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H1:IC31 vaccination is safe and induces long-lived TNF-α^+IL-2^+CD4 T cell responses in *M. tuberculosis* infected and uninfected adolescents: A randomized trial

Helen Mearns [a,1], Hennie D. Geldenhuys [a,1], Benjamin M. Kagina [a,2], Munyaradzi Musvosvi [a], Francesca Little [b], Frances Ratangee [a], Hassan Mahomed [a,3], Willem A. Hanekom [a], Søren T. Hoff [c], Morten Ruhwald [c], Ingrid Kromann [c], Peter Bang [c], Mark Hatherill [a], Peter Andersen [c], Thomas J. Scriba [a,*], the THYB04 study group Virginie Rozot [a], Deborah A. Abrahams [a], Katya Mauff [b], Erica Smit [a], Yolande Brown [a], E. Jane Hughes [a,4], Edward Makgotlho [a,5], Alana Keyser [a], Mzwandile Erasmus [a], Lebohang Makhethe [a], Hadn Africa [a], Charles Hopley [a], Marcia Steyn [a]

[a] South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine and Division of Immunology, Department of Pathology, University of Cape Town, South Africa
[b] Department of Statistical Sciences, University of Cape Town, South Africa
[c] Statens Serum Institut, Copenhagen, Denmark

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ABSTRACT

Background: Control of the tuberculosis epidemic requires a novel vaccine that is effective in preventing tuberculosis in adolescents, a key target population for vaccination against TB.
Methods: Healthy adolescents, stratified by *M. tuberculosis*-infection status, were enrolled into this observer-blinded phase II clinical trial of the protein-subunit vaccine candidate, H1:IC31, comprising a fusion protein (H1) of Ag85B and ESAT-6, formulated with the IC31 adjuvant. Local and systemic adverse events and induced T cell responses were measured after one or two administrations of either 15 μg or 50 μg of the H1 protein.
Results: Two hundred and forty participants were recruited and followed up for 224 days. No notable safety events were observed regardless of H1 dose or vaccination schedule. H1:IC31 vaccination induced antigen-specific CD4 T cells, co-expressing IFN-γ, TNF-α and/or IL-2. H1:IC31 vaccination of *M.tb*-uninfected individuals preferentially drove the emergence of Ag85B and ESAT-6 specific TNF-α^+IL-2^+CD4 T cells, while H1:IC31 vaccination of *M.tb*-infected individuals resulted in the expansion of Ag85B-specific but not ESAT-6–specific TNF-α^+IL-2^+CD4 T cells.
Conclusions: H1:IC31 was safe and immunogenic in uninfected and *M.tb*-infected adolescents. Two administrations of the 15 μg H1:IC31 dose induced the greatest magnitude immune response, and was considered optimal (South African National Clinical Trials Register, DoH-27-0612-3947; Pan African Clinical Trial Registry, PACTR201403000464306).

* Corresponding author at: SATVI, University of Cape Town, Wernher and Beit Building, Anzio Road, Observatory 7925, South Africa.
E-mail address: Thomas.scriba@uct.ac.za (T.J. Scriba).
[1] These authors contributed equally to this work.
[2] Current Affiliation: Vaccines for Africa Initiative, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa.
[3] Current Affiliation: Division of Community Health, Department of Interdisciplinary Science, Faculty of Medicine and Health Sciences, Stellenbosch University and Metro District Health Services, Western Cape Government: Health.

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1. Background

A core component of the global strategy to control tuberculosis (TB) is the development of efficacious vaccines that prevent infection and disease in adolescents and adults [1–4]. Bacille Calmette Guerin (BCG), the only licenced TB vaccine, has been widely administered for decades, but has been ineffective in controlling the worldwide epidemic [2]. Strategies involving a number of candidates are being assessed with heterologous vaccination of adolescents or adults to boost the immunity induced by either BCG, *Mycobacterium tuberculosis (M.tb)* or environmental mycobacterial infection thought most likely to have the greatest impact on the epidemic [1,3].

Adolescent and adult populations are at particular risk of TB disease. Adolescents typically manifest with adult-type pulmonary TB disease [5], which is primarily responsible for transmission of *Mycobacterium tuberculosis* (*M.tb*). Vaccination with a protective vaccine in adolescents could interrupt disease transmission in both the target population and the population at large [6]. In settings where TB is endemic, a large proportion of adolescents have already been exposed and/or infected with *M.tb* [7,8]. Prior *M.tb* or nontuberculous mycobacteria infection can interfere with vaccination, as described for live mycobacterial vaccines [9], or potentially compromise or enhance the antigen-specific response to vaccination [10,11]. Additionally, prior exposure may increase reactogenicity to vaccination due to a hypersensitivity reaction [3]. Vaccination of adolescents thus requires a preventive vaccine that is safe, immunogenic and efficacious in *M.tb* infected and uninfected persons.

H1 is a subunit fusion protein of *M.tb* antigens ESAT-6 and Ag85B, which is being developed as a pre- and post-exposure vaccine candidate for adolescents and young adults by Statens Serum Institut (SSI, Copenhagen). For clinical development in humans, H1 has been formulated in the two-component adjuvant IC31® (Intercell AG/Valneva) composed of the cationic polyaminoacid KLK, and the oligodeoxynucleotide ODN1a. In preclinical murine and guinea pig models, in comparison to BCG alone, H1:IC31 was safe and induced protective immune responses against *M.tb* challenge [12]. Subsequently, four clinical trials with H1:IC31 have been completed; three phase I trials [13,14] (unpublished data) and a phase II multicentre trial in HIV infected participants [15]. These studies demonstrated that a two-dose regimen with 50 μg of H1 protein in IC31 (500 nmol KLK and 20 nmol ODN1a) was safe in BCG vaccinated and unvaccinated adults, as well as persons with evidence of previous *M.tb* infection, known HIV seropositivity and individuals previously treated for TB. The vaccine also induced strong and persistent antigen-specific Th1 immune responses.

In this large phase II trial, we set out to determine the safety and tolerability of H1:IC31 and define the characteristics of induced immune responses in uninfected and *M.tb*-infected adolescents from a setting where TB is endemic. We explored effects of vaccine antigen dose, administration of one or two H1:IC31 vaccinations and determined the effects of pre and post *M.tb*-exposure vaccination on safety and immunogenicity of H1:IC31.

2. Methods

2.1. Trial design and participants

This was a phase II, single centre, randomized, observer-blinded (blinded to the subjects and those recording adverse events/drawing, processing blood samples and performing primary analyses), placebo-controlled clinical trial to evaluate safety and immunogenicity of H1:IC31 in healthy adolescents, who had received routine BCG at birth (Protocol available in supplementary material). We aimed to enrol 240 adolescents for randomization into one of four study groups. Group 1 received 2 vaccinations of 15 μg of H1, while Group 2 received 2 vaccinations of 50 μg of H1, 56 days apart. Groups 3 and 4 received only one vaccination of 15 μg, or 50 μg of H1, respectively and saline placebo on day 56. All H1 doses were formulated in a standard concentration of IC31 adjuvant (500 nmol KLK, 20 nmol ODN1a). At enrolment, participants were randomized into one of the study groups. Randomisation procedures took into account that the first half (120) of subjects enrolled should be Quantiferon Gold In-tube negative (QFT−) and the second half (120) QFT-positive (QFT+).

Prior to screening, participants and their guardians underwent an in-depth informed consent process. Only adolescents who provided written assent and whose legal guardians provided written consent, were screened for enrolment. The study was approved by University of Cape Town Faculty of Health Sciences Human Research Ethics Committee and South African Medicines Control Council (South African National Clinical Trials Register, DoH-27-0612-3947; Pan African Clinical Trial Registry, PACTR201403000464306) and conducted in accordance with Helsinki Declaration and Good Clinical Practices at the South African Tuberculosis Vaccine Initiative (SATVI) in the Breede Valley region, Western Cape, South Africa.

2.2. Inclusion and exclusion criteria

Participants were healthy adolescents aged 12–18 years. Adolescents were excluded if medical abnormalities were identified at screening through medical history and examination, chest X-ray, urine dipstick tests and safety blood tests (chemistry and haematology). Adolescents were excluded if found to be HIV-positive, or had evidence of previous TB disease, or pregnant, as were female participants who were sexually active and not willing to use contraception during the trial period.

2.3. Vaccination

H1:IC31 was administered on day 0 and/or day 56 by intramuscular injection into the deltoid area on alternate arms using syringe and needle (22–25 gauge 1–1.5 in.). Vaccine was stored in a temperature monitored freezer in the SATVI Pharmacy at <−15 °C and protected from light. Amongst study personnel, only pharmacists were not blinded.

Follow-up visits were conducted 1 and 14 days after each vaccination as well as on days 70, 112, 168 and 224 to examine the injection site, obtain a history of adverse events, perform physical examination, and review the participant diary card. Blood samples for safety assessments were taken at baseline (day 0) and all post-vaccination visits except Day 1 and Day 57. All adverse events (AE) and serious adverse events (SAEs) were recorded and reported. AEs were graded by an investigator for: type, causality, seriousness, severity and outcome using FDA's Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers (2005).

2.4. Immunogenicity assays

Venous blood was collected on days 0, 14, 56, 70, 112 and 224 for the trial's primary immunogenicity endpoint, the IFN-γ ELISpot on PBMCs and the secondary immunogenicity endpoint, a qualified whole blood intracellular cytokine staining (WBA-ICS) assay [16,17]. Detailed methods are available as supplementary data.

2.5. Data analysis

Data were analysed with Stata (v11 StataCorp), Prism (v 6.0f GraphPad Software Inc.) or R [18]. Flow cytometry analyses were

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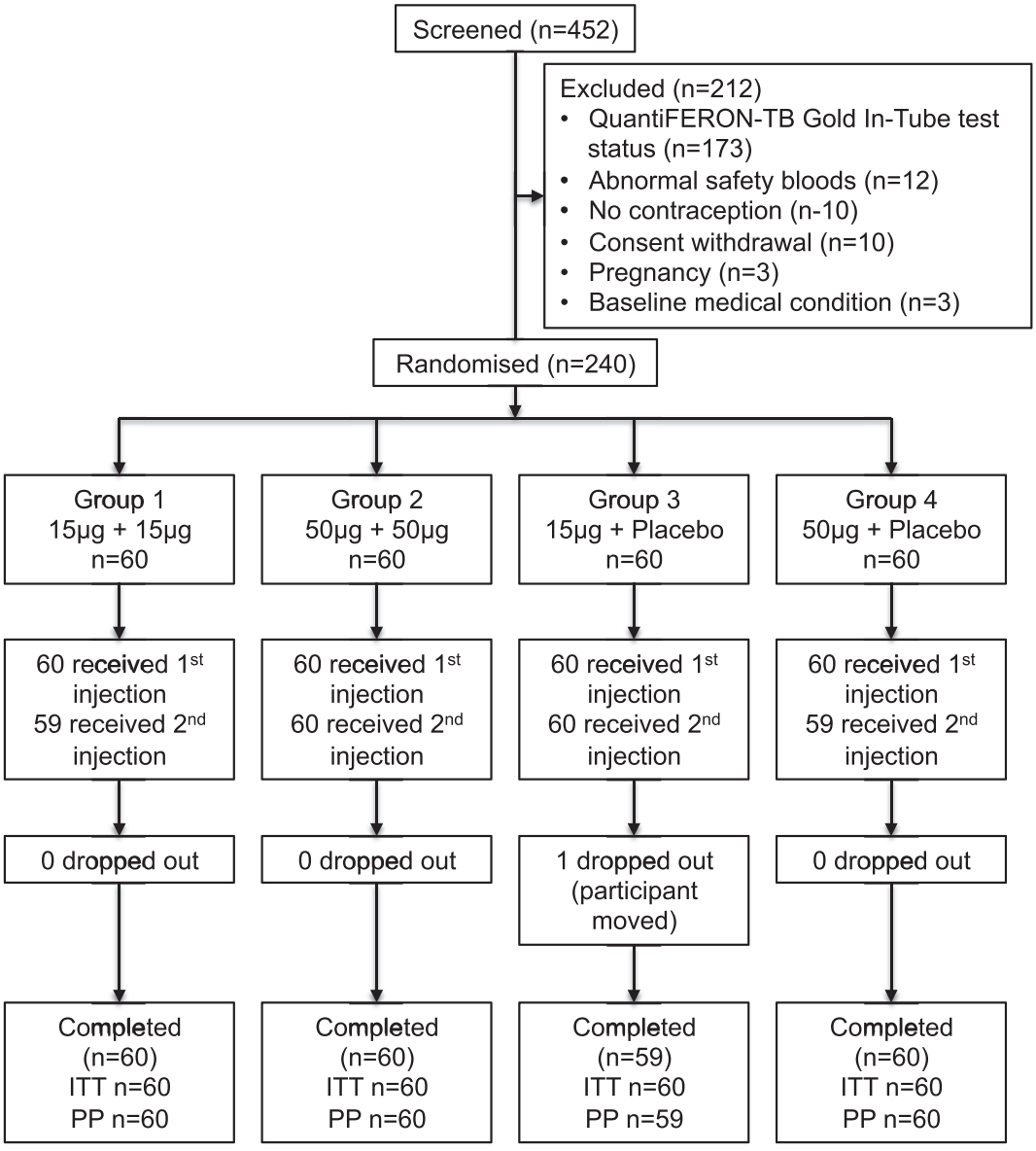

Fig. 1. Consort diagram of participants assessed for eligibility (screened), enrolled and randomized to the 4 groups and vaccinated. IC31 was at standard concentration across groups. (ITT: Intention to treat, PP: Per protocol). QFT− participants were enrolled first to allow safety assessment in uninfected adolescents before enrolment and vaccination of QFT+ participants. As a result, QFT+ participants were excluded during enrolment of the first 120 QFT− participants. Similarly, QFT− participants were excluded during enrolment of the final QFT+ 120 participants.

performed with FlowJo (v 9.6 TreeStar Inc.), Pestle (NIAID, Bethesda, USA) and Spice (NIAID, Bethesda, USA) [19]. Comparative analyses of AE frequencies between placebo and vaccine groups were performed using a chi-square test of independence. Longitudinal immune response data for each group and QFT status were modelled using linear mixed effects (LME) models, fitted with time as a polynomial trend instead of as a categorical variable. Appropriate transformations of the response variables were used to satisfy the underlying distributional assumptions. Models included interactions between the group indicator and the polynomial time trends. These mixed models estimate treatment and time effects correctly by incorporating both the between- and within-subject variability and calculate robust standard errors of treatment comparisons at different time points using information from all time points [20]. Due to the difficulty in interpreting polynomial time trends, we chose to report the results from these models as estimated means and 95% confidence intervals (CI). This representation also focuses on the estimation of trends rather than multiple comparisons of means at different times points just using p-values. Differences between timepoints and/or groups can be considered significantly different if the 95% CI did not overlap. However, this approach is subject to increased chance of false positives due to multiple comparisons. Where exact p-values were reported in figures the statistical test is indicated in the figure legend. P-values below 0.05 were considered significant. Where appropriate, the Bonferroni method was used to correct for multiple comparisons. In these cases adjusted p value thresholds are indicated in each figure legend.

3. Results

3.1. Participants

Of the 452 participants who signed consent and were screened, 212 were excluded and 240 were included in the trial (Fig. 1). The median age of participants was 15 years, 64% were female, and the majority (85%) were of mixed race (known as Coloured in South

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Table 1
Demographic and baseline characteristics by study group.

Demographics	Total n = 240	Group 1 (15 μg + 15 μg) n = 60	Group 2 (50 μg + 50 μg) n = 60	Group 3 (15 μg + Placebo) n = 60	Group 4 (50 μg + Placebo) n = 60	
Age (years)						p = 0.574
Median (IQR)	15 (14–16)	15 (14–16)	15 (14–16)	15 (14–16)	15 (14–16)	
Range	12–18	13–18	12–18	13–18	13–18	
Gender, n (%)						p = 0.143
Male	86 (36)	28 (47)	16 (27)	22 (37)	20 (33)	
Female	154 (64)	32 (53)	44 (73)	38 (63)	40 (67)	
Ethnicity, n (%)						p = 0.349
Black	26 (11)	11 (18)	3 (5)	5 (8)	7 (12)	
Caucasian	7 (3)	3 (5)	2 (3)	1 (2)	1 (2)	
Coloured	205 (85)	45 (75)	54 (90)	54 (90)	52 (86)	
Other	2 (1)	1 (2)	1 (2)	–	–	
Body mass index						p = 0.310
Median (IQR)	20 (18–23)	20 (18–23)	21 (18–25)	19 (18–23)	20 (18–23)	
Range	14–42	15–35	15–42	14–35	14–41	
QFT status, n (%)						p = 0.402
Positive	120	25	33	33	29	
Negative	120	35	27	27	31	

IQR = interquartile range; QFT = QuantiFERON status; p-value is calculated using Chi-Square test for independence or ANOVA across all four study groups.

Africa) (Table 1). The first participant was enrolled on 5 September 2012 and the final study visit was on 19 December 2013. Most exclusions were due to mismatched QFT status (for safety reasons QFT− participants were enrolled before QFT+ participants). All 240 received their first vaccination. Two participants did not receive their second vaccination; one because of concomitant unrelated anti-depressant therapy and the other due to the late discovery of participation in a previous clinical trial. None of the participants were lost to follow up, although one participant moved out of the study area and was not available for the final study visit. There were no significant differences between the four study groups in median age, gender and ethnicity distribution, or baseline body mass index (BMI) and QFT status (Table 1).

3.2. Safety and reactogenicity

Two-hundred and twenty-four (224, 93%) participants experienced at least one AE (Table 2). The number of participants with at least one AE did not differ between study groups by frequency, AE type, severity, causal relationship, outcome, baseline QFT status, or by 1st or 2nd vaccination (Table 2). The most commonly reported AEs were those at the injection site (167, 70% with at least one AE), followed by systemic AEs (157, 65%) and laboratory AEs (144, 60%). The most commonly reported injection site AE was tenderness (42% of all injection site reactions) and the most common systemic AE was headache (31%) (Supplementary Table 1). Laboratory AEs were diverse, with elevated creatinine kinase most frequently reported (41%).

Severe AEs occurred in a minority (30, 13%) of participants, of which 16 (7% of all participants) were related to vaccination. Among the latter, headache, tenderness at injection site and fatigue (8% of all severe AEs each) were most commonly reported.

No vaccine related serious AEs (SAEs) occurred. Nineteen unrelated SAEs occurred in 12 participants. The most common SAEs were suicide attempt by intentional drug overdose (4 SAE cases, 1.7%), occurring 31, 48, 50 and 167 days after most recent vaccination (Supplementary Table 2).

3.3. H1:IC31 was immunogenic at the 15 and 50 μg doses

To determine whether H1:IC31 is immunogenic and investigate the role of vaccine dose and homologous boosting, we compared T cell responses to the H1 fusion protein in QFT− and QFT+ adolescents. A positive immune response, measured by IFN-γ ELISpot assay, was defined as a post-vaccination response significantly higher than baseline. Vaccination with the 15 μg dose (Groups 1 and 3; Fig. 2A & C and Suppl. Fig. 1) boosted H1-specific responses 2 weeks post-vaccination in QFT+ and QFT− individuals. However, only QFT− individuals in Group 1 maintained these IFNγ responses above baseline until the final study time point (Fig. 2) This was not observed in any other group (Fig. 2). Vaccination with the higher dose (50 μg Groups 2 and 4) boosted H1-specific responses only in QFT− individuals at 2 weeks post-vaccination (Fig. 2). Two H1:IC31 administrations with the 50 μg dose did not result in a durable H1-specific immune response at Day 224 (Fig. 2).

The durable IFNγ responses observed in Group 1 recipients of the 15 μg dose were attributable to maintained frequencies of Ag85B-specific responses (Fig. 2). ESAT-6-specific cells, by contrast, were not maintained at frequencies exceeding pre-vaccination levels. In line with this, only 18 of 120 adolescents who were QFT− at baseline converted to QFT+ after H1:IC31 vaccination. These IFNγ ELISpot assay results suggest that both 15 μg and 50 μg of H1:IC31 are immunogenic in infected and uninfected persons. However, durable memory responses were only induced after two administrations of the 15 μg dose of H1:IC31 in QFT− individuals.

3.4. H1:IC31 vaccination induces long-lived Th1 CD4 T cells

To better understand the immune response to H1:IC31 beyond the single readout provided by IFNγ ELISpot assay, we measured co-expression patterns of the Th1 cytokines, IFN-γ, TNF-α, IL-2, as well as IL-17 by Ag85B and ESAT-6-specific CD4 and CD8 T cells using a whole blood ICS assay. H1:IC31 vaccination primarily induced antigen-specific CD4 T cells expressing the Th1 cytokines (Fig. 3, Supp. Fig. 3).

Ag85B-specific and ESAT-6-specific Th1 cell responses were maintained at levels exceeding baseline up to Day 224, but only in QFT− adolescents who received two administrations of either dose of H1:IC31 (Fig. 3A & B). By contrast, responses to either antigen were also not maintained in QFT+ adolescents, irrespective of vaccine dose (Fig. 3A & B). Similarly, single administrations of either dose of H1:IC31 did not result in responses that persisted up to Day 224 in QFT− or QFT+ adolescents.

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Table 2
Number of participants (n, %) with at least one adverse event (AE) by study group and category of AE. For percentage proportion the denominator is n in each column. For all categories, p-values were >0.6.

Category of AE	Total n = 240	Group 1 (15 μg + 15 μg) n = 60	Group 2 (50 μg + 50 μg) n = 60	Group 3 (15 μg + Placebo) n = 60	Group 4 (50 μg + Placebo) n = 60	
All AEs (n, %)	224 (93)	55 (92)	56 (93)	57 (95)	56 (93)	
AE Type (n, %)						p = 0.946
Injection site	167 (70)	44 (73)	46 (77)	39 (65)	38 (63)	
Systemic	157 (65)	44 (73)	43 (72)	31 (52)	39 (65)	
Laboratory	144 (60)	34 (57)	38 (63)	34 (57)	38 (63)	
Severity (n, %)						p = 0.898
Mild	222 (93)	54 (90)	56 (93)	57 (95)	55 (92)	
Moderate	94 (39)	26 (43)	25 (42)	22 (37)	21 (35)	
Severe	30 (13)	8 (13)	6 (10)	6 (10)	10 (17)	
Not graded	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Causality (n, %)						p = 0.686
Not vaccine related	156 (65)	41 (68)	40 (67)	39 (65)	36 (60)	
Possibly related	139 (58)	35 (58)	41 (68)	27 (45)	36 (60)	
Probably related	32 (13)	8 (13)	14 (23)	6 (10)	4 (7)	
Definitely related	167 (70)	44 (73)	46 (77)	39 (65)	38 (63)	
Outcome (n, %)						p = 0.882
Recovered	242 (101)	58 (97)	68 (113)	58 (97)	58 (97)	
On-going	56 (23)	13 (22)	15 (25)	15 (25)	13 (22)	
Unknown	2 (1)	1 (2)	0 (0)	0 (0)	1 (2)	
Baseline Quantiferon status (n, %)						p = 0.453
Negative	112 (47)	32 (53)	25 (42)	26 (43)	29 (48)	
Positive	112 (47)	23 (38)	31 (52)	31 (52)	27 (45)	
Vaccination (n, %)						p = 0.774
After first vaccination	200 (83)	51 (85)	49 (82)	50 (83)	50 (83)	
After second vaccination	190 (79)	50 (83)	53 (88)	47 (78)	40 (67)	

p-value was calculated using Chi-Square test for independence.

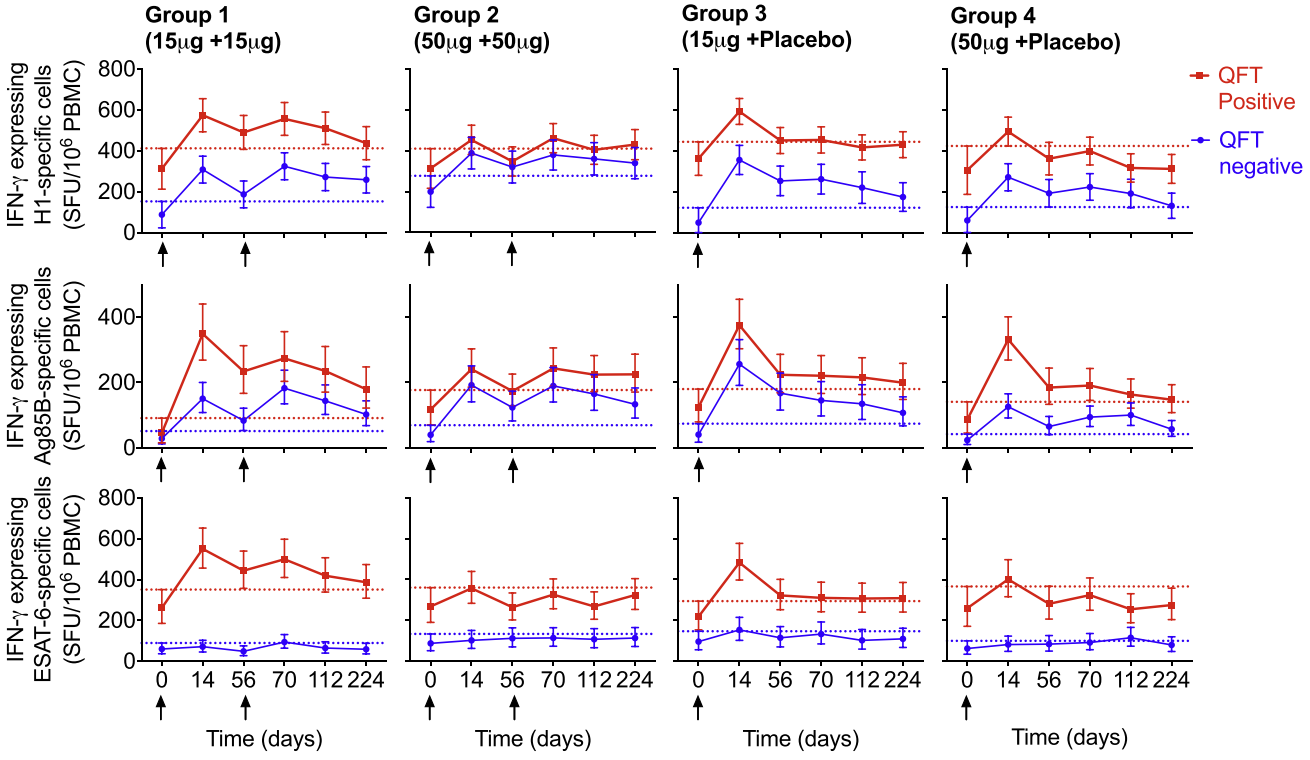

Fig. 2. H1:IC31 is immunogenic in *M.tb*-infected and uninfected adolescents. The upper panel illustrates longitudinal kinetics of frequencies of H1-specific interferon-γ (IFN-γ)-expressing cells, quantified by enzyme-linked immunospot (ELISpot) assay in adolescents who received one (Groups 3 and 4) or two (Groups 1 and 2) H1:IC31 administrations. The middle and lower panels depict Ag85B-specific (middle) and ESAT-6 specific (bottom) interferon-γ (IFN-γ)-expressing cells. Shown are means and 95% CI, modelled using linear mixed effects according to regimen, dose and QFT status. Raw (not modelled) data of the longitudinal responses are in Supplementary Fig. 1. Dashed horizontal lines represent the upper 95% CI of the baseline (day 0) response in QFT+ (red) or QFT− (blue) participants in each group. Post-vaccination responses with 95% CI (error bars) that do not cross the dashed line were considered significantly higher than the baseline. PBMC, peripheral blood mononuclear cells; SFU, spot-forming units. Arrows indicate vaccine administrations.

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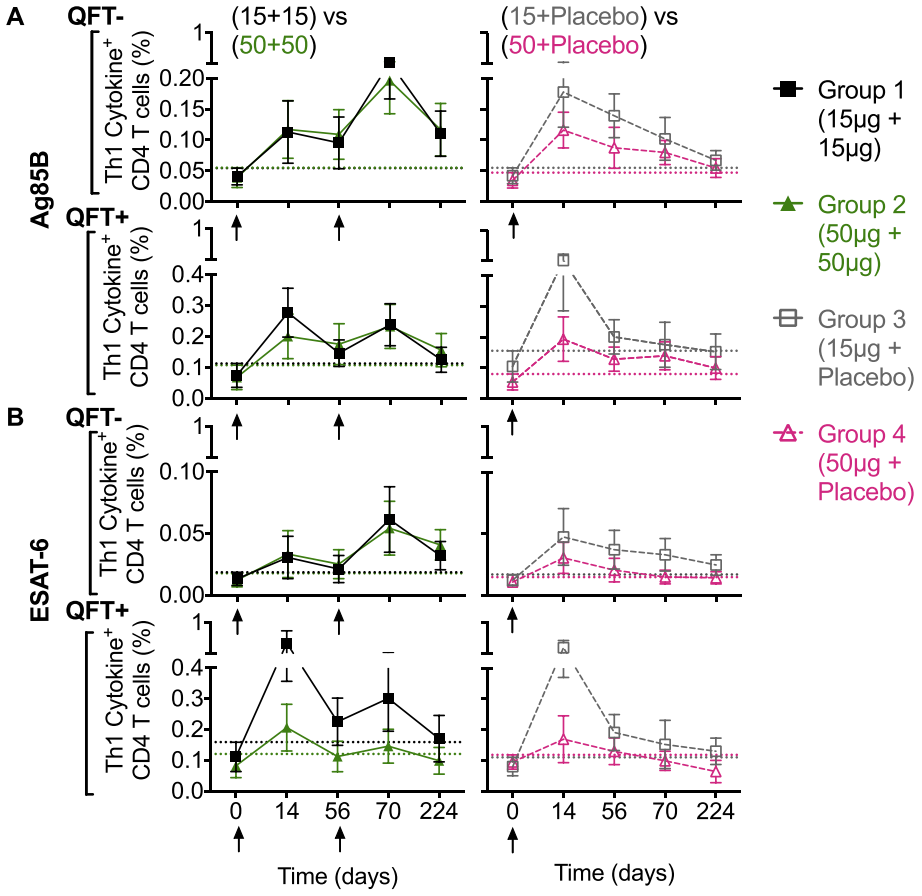

Fig. 3. H1:IC31 induces Th1 CD4 T cell responses with different magnitudes and kinetics in QFT+ and QFT− individuals. Longitudinal kinetics of H1-specific cytokine-expressing CD4$^+$ T cells, modelled using linear mixed effects. Shown are mean (error bars denote 95% CI) frequencies of Th1 cytokine$^+$ CD4 T cells expressing IFN-γ, TNF-α and/or IL-2 detected by intracellular cytokine staining following stimulation of whole blood with overlapping peptide pools of either Ag85B or ESAT-6. (A) Ag85B or (B) ESAT-6-specific CD4 T cell responses induced by one or two administrations of 15 µg or 50 µg doses of H1:IC31 in QFT+ (bottom) and QFT− (top) adolescents. Flow cytometry hierarchical gating strategy and raw data of the longitudinal responses are in Supplementary Figs. 2 and 3. Dashed horizontal lines represent the upper 95% CI of the baseline (day 0) response in each group. Post-vaccination responses with 95% CI (error bars) that do not cross the dashed line were considered significantly higher than the baseline response. Arrows indicate vaccine administrations.

Undetectable or very low, and only transiently raised, frequencies of antigen-specific IL-17-expressing CD4 T cells and Th1 cytokine-expressing CD8 T cells were detected after H1:IC31 vaccination (Supplementary Figs. 4 and 5). These responses were not analysed further.

In summary, either dose of H1:IC31, when administered twice, induced long-lived Ag85B-specific and low levels of ESAT-6-specific CD4 T cell responses in QFT− individuals, which were not observed in adolescents with underlying *M.tb*-infection.

3.5. H1:IC31 modifies the cytokine co-expression profile of CD4 T cell responses in M. tuberculosis infected and uninfected adolescents

To determine if H1:IC31 induced qualitative changes in Ag85B and ESAT-6 responses and how QFT status influenced this, we mapped the kinetics of Th1 cytokine co-expression patterns in Group 1 adolescents (Fig. 4A). Most Th1 cytokine-expressing CD4 T cell subsets increased after vaccination. However, only subsets that expressed IL-2 alone or in combination with TNF-α and/or IFN-γ were maintained up to Day 224 (Fig. 4A). This pattern was most prominent for Ag85B-specific CD4 T cells in QFT− and QFT+ adolescents, but was also observed for ESAT-6-specific cells in QFT− adolescents. Relative proportions of cytokine co-expression subsets within the Th1 cytokine$^+$ response were clearly dominated by IFN-γ-expressing CD4 T cell subsets at baseline, likely primed by BCG and/or environmental mycobacteria in QFT− persons, or by *M.tb*-infection in QFT+ persons (Fig. 4B). However, following H1:IC31 vaccination, subsets that co-expressed IL-2, especially TNF-α$^+$IL-2$^+$ CD4 T cells, were disproportionally enriched at the expense of IFN-γ-only expressing CD4 T cells up to Day 224 (Fig. 4B). These data suggest that H1:IC31 induces a qualitative change in the CD4 T cell response to one consistent with central memory cells.

When we analysed longitudinal changes in proportions of the TNF-α$^+$IL-2$^+$ CD4 T cell subset that persisted up to Day 224 in all trial study groups, increases in this Ag85B-specific cell subset were observed irrespective of H1:IC31 dose or vaccination schedule in QFT− and QFT+ participants, except for those in Group 4. By contrast, ESAT-6-specific TNF-α$^+$IL-2$^+$ CD4 T cells in QFT+ individuals remained low and unchanged following vaccination. ESAT-6-specific responses were too low in the majority of QFT−individuals for meaningful interpretation (Fig. 5).

In sum, H1:IC31 vaccination drives the differentiation of long-lived Ag85B and ESAT-6-specific CD4 T cells in participants without *M.tb* infection. However, in participants with underlying *M.tb* infection, the vaccine modified long-lived Ag85B, but not ESAT-6-specific CD4 T cell responses.

4. Discussion

We report results from a large phase II trial of the H1:IC31 vaccine candidate in BCG vaccinated, healthy *M.tb*-infected or

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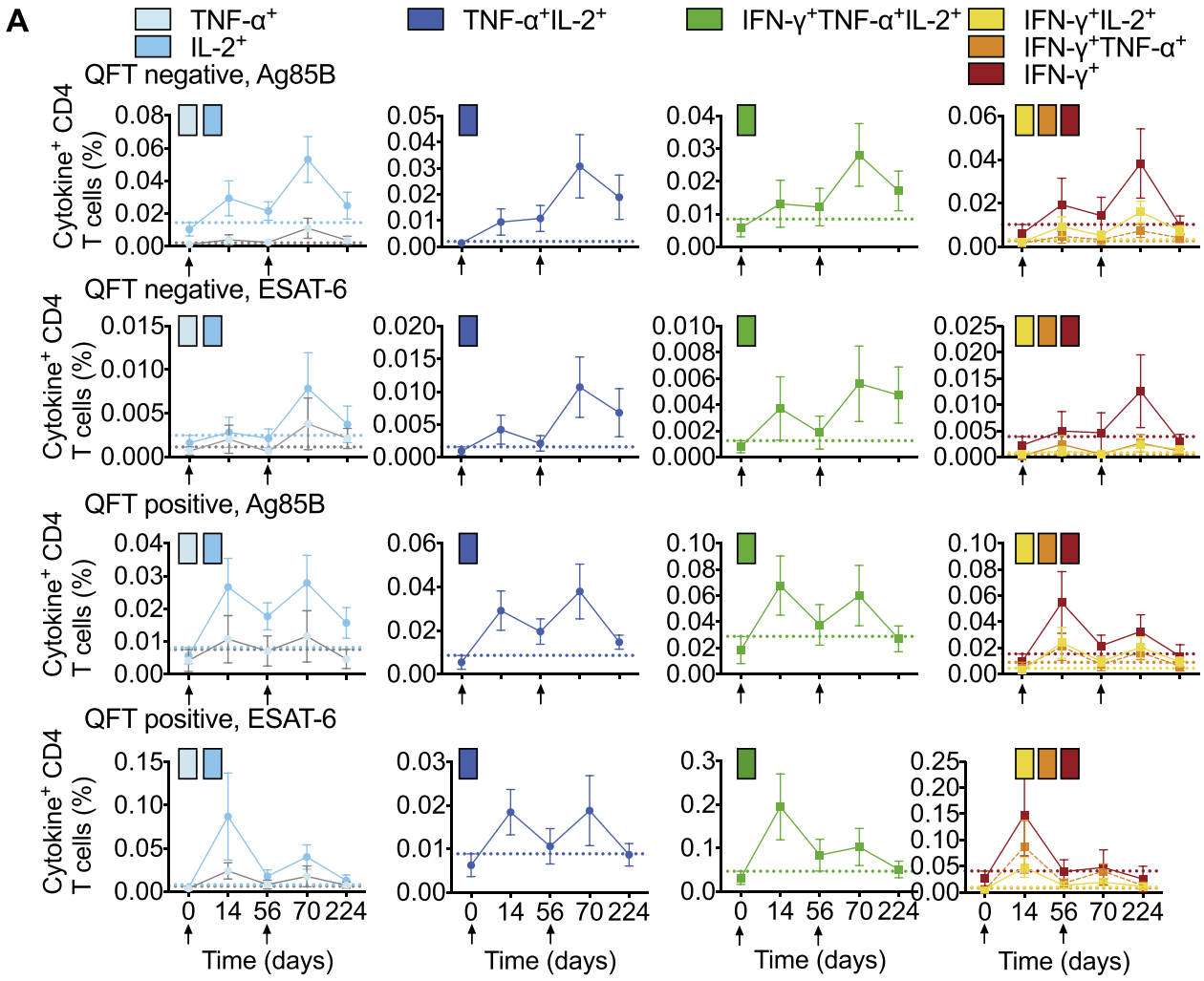

Fig. 4. H1:IC31 induces qualitative changes in cytokine-expressing antigen-specific CD4 T cells. (A) Longitudinal kinetics of the different Th1 cytokine-expressing subsets of Ag85B or ESAT-6-specific CD4$^+$ T cells. Mean frequencies (error bars denote 95% CI), modelled using linear mixed effects are shown in Group 1 participants, who received 2 vaccinations of 15 μg H1:IC31 on days 0 and 56. Dashed horizontal lines represent the upper 95% CI of the baseline (day 0) response for each cytokine-expressing cell subset. Post-vaccination responses with 95% CI (error bars) that do not cross the dashed line were considered significantly higher than the baseline response. Arrows indicate vaccine administrations. (B) Pie charts representing median proportions of Th1 cytokine co-expressing cells subsets for QFT− or QFT+ adolescents in Group 1 at baseline (day 0) or at the end of follow-up (day 224). Exact p values, determined by Wilcoxon signed rank test ($p < 0.05$), are shown in the table. P-values below 0.002 were considered significant after adjustment for multiple comparisons using the Bonferroni method, indicated in red in the table and as asterisks in the pie charts. No pie chart is shown for ESAT-6 responses in QFT− participants because their responses were too low to accurately calculate proportions.

uninfected adolescents from a TB endemic setting. The vaccine was well tolerated and had an acceptable safety profile. No differences in local or systemic adverse events were noted between *M.tb*-infected and uninfected adolescents and neither vaccine dose nor vaccination schedule had a marked effect. H1:IC31 was immunogenic and predominantly induced long-lived CD4 T cells that co-expressed IFN-γ, TNF-α and/or IL-2 against Ag85B, and to a lesser extent ESAT-6. Vaccine dose (15 μg or 50 μg) had a minimal effect on the magnitude and longevity of the Th1 response, however two H1:IC31 vaccinations resulted in greater long-term

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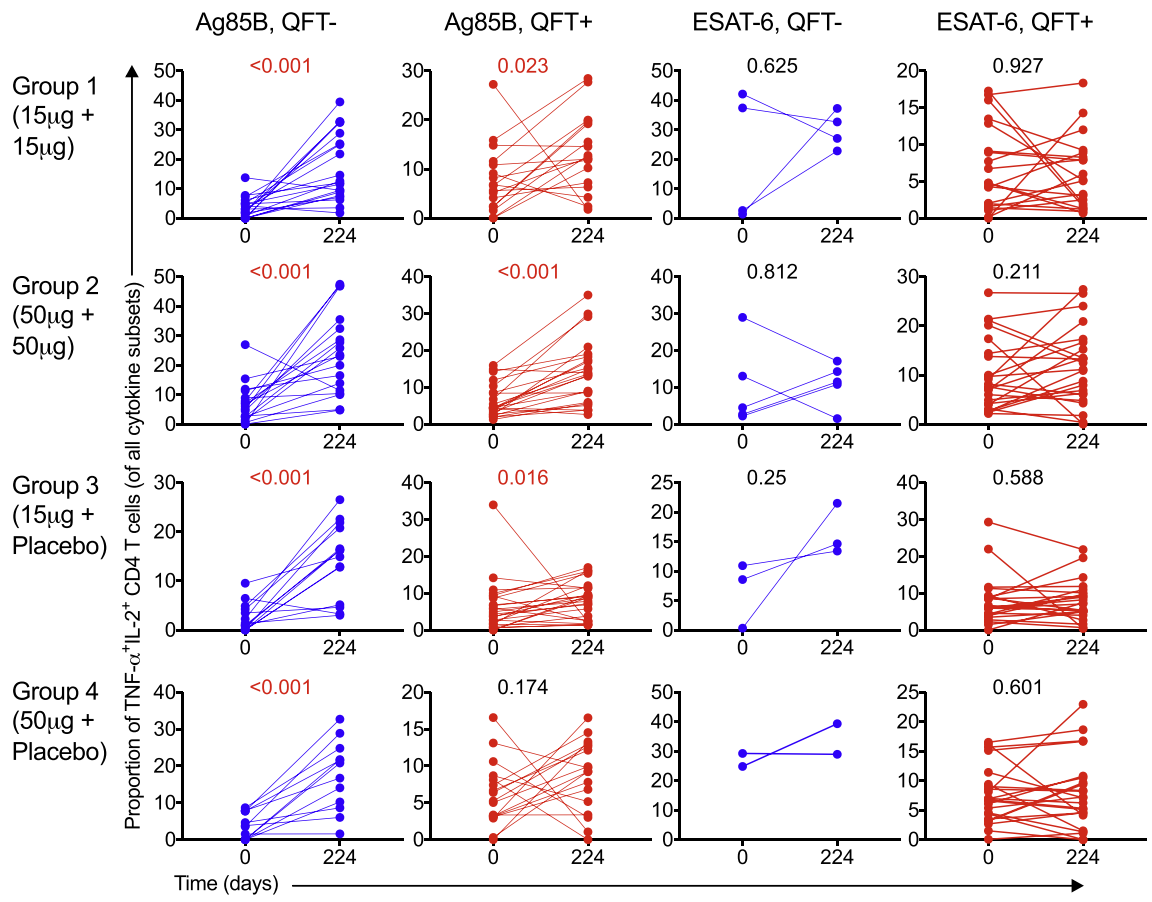

Fig. 5. H1:IC31 vaccination induces long-lived Ag85B-specific TNF-α^+IL-2^+ CD4 T cells. Relative proportions of Ag85B and ESAT-6 specific TNF-α^+IL-2^+-expressing CD4 T cells out of the total Th1 cytokine$^+$ CD4 T cell responses measured at baseline (day 0) and final study time point (day 224) in Groups 1–4, stratified by QFT status. P-values were determined by Wilcoxon-matched pairs signed rank test and a $p < 0.05$ was considered significant, indicated in red.

maintenance of antigen-specific T cell frequencies, up to 224 days following vaccination. Our data suggest that the favoured vaccination strategy in an adolescent population would be two H1:IC31 vaccinations at the 15 μg dose.

Participant retention of the 240 adolescents was high throughout the trial. Frequencies of adverse events in this large study population were consistent with previous trials of the H1:IC31 vaccine, and of the related H4:IC31 and H56:IC31 fusion protein vaccine candidates [11,13–15,21]. Most systemic adverse events were for headache, fatigue and myalgia (31%, 20%, and 16% respectively, supplementary Table 1) and there was no trend for abnormalities in safety laboratory tests. This is consistent with previous experience with this and other subunit adjuvanted vaccines and likely represents a mild transient flu-like vaccination response [10,11,15,21]. The favourable safety profile is notable in this study population with high levels of *M.tb* exposure, a very high prevalence of *M.tb* infection, and an annual risk of TST or QFT conversion as high as 14% [22]. QFT status did not affect the frequency of AEs, as had been noted in an adolescent trial of another adjuvanted protein vaccine, M72/AS01$_E$ [10]. There were no reported episodes of bradycardia, which had been reported in five out of 25 participants in a recent adult trial of the H56:IC31 vaccine [11].

Given the large global population of *M.tb*-infected persons, a critical component of the interventions to eliminate TB by 2050 [4,23] is a vaccine candidate suitable for individuals with and without pre-existing *M.tb* infection. Our data showed that adolescents with *M.tb* infection had both a more rapid and higher magnitude of specific T cell responses after H1:IC31 vaccination, compared with QFT− adolescents. This was not surprising given that QFT+ individuals typically have higher and more activated T cell responses at baseline, likely due to ongoing *in vivo* exposure to *M.tb* antigens.

H1:IC31-primed low levels of de novo ESAT-6-specific CD4 T cells in QFT− adolescents without prior *M.tb* infection and 18 of the 120 QFT− adolescents converted to QFT+ after vaccination. It is difficult to discern whether these conversions resulted from new *M.tb* infections or if these conversions were vaccine induced. An ESAT-6-free IGRA currently under development at the Statens Serum Institut will be of value in future trials of ESAT-6-containing vaccines. The effects of *M.tb* infection on magnitude and kinetics of antigen-specific T cell responses were consistent with those observed after H56:IC31 vaccination of adults and M72/AS01$_E$ vaccination of adolescents from the same region [10,11].

Prior *M.tb* infection also had an impact on the character of the immune response. Adolescents with prior *M.tb* infection had lower proportions of IL-2-expressing, and high proportions of IFN-γ-expressing, antigen-specific CD4 T cells following vaccination when compared with *M.tb*-uninfected adolescents. These data confirm previous findings from a similar trial of H56:IC31 [11] and suggest that underlying *M.tb* infection is associated with CD4 T cell differentiation from an IL-2-expressing central memory response to an effector memory/effector response [24]. It was notable that relative proportions of TNF-α^+IL-2^+ cells within the cytokine-expressing CD4 T cell response increased consistently over time after H1:IC31 vaccination. Such a TNF-α^+IL-2^+ CD4 T cell subset

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was also induced by H1:CAF01 vaccination in mice and had characteristics consistent with long-lived central memory cells [25]. In this model, the vaccine-induced T cell response was associated with long-lived protective immunity against *M.tb* in pre-exposure [25–27] as well as post-exposure mouse models [28].

We also observed marked differences in the response kinetics, longevity and cytokine co-expression profile between H1:IC31-induced Ag85B and ESAT-6-specific CD4 T cells; differences particularly pronounced in *M.tb*-infected adolescents. This finding is consistent with a detailed transcriptional, functional and phenotypic analysis of Ag85B and ESAT-6-specific CD4 T cells performed on samples from Group 1 of the trial presented here. These analyses suggested that on-going ESAT-6-expression by *M.tb* in QFT+ persons chronically stimulates ESAT-6-specific CD4 T cells to a more differentiated state than Ag85B (Moguche AO and Musvosvi M. et al., unpublished). Taken together, our data suggest that it may be particularly challenging to modify ESAT-6-specific CD4 T cells in persons with underlying *M.tb* infection through vaccination. These data suggest that ESAT-6 may be more effective as a vaccine antigen in QFT− than in QFT+ individuals.

In some previous clinical trials of fusion or polyprotein vaccines immune responses to individual mycobacterial antigens have not been measured separately, but instead as a combined response to the polyprotein. Our data showing distinct characteristics of Ag85B and ESAT-6-specific responses illustrate the importance of characterizing T-cell responses to individual antigens separately.

In conclusion, the H1:IC31 vaccine was safe and well tolerated and adverse event profiles were not affected by dose or vaccination schedule in healthy QFT-positive and QFT-negative adolescents. Our results suggest that 2 administrations of the 15 μg dose of H1:IC31 may be the optimal strategy to secure immunity in the target population including *M.tb*-infected and uninfected adolescents.

Conflict of interest

The authors declare no conflict of interest. The authors do not have a commercial or other association that might pose a conflict of interest.

Meetings where this information has been presented

Keystone Symposia: Host Response in Tuberculosis, January 22–27, 2015. Santa Fe, New Mexico, USA. Poster Presentation.

Keystone Symposia: Cell Biology and Immunology of Persistent Infection, January 31-February 4, 2016, Banff, Alberta, Canada. Poster Presentation.

5th Conference of the South African Immunology Society, March 6–9, 2016. Johannesburg, South Africa. Oral Presentation.

Contributions

HM and HG contributed equally to this work.
SSI was the sponsor of the study

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Author's contributions

HDG, HMo, WAH, STH, IK, PB, MH, PA and TJS designed the study, HDG, FR conducted the clinical work, ES, YB, AK, ME, LM, DAA, EM, VR, BMK, HMe, MM and TJS performed the immunology assays and analyses, KM, FL, HMe and HDG performed statistical analyses, and HMe drafted the article and revised it, HDG, STH, MR, IK, PB and TJS critically revised the manuscript. All authors reviewed and approved the final manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.11.023.

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