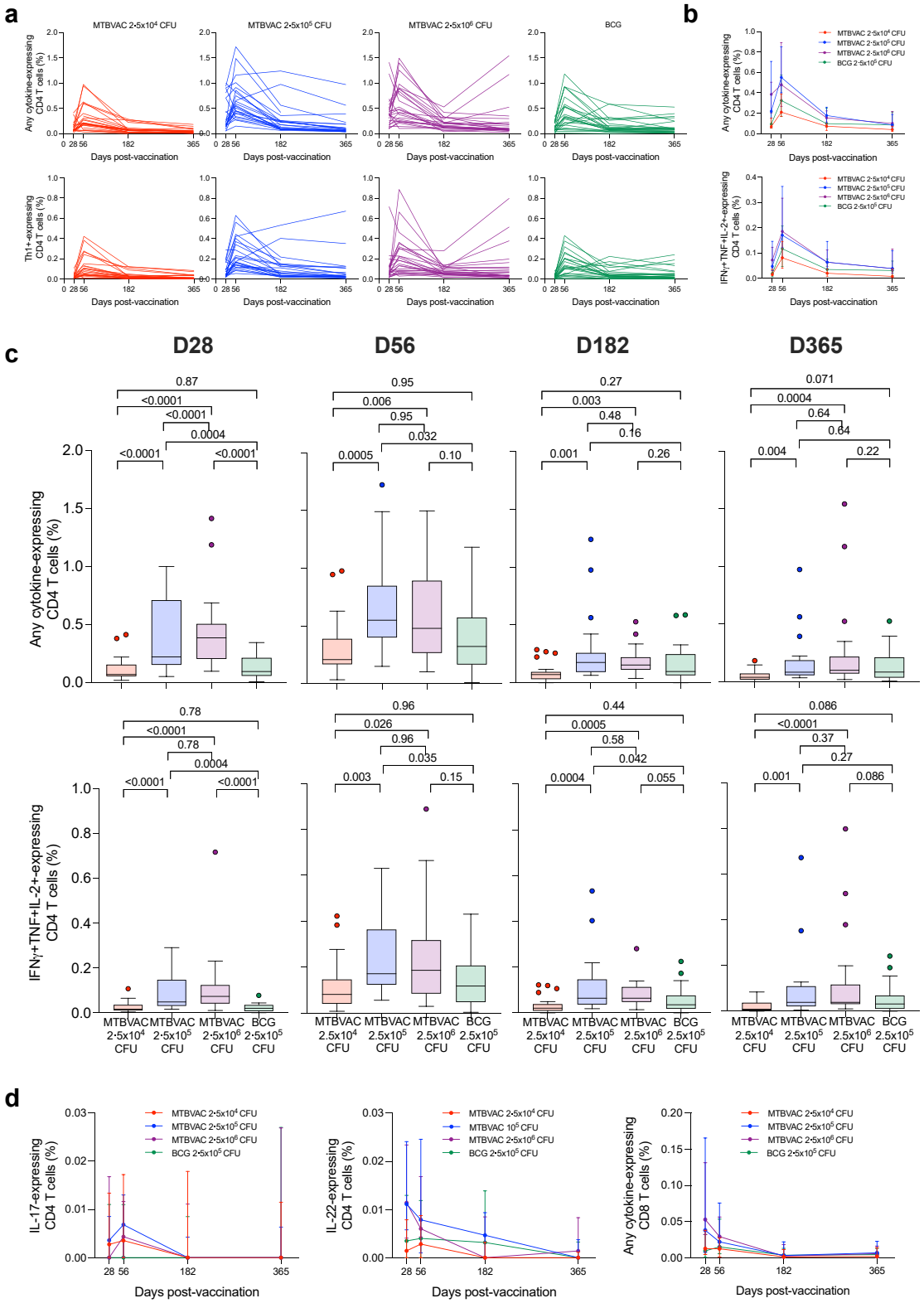

***Fig. 3*: Kinetics of cytokine-expressing MTBVAC-specific T cell responses in newborns vaccinated with MTBVAC or BCG.** Frequencies of MTBVAC-specific CD4 T cells measured after MTBVAC stimulation by whole blood intracellular cytokine staining assay in MTBVAC or BCG

improved safety, as compared to the current licensed vaccine BCG; as well as efficacy equal to or greater than 80% compared to baseline incidence, or superior efficacy compared to BCG in preventing TB disease, including severe, disseminated TB, TB meningitis and pulmonary TB, in infants and young children.[6] A new infant TB vaccine with an improved safety profile, particularly in regard to the propensity of BCG to result in an erythematous, indurated injection site reaction, would be a major advance for vaccine acceptability. Although not tested in this study, an improved injection site safety profile that extended to reduced risk of local, regional, and disseminated vaccine disease, which is currently a major concern for infants with inherited and acquired immune deficiencies, such as untreated HIV infection, would have significant benefit for global child health. This phase 2a dose escalation trial demonstrated that the MTBVAC 2.5×10^5 CFU dose is less reactogenic compared with BCG, thus meeting the WHO Preferred Product Characteristics in terms of safety.

Note that this phase 2a study was not powered to evaluate efficacy of MTBVAC compared to BCG, although rigorous TB screening and standardised investigation of possible TB disease was conducted throughout the study. Four of the eight infants diagnosed with unconfirmed pulmonary TB were BCG recipients; four received the lowest MTBVAC dose of 2.5×10^4 CFU; and no cases of TB were diagnosed in infants receiving the 2.5×10^5 or 2.5×10^6 CFU MTBVAC doses. Two of these eight cases, one each in the BCG and MTBVAC groups, had a single trace positive Xpert MTB/RIF Ultra result on induced sputum. Neither the revised consensus diagnostic classification of childhood TB,[19] nor the protocol classification, include trace positive Xpert MTB/RIF Ultra results, so all TB cases were considered unconfirmed by the investigators. Further, although an infant BCG recipient was diagnosed and treated for suspected TB meningitis nine days post vaccination, the benevolent clinical course and lack of any neurological sequelae cast doubt on the likelihood of this diagnosis. The overall rate of unconfirmed TB cases seen in the BCG (21%) and MTBVAC (5%) dose groups is reassuring for future efficacy evaluation of MTBVAC, although the observed difference is driven by a higher than expected rate in the BCG arm, rather than a lower than expected rate in the MTBVAC arm, when compared to the 3.3% TB incidence previously observed in this population.[20] We cannot speculate on the reason for the high rate of unconfirmed TB diagnoses in the BCG arm, nor on the possible influence of the SARSCoV2 pandemic. There has been no increased vigilance in TB surveillance since the earlier trials in this population; and observer bias is not suspected as clinicians were blinded to both study assignment and QFT results. Six of the eight participants diagnosed with pulmonary TB were started on treatment prior to the onset of the SARSCoV2 pandemic in March 2020 The single BCG recipient who was QFT positive at day 56 was not diagnosed with TB during the study.

We do acknowledge the risk of sparse data bias, uncontrolled random confounding due to baseline imbalances e.g. sex as well as no confounding adjustment due to small sample size as limitations of our study. Additionally, this trial being designed to address MTBVAC dose-selection based on safety and immunogenicity data, results were analysed in the modified intention-to treat (mITT) cohort rather than the ITT cohort not carrying out a sensitivity analysis using multiple imputations or the estimand framework. This might be subject to selection bias, although we do not believe it would impact the dose-selection.

Here, we observed that MTBVAC was highly immunogenic and induced antigen-specific CD4 T cells that primarily expressed Th1 cytokines. At the early post-vaccination time point, on Day 28, the induced response was associated with MTBVAC dose and the two higher doses of MTBVAC induced significantly higher responses than BCG. Thereafter, on days 56, 182, and 365, responses to the 2.5×10^6 CFU and 2.5×10^5 CFU doses were not different to each other, although responses to these higher doses remained significantly higher than the lowest 2.5×10^4 CFU dose of MTBVAC. MTBVAC did not induce noteworthy antigen-specific Th1 CD8 T cells or Th17 or Th22 CD4 T cell responses. These results are consistent with previous phase 1–2 trials that showed MTBVAC to be highly immunogenic, with the induction of robust and durable MTBVAC-specific Th1 responses in Swiss adults with low exposure to mycobacteria[13]; in *Mtb*-unsensitised and *Mtb*-sensitised South African adults and in a previous study in South African infants.[14]

vaccinees. (a) Individual longitudinal trajectories of CD4 T cell responses, categorized by treatment arm and dose at the indicated time points. Each line represents one participant. The top panel shows frequencies of CD4 T cell responses expressing any combination of IFN-γ IL-2, TNF, IL-17 A, or IL-22 (Any cytokine-expressing CD4 T cells) and the bottom panel shows polyfunctional IFN-γ+IL-2+TNF+CD4 T cells. (b) Median longitudinal trajectories of any cytokine-expressing CD4 T cells (top) and polyfunctional Th1 T cells (bottom) are shown. Error bars denote IQRs. (c) Frequencies of CD4 T cell responses expressing any combination of IFN-γ, IL-2, TNF, IL-17 A, or IL-22 (top row) and of polyfunctional IFN-γ+IL-2+TNF+CD4 T cells (bottom row) in newborns. Horizontal lines represent medians, boxes the IQR, and whiskers denote 1.5 times the IQR from Q1 and Q3; dots show outliers beyond the whiskers. P-values were obtained using a linear mixed model, and adjusted using Holm's multiplicity method. Adjustments were made within each visit. (d) Median longitudinal trajectories of IL-17 or IL-22 expressing MTBVAC-specific CD4 T cells, or any cytokine-expressing MTBVAC-specific CD8 T cells (right). Numbers of infants included in the immunogenicity dataset are presented in the Consort diagram in Fig. 1.

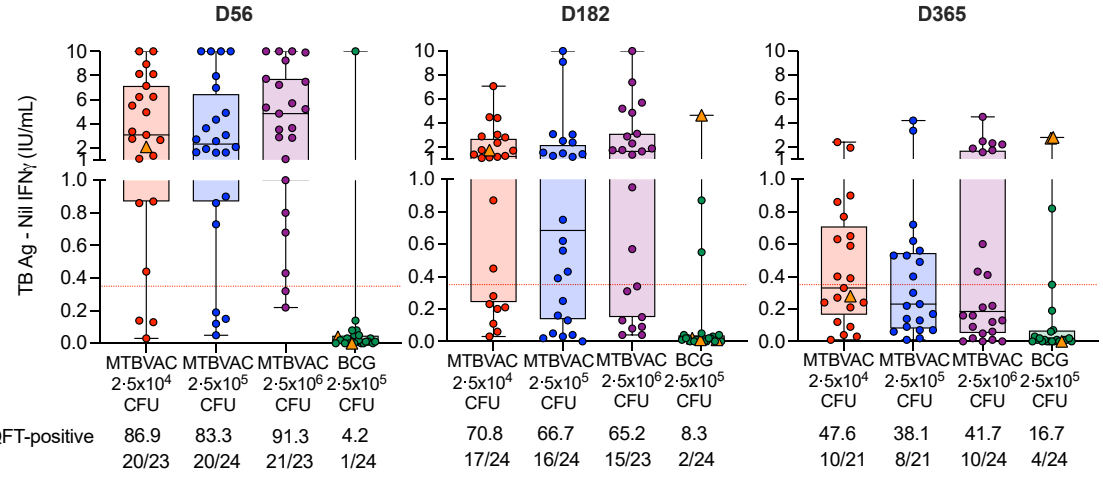

Fig. 4: **IFN-γ responses measured by QFT in newborns vaccinated with MTBVAC or BCG.** IFN-γ concentrations (IU/mL) measured by QFT-TB Gold Plus assay, categorised by timepoint post-vaccination. The higher IFN-γ concentration between TB1 and TB2, after subtraction of the Nil condition, is shown. The dotted horizontal lines indicate the positivity cut-off (0.35 IU/mL) of the assay. Horizontal lines represent medians, boxes the IQR, and whiskers the range. The percent and number of QFN-positive participants at each time point for each cohort are indicated below each graph. Orange triangles represent QFT results of infants diagnosed with TB at TB investigation or at the closest timepoints to TB investigation when it occurred between scheduled visits. Numbers of infants included in the immunogenicity dataset are presented in the Consort diagram in Fig. 1.

Confirming our previous observation,[14] more than 80% of infants who received MTBVAC vaccination converted to a positive QFT test by D56. By contrast, at this time point only 1 out of 24 infants who received BCG converted to a positive QFT. These IFN-γ responses to ESAT-6 and CFP-10 were transient and waned during follow-up and, at day 365, more than half of the infants vaccinated with MTBVAC had reverted to a negative QFT. This QFT conversion phenomenon is similar to the false-positive tuberculin skin tests seen after BCG administration and demonstrate MTBVAC-specific induction of a strong immune response to the RD-1 antigens ESAT-6 and CFP-10. Studies performed in mice and non-human primates have demonstrated a correlation between ESAT-6 and CFP-10 vaccine-induced T cell responses and protection against mycobacterial challenge.[10,12] Therefore, it is encouraging to observe these same responses in MTBVAC-vaccinated infants, although results from an efficacy trial will be required to inform protection in humans.

A clear limitation of MTBVAC vaccine-induced QFT conversion is the lack of ability to differentiate a positive test due to wild-type *Mtb* sensitisation from that induced by MTBVAC during the first ~6 months post-vaccination. This situation is analogous to that faced by HIV vaccine developers, in which HIV vaccine-induced seropositivity confounds interpretation of subsequent HIV antibody tests. It should be noted that several novel TB vaccine candidates in the development pipeline also include ESAT-6 or CFP-10 and would be affected by the same issue. This limitation should not be seen as an insurmountable challenge, since national programmatic TB control guidelines in high burden countries require a known TB contact to trigger provision of TB preventive therapy (TPT), rather than the result of IGRA testing. However, an alternative test of *Mtb* sensitisation that is independent of MTBVAC vaccination might facilitate acceptance by TB programme staff and policy makers. This approach has been used in the context of other ESAT-6-containing subunit vaccines, with the development of an ESAT-6-free IGRA.[21,22] A CFP-10-free IGRA is currently being developed as a companion assay for MTBVAC (*data not shown*).

In conclusion, we have shown that MTBVAC is safe and well-tolerated at all three dose levels in infants, with the 2.5×10^5 CFU MTBVAC dose being less reactogenic and more immunogenic than the licensed BCG vaccine. On the basis of concordance with WHO recommendations, the 2.5×10^5 CFU MTBVAC dose was selected for phase 3 efficacy evaluation compared to BCG vaccination in infants.

Contributors

BF, JT, IM, ER, EP, JD, CM, DM, MT, TJS, MH designed the study. MT, HG, AKL, SM, JS, NT recruited participants and collected data. VR, CI, NB, CY, JGA, NA, DM, CM collected an analysed laboratory data. VR, CY, TJS designed the figures. HM, RM, TJS, VR, MH, MT, CI, IM, CM analysed data and interpreted results. MF, NB, AV, IM provided operational support. MT, VR, TJS and MH drafted the manuscript. MTBVAC 202 study team recruited, consented participants and collected follow up data, and laboratory samples, conducted quality control and quality assurance. MT, VR and CI contributed equally. TJS and MH contributed equally. All authors had full access to the data, and reviewed, revised and gave final approval of the manuscript before submission.

Data sharing statement

Individual participant data that underlie the results reported in this Article will be available upon request, after de-identification and publication. The full study protocol and clinical and immunological statistical analysis plans are available on ZivaHub (DOI 10.25375/uct.28387955).

Declaration of interests

ER, JT, EP, JD and IMJ are employed by Biofabri, holders of the EDCTP grant RIA2016V-1637.

CM, NA and JGA are employed by University of Zaragosa, the patent holder of "Tuberculosis Vaccine". NA, JGA and CM declare support to attend MTBVACN3 kickoff meeting in Baiona Spain in 2022 paid by the organisers of the meeting with EDCTP funding. MH declares consultancy for WHO, member of advisory committee TBVI, grant holder for clinical trials through his institution, University of Cape Town. TJS declares grants from Biofabri, EDCTP, NIH CDMRP to his institution, University of Cape Town. MTBVAC202 study group funded by EDCTP grant to institution University of Cape Town. BF received consulting fees as a clinical adviser for the development of MTVAC—payment to BFL Conseils SAS. BF also received support from TBVI to attend meetings related to the study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105628.

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