Vaccine 33 (2015) 4130–4140

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

First-in-human trial of the post-exposure tuberculosis vaccine H56:IC31 in *Mycobacterium tuberculosis* infected and non-infected healthy adults

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 7 June 2015
Accepted 8 June 2015
Available online 19 June 2015

Keywords:
Tuberculosis
Vaccine
H56
Latent
Clinical trial

ABSTRACT

Background: H56:IC31 is a candidate tuberculosis vaccine comprising a fusion protein of Ag85B, ESAT-6 and Rv2660c, formulated in IC31 adjuvant. This first-in-human, open label phase I trial assessed the safety and immunogenicity of H56:IC31 in healthy adults without or with *Mycobacterium tuberculosis* (*M.tb*) infection.
Methods: Low dose (15 μg H56 protein in 500 nmol IC31) or high dose (50 μg H56, 500 nmol IC31) vaccine was administered intramuscularly thrice, at 56-day intervals. Antigen-specific T cell responses were measured by intracellular cytokine staining and antibody responses by ELISA.
Results: One hundred and twenty-six subjects were screened and 25 enrolled and vaccinated. No serious adverse events were reported. Nine subjects (36%) presented with transient cardiovascular adverse events. The H56:IC31 vaccine induced antigen-specific IgG responses and Th1 cytokine-expressing $CD4^+$ T cells. *M.tb*-infected vaccinees had higher frequencies of H56-induced $CD4^+$ T cells than uninfected vaccinees. Low dose vaccination induced more polyfunctional (IFN-γ^+TNF-α^+IL-2^+) and higher frequencies of H56-specific $CD4^+$ T cells compared with high dose vaccination. A striking increase in IFN-γ-only-expressing $CD4^+$ T cells, displaying a $CD45RA^-CCR7^-$ effector memory phenotype, emerged after the

Abbreviations: M.tb, *Mycobacterium tuberculosis*; TB, tuberculosis.

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http://dx.doi.org/10.1016/j.vaccine.2015.06.051
0264-410X/

second high-dose vaccination in *M.tb*-infected vaccinees. TNF-α^+IL-2^+ H56-specific memory $CD4^+$ T cells were detected mostly after low-dose H56 vaccination in *M.tb*-infected vaccinees, and predominantly expressed a $CD45RA^-CCR7^+$ central memory phenotype. Our results support further clinical testing of H56:IC31.

1. Introduction

Bacillus Calmette–Guerin (BCG) is widely used to prevent severe tuberculosis (TB) disease in infants [1], yet the global TB burden remains extremely high. One-third of the global population is thought to harbour latent *Mycobacterium tuberculosis* (*M.tb*) infection [2] representing a massive reservoir of potential TB disease. New and effective vaccination strategies, particularly those targeting the *M.tb*-infected, are required to significantly impact the epidemic [3]. Epidemiological modelling suggests that TB elimination may be best achieved with an effective vaccination strategy, coupled with better diagnostics and more effective treatment of TB patients [4].

Fourteen novel vaccine candidates have reached clinical phases of development. Amongst these is H56:IC31, a novel subunit vaccine consisting of an Ag85B, ESAT-6 and Rv2660c fusion protein formulated in IC31 [5] a two-component adjuvant of anti-microbial peptide (KLK) and oligodeoxynucleotide (ODN1a), a Toll-like receptor nine (TLR9) agonist [6]. H56:IC31 is designed as a post-exposure vaccine specifically targeted at the *M.tb*-infected population. Preclinical studies show that IC31 polarizes naïve T cells to Th1 cells [7], which are thought to be important in immunity against *M.tb*. Preclinical and clinical studies with similar IC31-adjuvanted vaccines have shown acceptable safety [8,9].

The antigens in H56 were selected to target several stages of the host–pathogen interaction. *M.tb* possesses several mechanisms to survive the harsh intracellular environment of the macrophage [10] and Ag85B [11,12], ESAT-6 [13] and Rv2660c [14] are thought to be necessary for intracellular survival. The gene encoding Ag85B but not ESAT-6 is present in BCG [15]. Although Rv2660c was originally found to be absent from BCG by DNA microarray hybridization [16], the locus was recently found to be present by analysis of genome sequences [15]. The mycolyl transferase Ag85B (Rv1886c) belongs to a family of proteins with fibronectin-binding capacity that enhance complement receptor-mediated *M.tb* phagocytosis [17]. Ag85B is immunogenic and frequently targeted by T cells [18]. ESAT-6 (Rv3875) belongs to the region of difference (RD1) family of *M.tb* proteins, encoded within the Esx-1 region, a type VII secretion system [19]. ESAT-6 is highly immunogenic and contains multiple T cell epitopes [20]. Increased Rv2660c transcripts in intracellular *M.tb* under nutrient stress suggest that Rv2660c contributes to intracellular survival [21]. Inclusion of Rv2660c in fusion protein constructs of ESAT-6 and Ag85B significantly enhanced vaccine-induced protection against *M.tb* in mice [5]. Low-level T cell responses to Rv2660c were reported in *M.tb*-infected macaques [14]. Humans with latent *M.tb* infection had higher Rv2660c-specific T cell responses than patients with TB disease [22,23]. *In vivo* activity of the Rv2660c protein is the subject of ongoing work [23–25].

Murine vaccination with H56 formulated with the Th1 adjuvant, CAF01 (without BCG prime), induced highly polyfunctional CD4 T cells that conferred better containment of late-stage *M.tb* infection than BCG [5]. In a latent *M.tb* model, H56:CAF01 vaccination after *M.tb* infection controlled reactivation and significantly lowered bacterial load compared with control mice that received adjuvant [5]. Despite these promising results in mice, the IC31 adjuvant has been the preferred adjuvant for clinical use and is at a more advanced stage of clinical development. In cynomolgus macaques, a BCG-prime, H56:IC31-boost strategy delayed clinical disease and reduced pathology and prevented reactivation of latent *M.tb* infection upon treatment with anti-TNF-α antibody [14].

In this first-in-human clinical trial, we assessed safety and tolerability of H56:IC31 and vaccine-induced T cell responses in healthy adults without or with *M.tb* infection.

2. Materials and methods

2.1. Study design and regulatory approvals

We conducted an open label phase I trial at the South African Tuberculosis Vaccine Initiative (SATVI) clinical site near Cape Town, South Africa. The trial was conducted in accordance with the Helsinki Declaration and Good Clinical Practice. The Medicines Control Council (MCC) of South Africa and the Human Research Ethics Committee of the University of Cape Town approved the study protocol (UCT HREC179/2011) and all subsequent amendments. The trial was registered on ClinicalTrials.gov (NCT01967134).

2.2. Study population

We enrolled healthy adults aged between 18 and 59 years. For inclusion, participants had to be HIV-negative, generally healthy, with no history of chronic medical conditions. Screening procedures included a medical history, physical examination and blood collection for baseline chemistry, haematology, and Hepatitis B and C serology. Adults included the study were presumed to be BCG immunized as BCG vaccination became compulsory at birth in South Africa in 1973 [26]. Participants with a reported TB contact or abnormal laboratory results were excluded. A chest X-ray was performed to exclude those with TB disease. We used the QuantiFERON Gold-In-Tube test (QFT, Cellestis Limited) to detect *M.tb* infection. H56:IC31 was first administered to QFT-negative participants and, after review of safety data, to QFT-positive participants.

2.3. Vaccinations

The H56 protein was formulated in a constant IC31 concentration of 500 nmol KLK and 20 nmol ODN1a. Three intramuscular H56:IC31 vaccinations were administered at 56-day intervals in the deltoid region of alternating arms.

Participants were stratified into three groups according to baseline QFT results. Group 1 comprised QFT-negative participants who received a dose of 50 μg of H56 protein in IC31 (denoted as 50/500). Participants in Groups 2 and 3 were QFT-positive and received 15 μg and 50 μg of H56 protein in IC31 (15/500; 50/500), respectively.

2.4. Follow-up and safety evaluation

Enrolment into each subsequent group was dependent on a satisfactory safety review from the previous group. A maximum of 3 individuals were vaccinated per day. The first two vaccinees in each group were observed for 180 min after each vaccination. Subsequently, vaccinees were observed for 60 min. For the first and second vaccination, vaccinees were evaluated for safety after 2, 7,

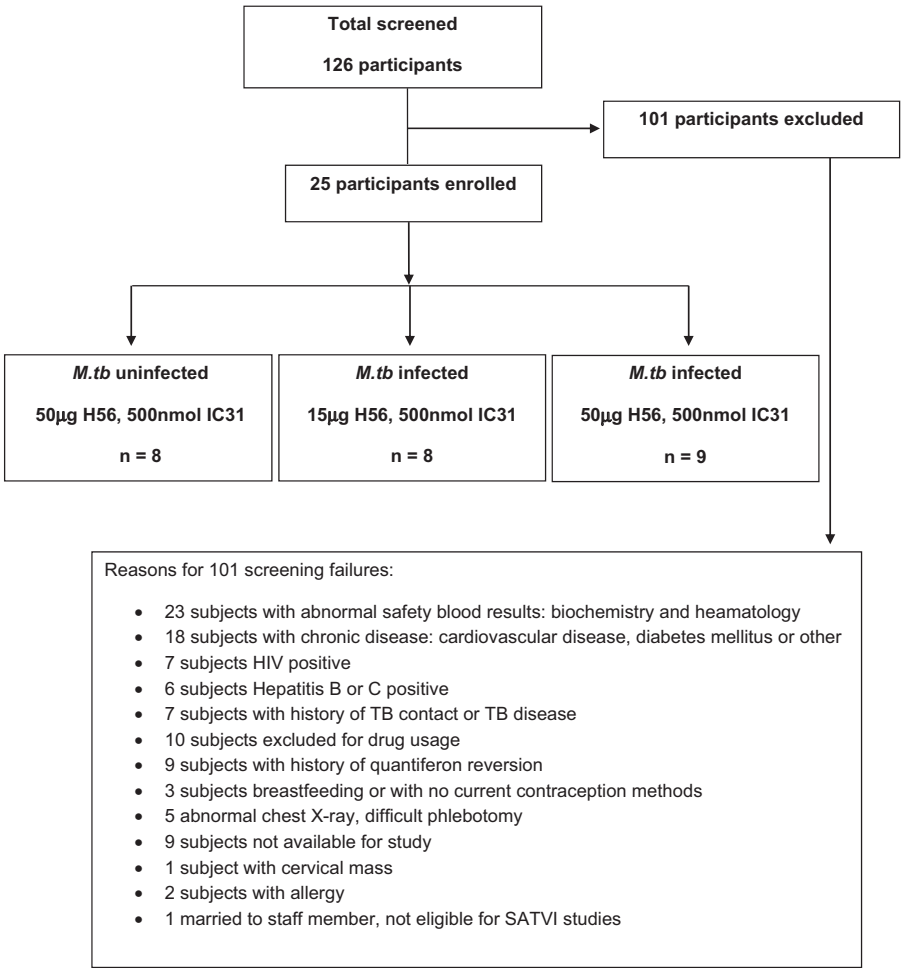

Fig. 1. Consort diagram of the participants assessed for eligibility, enrolled and vaccinated.

14, and 28 days. For the third vaccination, safety follow-up was on days 7, 14, 28 and 98. Participants were given a thermometer and diary card to record any AEs. Solicited and unsolicited AEs were recorded by study nurses until day 28 post-vaccination and vaccine-relatedness was assessed by the study physician. Blood and urine samples were collected at predefined visits for serum chemistry and haematology, as well as urinalysis. All vaccinees were followed up for safety outcomes for 210 days.

2.5. Immunogenicity evaluation

Venous blood was collected for immunology at baseline and on days 14, 56, 70, 112, 126 and 210. Immunogenicity assays included IgG antibody ELISA and whole blood intracellular cytokine staining assay (WB-ICS). Detailed methods are available as supplementary data.

2.6. Data analysis

Analyses were performed using MS Excel or GraphPad Prism (v.5.0a). To calculate frequencies of participants with H56-specific IgG antibody responses, we applied a responder cut-off derived as the mean EC_{50} of IgG responses measured before vaccination, plus 3 standard deviations.

Analyses of flow cytometry data were performed using FlowJo (9.6.2, TreeStar) using predesigned gating template to yield predefined outcomes. Detailed analysis procedures are available as supplementary data.

3. Results

3.1. Participants

Between September 2011 and January 2012 we screened 126 adults and enrolled 25. The main reasons for screening failures were abnormal safety blood results and chronic diseases (Fig. 1). We enroled 8 *M.tb*-uninfected participants into Group 1; and 8 and 9 *M.tb*-infected participants into Groups 2 and 3, respectively. Participants in all groups were either Black African or mixed race and the gender distribution was similar. However, Group 2 had more females than the other groups (Table 1).

All participants completed the scheduled study visits, with the exception of one in Group 2, who was lost to follow up after 119 days. Three participants did not receive the 3rd vaccine dose, either because of prior adverse events (pulse <55 bpm on vaccination day and large swelling at the injection site for a subject in Groups 1 and 3, respectively), or, in the other case, due to prednisone treatment for acute asthma (Group 2). The large injection site AE reported had a diameter of swelling and redness of 70 mm, which resolved within 72 h, with no lymphadenopathy noticed.

3.2. Safety

AEs were graded using the FDA toxicity grading scale for healthy volunteers as mild, moderate or severe and assessed for their relationship to vaccination. All participants experienced at least one AE within 28 days post-vaccination. More participants reported

Table 1
Demographic characteristics of enrolled participants.

	Group 1 *M.tb* uninfected participants: *n* = 8	Group 2 *M.tb* infected participants: *n* = 8	Group 3 *M.tb* infected participants: *n* = 9
Dose	50 μg H56/500 nmol IC31	15 μg H56/500 nmol IC31	50 μg H56/500 nmol IC31
Male, *n* (%)	4 (50%)	2 (25%)	4 (44%)
Median age in years (range)	32 (19–38)	30.5 (22–47)	29 (22–43)
Ethnicity			
Black African	3 (37.5%)	1 (12.5%)	1 (11.1%)
Mixed race	5 (62.5%)	7 (87.5%)	8 (88.9%)
Median BMI Kg/m^2 (range)	25.1 (21.0–35.0)	27.3 (20.0–38.0)	25.2 (17.4–34.8)

local AEs after the second vaccination in all study groups (Table 2). No serious AEs were reported. No differences in AE incidence or severity between *M.tb*-infected and uninfected participants were observed.

Flu-like symptoms were reported after vaccination. Fever was not reported (all axillary temperatures recorded were below 38 °C). The most common AEs were injection site pain (18), injection site warmth (17), fatigue (9), headache (8), injection site swelling (7), upper respiratory tract infection (7), and red blood cells in the urine (6). The median sizes of swelling after the 3 doses at the injection site were 10 mm (minimum 10; maximum 10), 8 mm (4;10) and 6.5 mm (4;70) in Groups 1, 2 and 3, while redness was 5 mm (2;8), 3 mm (3;4) and 5 mm (4;70) in Groups 1, 2 and 3, respectively. Other AEs included abnormalities of laboratory safety results, with none definitely vaccine-related (Table 2 and Supplementary Table 1). AEs for most subjects were mild or moderate in severity. At the H56 dose of 50 μg/500 nmol, *M.tb*-uninfected participants experienced more AEs than *M.tb*-infected participants (Table 3). These AEs were decreased haemoglobin, increased ALT, chills, injection site erythema, and sinusitis.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.06.051

Five participants demonstrated bradycardia within 60 min post-vaccination (Table 2 and Supplementary Table 1), three in Group 1 and two in Group 2. All bradycardia cases were asymptomatic and four occurred after the first vaccination in different individuals. One episode of severe bradycardia (<45 bpm) occurred in Group 1. In all participants, bradycardia resolved within 3 h post vaccination. A case of severe hypertension (159/100 mm/Hg) was also reported in an *M.tb*-infected participant in Group 2 (who did not have bradycardia), which was asymptomatic. All bradycardia cases were followed

Table 2
Participants with solicited and unsolicited adverse events within 28 days of vaccination, irrespective of relatedness to the vaccine.

	M.tb uninfected; H56 50/500			*M.tb* infected; H56 15/500			*M.tb* infected; H56 50/500		
	Dose 1 *N* = 8	Dose 2 *N* = 8	Dose 3 *N* = 7	Dose 1 *N* = 8	Dose 2 *N* = 8	Dose 3 *N* = 7	Dose 1 *N* = 9	Dose 2 *N* = 9	Dose 3 *N* = 8
Participants with at least 1 AE, *n* (%)	8 (100)	7 (87.5)	6 (85.7)	8 (100)	7 (87.5)	7 (87.5)	9 (100)	8 (88.9)	4 (50)
Participants with local injection site AEs (number of participants with AEs/total number of participants in group, %)									
Erythema	-	-	-	-	-	-	1 (11)	1 (11)	-
Pain	4 (50)	4 (50)	4 (57.1)	6 (75)	6 (75)	2 (28.6)	3 (33.3)	6 (66.7)	-
Pruritus	-	1 (12.5)	-	-	-	-	-	-	-
Swelling	1 (12.5)	-	-	1 (12.5)	1 (12.5)	-	3 (33.3)	2 (22.2)	-
Ulceration	-	-	-	-	-	-	-	-	-
Drainage	-	-	-	-	-	-	-	-	-
Warmth	4 (50)	2 (25)	2 (28.6)	3 (37.5)	5 (62.5)	-	4 (44.4)	-	-
Participants with systemic AEs (number with AEs/total number of participants in group, %)									
Infections	-	1 (12.5)	2 (28.6)	3 (37.5)	3 (37.5)	3 (42.9)	1 (11)	1 (11)	1 (12.5)
Rhinitis	-	1 (12.5)	1 (14)	2 (25)	-	1 (14)	-	-	-
Upper respiratory infections	-	--	1 (14)	1 (12.5)	3 (37.5)	1 (14)	1 (11)	-	-
Nervous System disorders	3 (37.5)	2 (25)	1 (14)	3 (37.5)	1 (12.5)	1 (14)	1 (11)	-	3 (37.5)
Dizziness	2 (25)	-	-	1 (12.5)	-	-	-	-	-
Headache[a]	2 (25)	2 (25)	1 (14)	1 (12.5)	1 (12.5)	1 (14)	1 (11)	-	3 (37.5)
Hypoesthesia	-	-	-	2 (25)	-	-	-	-	-
Cardiac disorders	2 (25)	2 (25)	-	2 (25)	-	-	-	-	-
Bradycardia[a]	2 (25)	2 (25)	-	2 (25)	-	-	-	-	-
Vascular disorder	3 (37.5)	1 (12.5)	1 (14)	2 (25)	-	-	1 (11)	-	-
Hot flush	1 (12.5)	-	-	-	-	-	-	-	-
Hypertension[a]	2 (25)	1 (12.5)	1 (14)	2 (25)	-	-	1 (11)	-	-
Gastro intestinal disorder	2 (25)			2 (25)	2 (25)	2 (28.6)	1 (11)	-	-
Musculoskeletal and connective tissue disorder	2 (25)	2 (25)	-	4 (50)	1 (12.5)	1 (14)	1 (11)	1 (11)	
Arthralgia	-	1 (12.5)	-	3 (37.5)	-	-	-	-	-
Back Pain	-	-	-	-	-	-	-	1 (11)	-
Joint stiffness	-	1 (12.5)	-	-	-	-	-	-	-
Musculoskeletal pain	-	-	-	-	-	-	1 (11)	-	-
Myalgia	2 (25)	-	-	[a]2 (25)	1 (12.5)	1 (14)	1 (11)	-	-
Pain in extremity	-	-	-	1 (12.5)	-	-	-	-	-
General disorders	3 (62.5)	3 (62.5)	1 (57.1)	2 (87.5)	1 (87.5)	4 (57.1)	2 (77.8)	1 (66.7)	-
Chills	-	-	-	-	-	-	2 (22)	-	-
Fatigue	3 (37.5)	3 (37.5)	1 (14)	2 (25)	1 (12.5)	4 (57.1)	2 (22)	1 (11)	-
Pyrexia	-	-	-	-	-	-	-	-	-
Laboratory abnormalities	5 (62.5)	5 (62.5)	-	-	-	3 (42.9)	3 (33.3)	3 (33.3)	1 (12.5)

[a] Participant with more than one AEs in the same category.

Table 3
Solicited and unsolicited adverse events graded by severity and assessed for vaccine relatedness within 28 days of vaccination.

	M.tb uninfected H56 50/500 *n* = 8	*M.tb* infected H56 15/500 *n* = 8	*M.tb* infected H56 50/500 *n* = 9
Total number of AEs (*n* = 225)	78 (35%)	88 (39%)	59 (26%)
Severity (number of AEs/total AEs in each group, %)			
Mild	68 (87)	85 (97)	51 (86)
Moderate	9 (12)	2 (2)	8 (14)
Severe[a]	1 (1)	1 (1)	0
Local and systemic adverse event in each group (n,%)			
Local	22 (28)	30 (38)	28 (35)
Systemic	56 (39)	58 (40)	31 (21)
Relationship to the vaccine (number of AEs/total AEs in each group, %)			
Related	23 (29)	30 (34)	26 (44)
Possibly related	29 (37)	26 (30)	22 (37)
Probably related	2 (3)	1 (1)	0
Unlikely related	21 (27)	29 (33)	11 (19)
Not related	3 (4)	2 (2)	0

[a] Severe: Hypertension (159/100 mm/Hg) and Bradycardia (43 bpm).

up by a physician and found to be healthy. The bradycardia and hypertension reactions were judged possibly vaccine-related, were transient, and resolved spontaneously within 24 h.

3.3. H56:IC31 induces antigen-specific IgG responses

To determine if H56:IC31 vaccination induces antigen-specific antibody responses we measured serum IgG levels against recombinant H56 (Ag85B-ESAT-6-Rv2660c) protein. Only one vaccinee in Group 1 and one in Group 3 had low-level, detectable H56-specific IgG after the first vaccination (Fig. 2A). The second H56 vaccination boosted the number of responders amongst *M.tb*-infected vaccinees in Groups 2 and 3 to five (63%) and six (67%), respectively, but not in *M.tb*-uninfected vaccinees in Group 1 (Fig. 2B). After the third H56 vaccination, serum H56-specific IgG had increased markedly and responder frequencies increased to 63%, 86% and 78% in Groups 1, 2 and 3, respectively (Fig. 2C and D).

3.4. H56 induces antigen-specific T cell responses

We also sought to determine if H56 induced a specific T cell response. We quantified antigen-specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, IL-2 and/or IL-17 by intracellular cytokine staining before and after vaccination (Supplementary Fig. 1). After restimulation with H56 fusion protein, Ag85B or ESAT-6 peptide pools, frequencies of $CD4^+$ T cells expressing any combination of cytokines (total $cytokine^+$ response), increased after vaccination in all groups and remained higher than pre-vaccination levels up to study day 210 (Fig. 3A–C). A notable feature was that H56-specific $CD4^+$ T cell responses in most QFT-positive participants increased rapidly and peaked on day 14. By contrast, in most QFT-negative participants H56-specific $CD4^+$ T cell responses peaked after the second vaccination at day 70.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.06.051

The response to Rv2660c was not durable; frequencies of total $cytokine^+$ Rv2660-specific $CD4^+$ T cells appeared to increase in some vaccinees after the first, second or third vaccination to low but

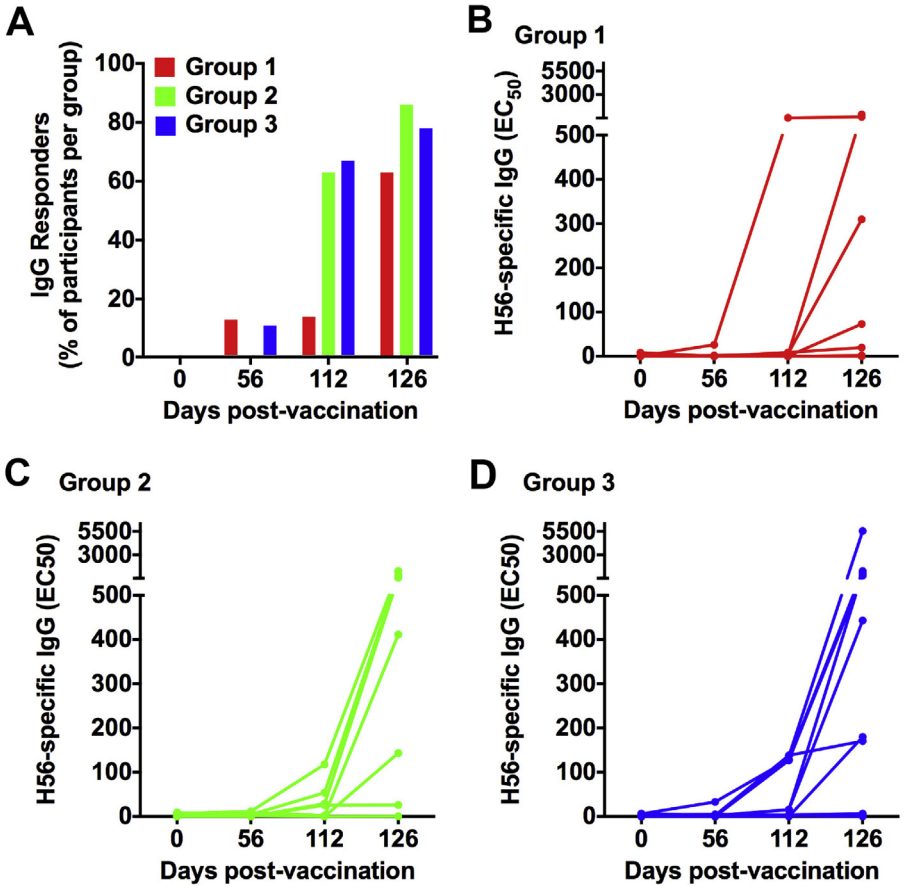

Fig. 2. H56:IC31 vaccination induced antigen-specific IgG responses. H56 was administered on days 0, 56 and 112. The cut-off for a positive IgG response was defined as the mean EC_{50} plus 3 standard deviations of H56-specific IgG responses on study day 0. (A) Percentage of vaccinees in each group with positive H56-specific IgG responses before vaccination, and on study days 56, 112 and 126. (B–D) Longitudinal H56-specific IgG responses in each individual vaccinee in Group 1 (B), Group 2 (C) and Group 3 (D).

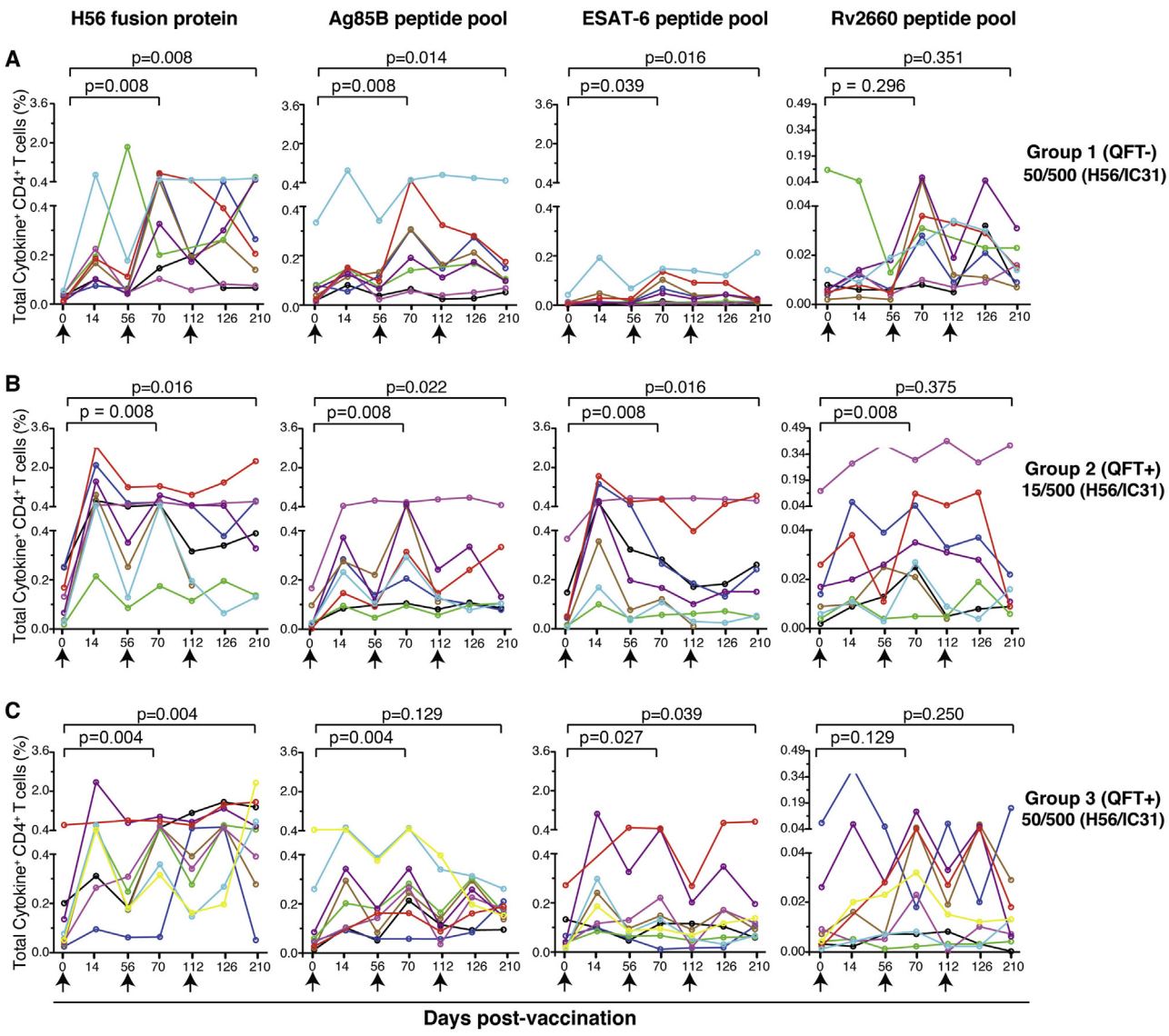

Fig. 3. Longitudinal kinetics of H56-specific cytokine-expressing CD4⁺ T cells. Frequencies of IFN-γ, TNF-α, IL-2 and/or IL-17-expressing CD4⁺ T cells detected by intracellular cytokine assay following stimulation of whole blood with H56-fusion protein or peptide pools of Ag85B, ESAT-6 or Rv2660c. Lines represent individual vaccinees in Group 1 (A), Group 2 (B) and Group 3 (C). Arrows represent each H56 vaccination. Wilcoxon signed rank test was used to compare pre-vaccination (day 0) and the last time point post-vaccination (day 210) to the specific responses. Note that the *y*-axis scales are not consistent for all plots.

detectable levels, but returned to pre-vaccination levels by day 210 (Fig. 3A–C). H56 vaccination did not induce detectable cytokine-expressing CD8⁺ T cells in any of the groups (data not shown). Subsequent analyses therefore focus on H56-specific CD4⁺ T cells only. Magnitudes of H56-specific IgG and CD4⁺ T cell responses observed after vaccination did not correlate and individuals who had no discernable antibody responses were not necessarily those with low CD4⁺ T cell responses.

3.5. Dose and prior mycobacterial sensitization affect frequencies and functions of H56-induced CD4⁺ T cells

Murine studies suggest that polyfunctional IFN-γ⁺TNF-α⁺IL-2⁺ and TNF-α⁺IL-2⁺ H56-specific CD4⁺ T cells conferred efficient control of *M.tb* [5,27,28]. We aimed to evaluate H56-induced response quality by analysing cytokine co-expression patterns. H56-specific CD4⁺ T cells expressed complex patterns of Th1 cytokines predominantly comprised of IFN-γ⁺TNF-α⁺IL-2⁺, TNF-α⁺IL-2⁺ or monofunctional IFN-γ⁺ cells (Fig. 4). Kinetics and relative proportions of these Th1 subsets differed between the study groups, suggesting that vaccine dose and mycobacterial sensitization affected response magnitude and functionality (Fig. 4). Polyfunctional IFN-γ⁺TNF-α⁺IL-2⁺ CD4⁺ T cells were the predominant subset early after H56 vaccination. *M.tb*-infected participants who received the lower vaccine dose (Group 2), had the highest and most durable polyfunctional CD4⁺ T cells, detectable two weeks post-vaccination (Fig. 4). IFN-γ⁺TNF-α⁺ CD4⁺ T cells were also induced in Group 1, but this response did not persist beyond two weeks. Groups 1 and 3 participants, who received the higher dose, had significantly lower frequencies of polyfunctional CD4⁺ T cells than Group 2.

Supplementary Fig. 3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.06.051

Low-level H56-specific TNF-α⁺IL-2⁺ CD4⁺ T cells were also induced in all groups, albeit at a slower kinetic; frequencies of this subset peaked on study day 70, after the second vaccination (Fig. 4). A striking increase in frequencies of monofunctional IFN-γ-expressing H56-specific CD4⁺ T cells emerged after the second high-dose H56 vaccination in *M.tb*-infected vaccinees (Group 3).

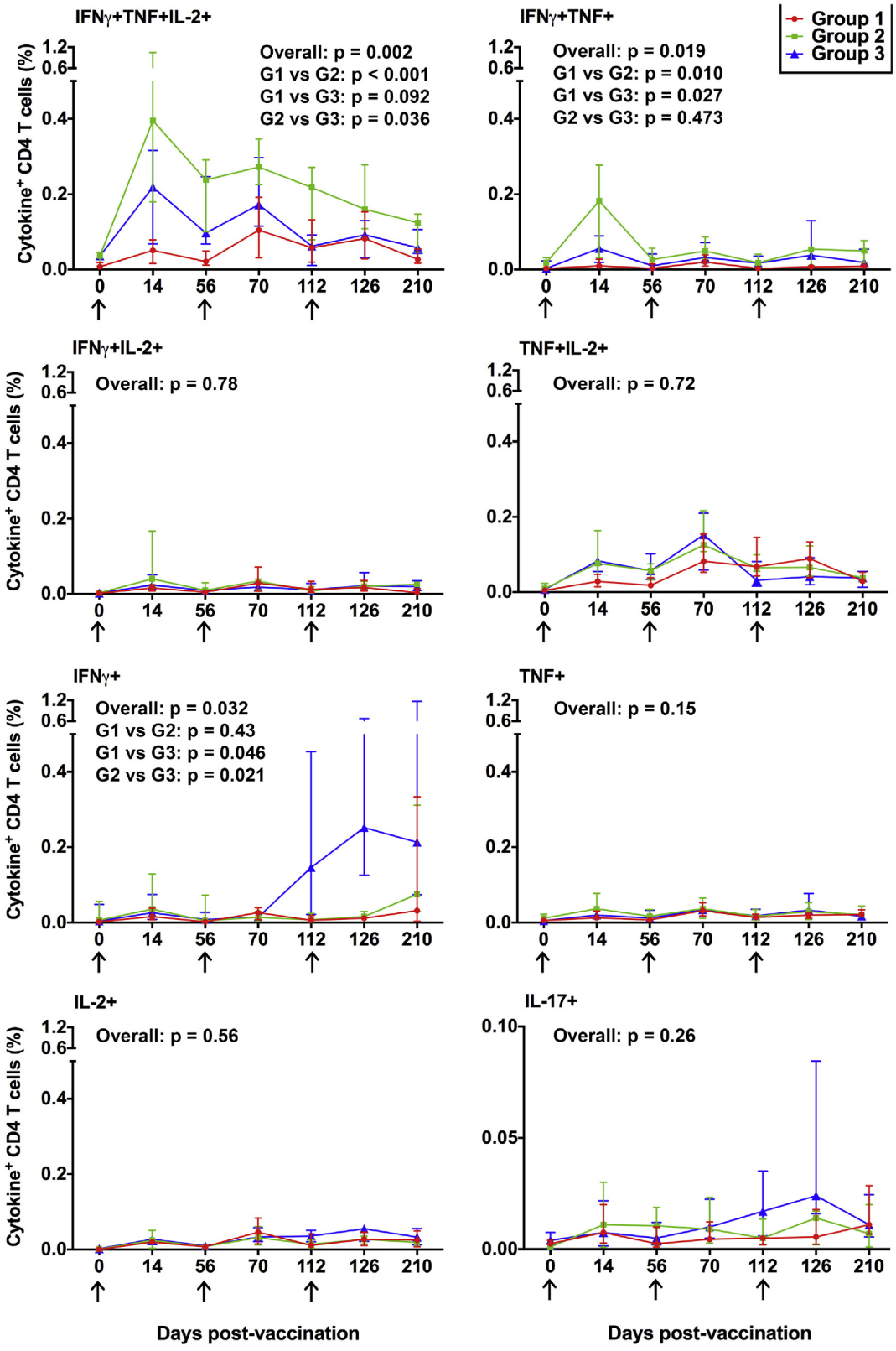

Fig. 4. Longitudinal kinetics of H56-specific CD4⁺ T cell subsets. Frequencies of H56-specific CD4⁺ T cells co-expressing IFN-γ, TNF-α, and/or IL-2 in Groups 1–3. Lines represent the median for each group and error bars represent the IQR. For each T cell subset area under the curve (AUC) values were compared using Kruskal–Wallis (overall effect) and, if $p < 0.05$, Mann–Whitney tests. Similar cytokine-expression profiles were observed when Ag85B and ESAT-6 peptide pools were used for re-stimulation (Supplementary Fig. 2).

Frequencies of all other subsets of Th1 cells were low and not different between groups, while IL-17-expressing CD4+ T cells were not induced (Fig. 4). These cytokine-expression patterns were also observed for ESAT-6 and Ag85B-specific CD4 T cells (Supplementary Fig. 2). Low-level Rv2660c-specific CD4+ T cells predominantly expressing TNF-α and IL-2 were also observed, mostly in Group 2, but these responses did not persist.

3.6. H56:IC31 modifies the pre-existing antigen-specific CD4+ T cell response

To dissect how H56:IC31 may modify pre-existing responses; we compared cytokine expression patterns at day 0, day 70, and at the end of follow-up. Before vaccination, H56 protein-specific CD4+ T cells in all groups were predominantly polyfunctional, but *M.tb*-infected participants had higher frequencies than *M.tb*-uninfected participants (Fig. 5A, $p = 0.012$). By comparison, pre-vaccination frequencies of mycobacteria-specific CD4+ T cells, detected upon BCG stimulation, were not different in *M.tb*-infected and uninfected participants (Supplementary Fig. 3, $p = 0.304$).

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.06.051

Frequencies of all antigen-specific CD4+ T cell subsets were boosted after two H56:IC31 vaccinations. In particular, frequencies and relative proportions of TNF-α+IL-2+ CD4+ T cells increased markedly and comprised a dominant subset of the total response on day 70 (Fig. 5B).

By the end of follow-up, the H56-specific CD4+ response had waned in all groups (Fig. 5C). In the low-dose, *M.tb*-infected group, polyfunctional CD4+ T cells remained the predominant subset, while cells expressing only IFN-γ dominated the response in the high-dose, *M.tb*-infected group. This dominant monofunctional IFN-γ+ pattern resembled the BCG-specific CD4+ T cell response in non-*M.tb* infected individuals (Supplementary Fig. 3).

3.7. Functional patterns of H56-specific response are associated with memory phenotype

We also investigated if functional heterogeneity of H56-specific CD4+ T cells was associated with memory phenotype. Antigen-experienced cells can be classified into long-lived CCR7 + CD45RA- central memory (T_{CM}) which predominantly express IL-2 and TNF-α, or CCR7-CD45RA-effector memory (T_{EM}) cells with rapid effector function, including high expression of IFN-γ [29,30]. We characterized memory phenotype of H56-induced IFN-γ+TNF-α+IL-2+, TNF-α+IL-2+ and IFN-γ+ CD4+ T cell subsets by measuring CCR7 and CD45RA co-expression after establishment of the memory response on day 210 (Fig. 6A and B). In all groups, H56-specific TNF-α+IL-2+ CD4+ T cells displayed a dominant T_{CM} phenotype, while IFN-γ+ expressing cells predominantly displayed a T_{EM} phenotype (Fig. 6C–E). Polyfunctional IFN-γ+TNF-α+IL-2+ CD4 T cells were evenly split amongst T_{CM} and T_{EM} (Fig. 6C–E). Our data suggest that vaccine dose and *M.tb* infection status did not markedly alter the memory phenotype expressed by distinct functional T cell subsets, but rather modulated the relative proportions of these subsets.

4. Discussion

We completed a first-in-human study of H56:IC31 in healthy adults without or with *M.tb* infection. H56:IC31 had an acceptable safety and local tolerability profile. No serious AEs were reported, irrespective of vaccine dose or *M.tb* infection. Transient cardiovascular AEs were reported as possibly related to vaccination. H56:IC31 vaccination induced antigen-specific IgG responses, and H56-specific CD4 T cells which persisted up to day 210. This CD4 T cell response comprised primarily ESAT-6 and Ag85B-specific cells; RV2660c-specific cells were detected at low frequencies and did not persist. Interestingly, higher frequencies of H56-specific polyfunctional CD4+ T cells were induced by the lower H56 dose.

All study participants experienced AEs, but most were mild or moderate and did not persist or require treatment. Previous clinical trials of an IC31-adjuvanted vaccine reported acceptable safety profiles [9,31] with bruising and stiffness at the injection site documented in the first trial [9]. In the second trial of the H1:IC31 vaccine, a higher number of *M.tb*-infected vaccinees experienced local AEs than *M.tb*-uninfected vaccinees [31]. We observed a similar number of systemic AEs in *M.tb*-infected and non-infected vaccinees. However, at the higher dose, *M.tb*-infected vaccinees reported fewer systemic reactions compared with the other groups. One possible explanation for this unexpected finding might be increased immunoregulation in those with latent *M.tb* infection, resulting in a reduced inflammatory response upon vaccination. This should be interrogated further.

Bradycardia was observed after vaccination in 5 of 25 participants and appeared more frequently in M.tb-uninfected participants. These reactions were transient, did not require medication and resolved spontaneously and were possibly related to vaccination. IC31-formulated vaccines have been tested previously in several trials in combination with SSI TB vaccine fusion proteins, H1 and H4. Previous clinical trials with H1:IC31 have not reported bradycardia AEs [9,31] and in a recent phase II trial of H1:IC31, performed in 240 adolescents at the SATVI Field Site, no bradycardia AEs were observed (personal communication, Geldenhuys et al.). However, bradycardia has been reported in clinical trials with H4:IC31; in a cumulative total of 198 subjects who have received H4:IC31, 4 (2%) cases of bradycardia as an AE related to investigational product have been reported, with the highest severity rated as mild. In one case (0.5%), bradycardia was reported as un-related to vaccination and rated as severe (Aeras-404 (H4:IC31) Investigators Brochure).

Bradycardia after administration of other vaccines has been previously observed in newborns and preterm babies after DTP vaccination [32] as well as in healthy adults in a clinical trial of AERAS-402 a recombinant adenovirus-35 TB vaccine [33]. The cases of severe hypertension might have been related to stress or discomfort during vaccination; as also reported for AERAS-402 [33].

Our finding that H56:IC31 induced durable Ag85B and ESAT-6-specific CD4+ T cell responses is consistent with results reported in cynomolgus macaques after BCG prime and H56:IC31 boost [14]. In these non-human primates, H56:IC31 also induced greater magnitudes of Ag85B and ESAT-6-specific T cells than those specific for Rv2660c. T cells targeting Rv2660c were also transient, while Ag85B-specific T cells persisted [14]. Some trial participants had pre-existing, low-level Rv2660c-specific responses, which did not markedly increase after H56:IC31 vaccination. This is consistent with reports of Rv2660c-specific T cell responses in persons with latent *M.tb* infection and/or TB disease [34] [22,35,36].

Cytokine co-expression patterns of H56-induced Th1 cells were influenced by *M.tb* infection status and vaccine dose. Frequencies of ESAT-6 and Ag85B-specific, and to a small extent, Rv2660c-specific CD4+ T cell responses, were higher in *M.tb*-infected than *M.tb*-uninfected vaccinees. Our results corroborate previous findings of greater vaccine-induced mycobacteria-specific T cell response frequencies in persons with prior mycobacterial sensitization, compared with unsensitized persons [37,38].

We observed that the lower H56 dose induced the highest frequencies of durable IFN-γ+TNF-α+IL-2+ CD4+ T cells. Given that such polyfunctional cells are less differentiated [39,40] and more long-lived [40,41] than IFN-γ-only expressing CD4+ T cells, we propose that the lower dose induced the most optimal response. This is in agreement with results from the mouse model [42]; mice

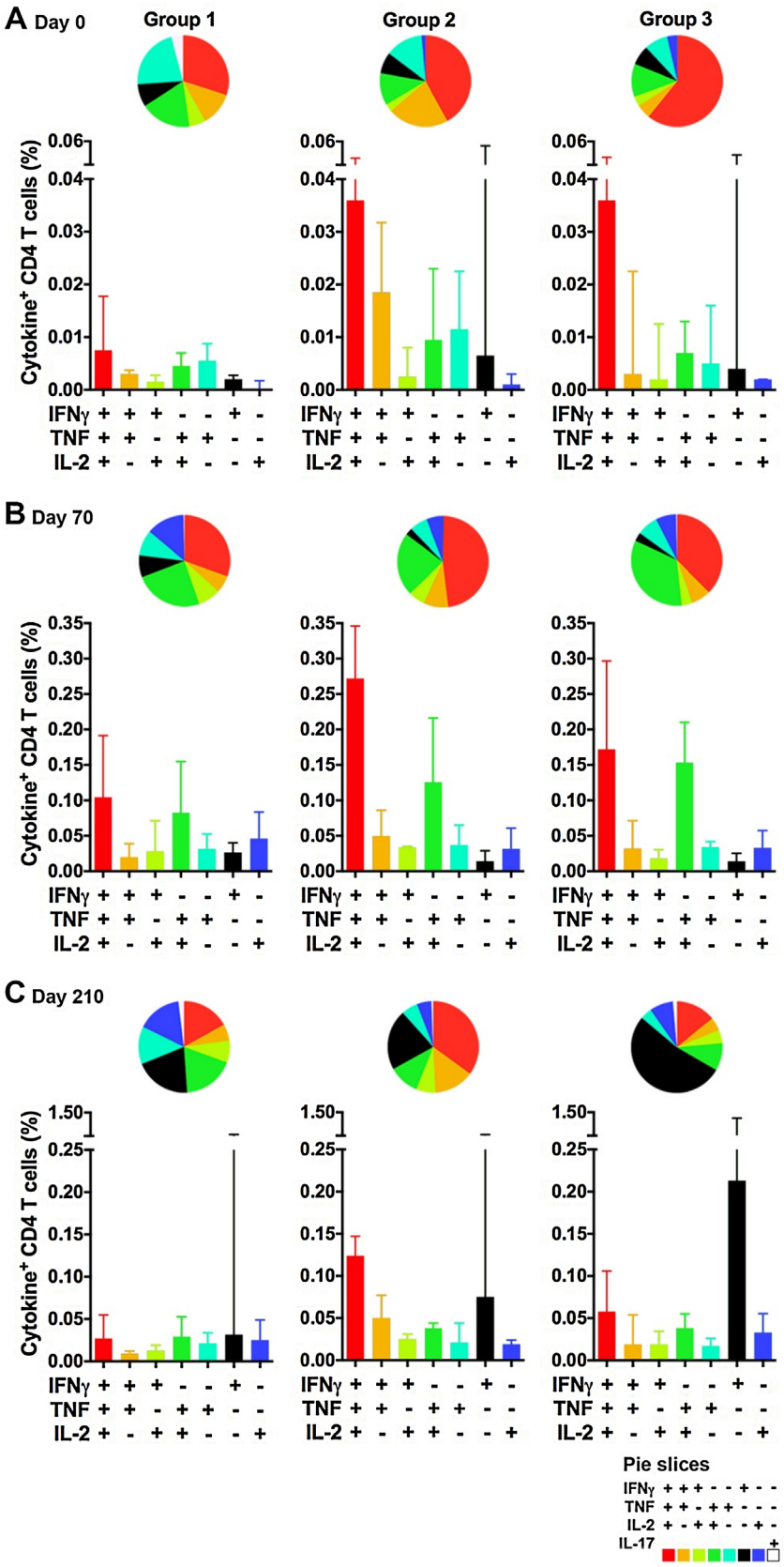

Fig. 5. Cytokine expression profiles of H56-specific CD4⁺ T cells before and after H56:IC31 vaccination. Participants received three intramuscular injections of H56:IC31 on study days 0, 56, and 112. H56-specific CD4⁺ T cells co-expressing IFN-γ, TNF-α, IL-2 or IL-17 were measured by intracellular cytokine assay following stimulation of whole blood with H56-fusion protein on days 0 (A), 70 (B) and day 210 (C). Pies represent median proportions of H56-specific CD4⁺ T cells co-expressing each of 7 possible combinations of IFN-γ, TNF-α and/or IL-2, or expressing IL-17. IL-17 was not co-expressed with Th1 cytokines. Bar graphs represent median frequencies of the possible combinations of IFN-γ, TNF-α and/or IL-2 co-expressing H56-specific CD4⁺ T cells. Error bars represent the upper IQR. Note that the *y*-axis scales for the different post-vaccination timepoints in panels A, B and C are different.

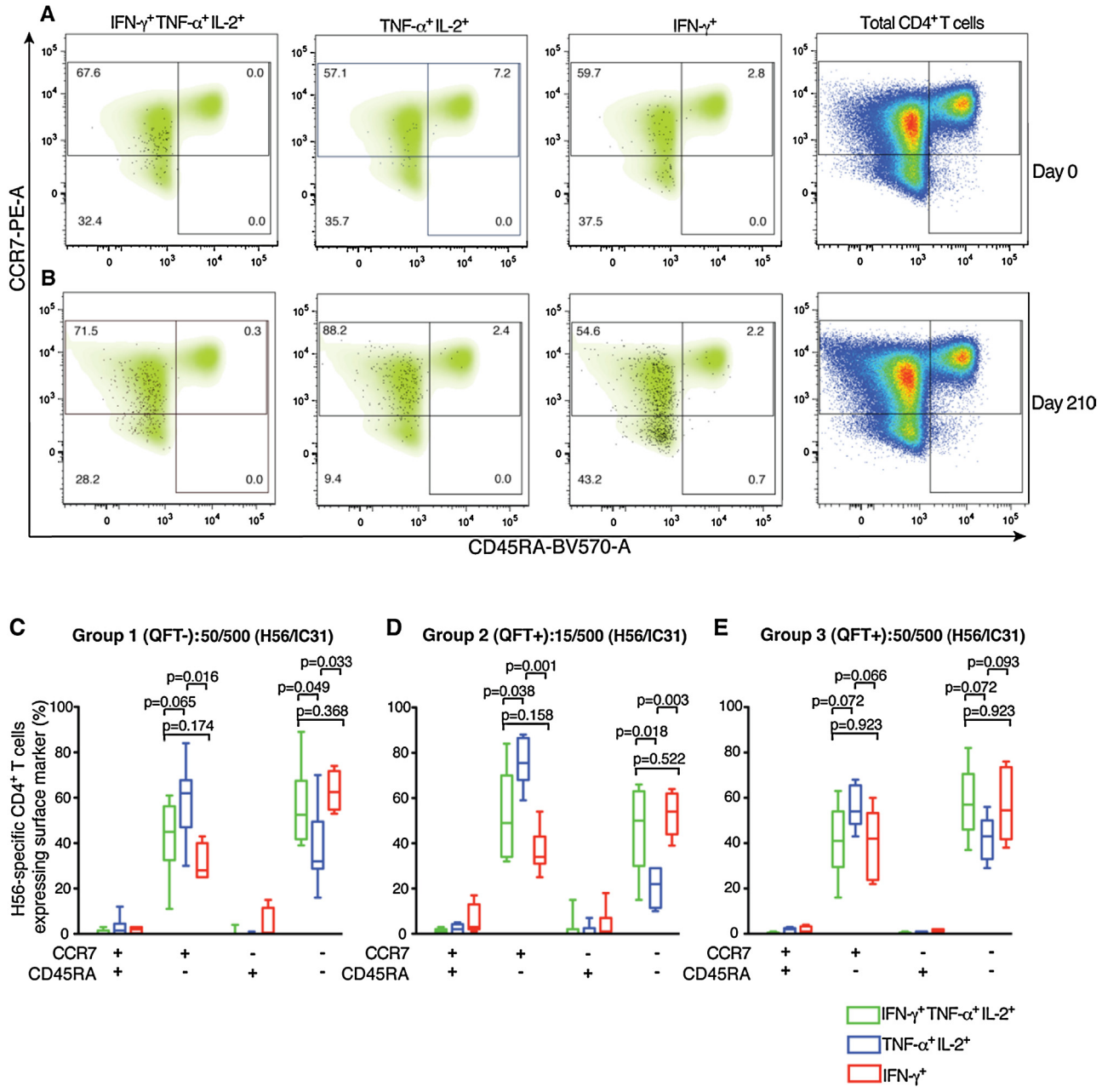

Fig. 6. Memory phenotypes of H56-specific cytokine-expressing CD4+ T cells. Flow cytometry plots of CCR7 and CD45RA memory marker expression by antigen-specific cytokine-expressing (black dots) and non-cytokine-expressing (green shading or pseudo plots) CD4+ T cells from a representative participant before H56 vaccination (A) and on the final study day (B). Relative proportions of H56-specific CD4+ T cells that expressed the possible combinations of CCR7 and CD45RA are represented as percentages. CCR7 and CD45RA co-expression by IFN-γ+TNF-α+IL-2+ (green), TNF-α+IL-2+ (blue), and IFN-γ+ (red) H56-specific CD4+ T cells measured on day 210 in participants from groups 1 (C), 2 (D) and 3 (E). Boxes represent the IQR, horizontal lines represent medians and whiskers represent the range. Proportions of cells expressing different combinations of memory markers were compared between the three cytokine-expressing subsets using the Kruskal–Wallis test and then, if $p < 0.05$, by the Mann–Whitney test. *P* values shown are from Mann–Whitney tests. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with vaccine-induced IFN-γ+TNF-α+IL-2+ and TNF-α+IL-2+ CD4+ T cells controlled *M.tb* better than mice with CD4+ T cells that predominantly expressed IFN-γ and little IL-2 [28]. This H56-induced functional profile is different to the predominantly monofunctional IFN-γ-expressing CD4+ T cells induced by newborn BCG vaccination [43,44]. Such mono-functional IFN-γ-expressing T_{EM} CD4+ T cells typically possess less profilerative capacity than the more polyfunctional, IL-2-expressing T_{CM} CD4+ T cells [45]. However, functional and phenotypic attributes that may correlate with protection can only be determined in the context of an efficacious vaccine.

The small sample size of this first-in-human Phase I trial was a necessary limitation of the study, which reduced statistical power. Nevertheless, we show that H56 was immunogenic in humans; that pre-existing mycobacteria-specific T cell responses were significantly boosted; and that the 15 μg H56 dose may be preferable. Our findings support larger clinical trials to test the safety, immunogenicity, and in future, efficacy of the H56:IC31 vaccine. The occurrence of bradycardia and hypertension should be carefully monitored in subsequent H56:IC31 trials.

Conflict of interest

None declared.

Author's contributions

PA, WAH, HM, MH, IK, STH, TJS, PB and ZS designed the study. AKKL, MDT, HG, ES, CK, and GDH conducted or supervised the clinical and fieldwork. STH, TJS, BMNK, CD, HA, LM, ES, YB, SS and JH performed or supervised the immunology experiments. AKKL, BMNK, BMcC, MAS, TJS and PA performed statistical analysis. AKKL, BMNK, PA and TJS interpreted the results and wrote the manuscript. All authors reviewed and gave input to the revisions and final manuscript.

Acknowledgements

The authors thank the volunteers participating in this study, and acknowledge the contributions of clinicians, nurses, and technicians at the South African Tuberculosis Vaccine Initiative. This study was funded by Aeras.

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