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Safety and immunogenicity of the novel H4:IC31 tuberculosis vaccine candidate in BCG-vaccinated adults: Two phase I dose escalation trials

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ABSTRACT

Background: Novel vaccine strategies are required to provide protective immunity in tuberculosis (TB) and prevent development of active disease. We investigated the safety and immunogenicity of a novel TB vaccine candidate, H4:IC31 (AERAS-404) that is composed of a fusion protein of *M. tuberculosis* antigens Ag85B and TB10.4 combined with an IC31® adjuvant.

Methods: BCG-vaccinated healthy subjects were immunized with various antigen (5, 15, 50, 150 μg) and adjuvant (0, 100, 500 nmol) doses of the H4:IC31 vaccine (n = 106) or placebo (n = 18) in two randomized, double-blind, placebo-controlled phase I studies conducted in a low TB endemic setting in Sweden and Finland. The subjects were followed for adverse events and $CD4^+$ T cell responses.

Results: H4:IC31 vaccination was well tolerated with a safety profile consisting of mostly mild to moderate self-limited injection site pain, myalgia, arthralgia, fever and post-vaccination inflammatory reaction at the screening tuberculin skin test injection site. The H4:IC31 vaccine elicited antigen-specific $CD4^+$ T cell proliferation and cytokine production that persisted 18 weeks after the last vaccination. $CD4^+$ T cell expansion, IFN-γ production and multifunctional $CD4^+$ Th1 responses were most prominent after two doses of H4:IC31 containing 5, 15, or 50 μg of H4 in combination with the 500 nmol IC31 adjuvant dose.

Conclusions: The novel TB vaccine candidate, H4:IC31, demonstrated an acceptable safety profile and was immunogenic, capable of triggering multifunctional $CD4^+$ T cell responses in previously BCG-vaccinated healthy individuals. These dose-escalation trials provided evidence that the optimal antigen-adjuvant dose combinations are 5, 15, or 50 μg of H4 and 500 nmol of IC31.

Conclusions: Trial registration: ClinicalTrials.gov, NCT02066428 and NCT02074956.

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1. Introduction

WHO has declared tuberculosis (TB) as a global health emergency and despite socioeconomic improvement, TB continues to cause a considerable number of deaths. A preventive TB vaccine could reduce the spread and severity of TB disease significantly. The bacillus Calmette-Guérin (BCG) vaccine provides incomplete protection against pulmonary TB and a boost with BCG does not consistently provide additional protection [1,2]. However, newborn BCG vaccine prevention is still a major global strategy for

Abbreviations: TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; BCG, bacillus Calmette-Guérin; MDR, multidrug-resistant; TST, tuberculin skin test; ICS, intracellular cytokine staining; FASCIA, Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood; AE, adverse events; INR, international normalized ratio; MHC, major histocompatibility complex.

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the control of TB, which underlines the importance of developing an improved TB vaccine that would more effectively boost BCG.

H4:IC31 (AERAS-404) is an investigational vaccine that is composed of two active components: the H4 antigen which is a fusion protein created from two *Mycobacterium tuberculosis* (Mtb)-antigens, antigen 85B (Ag85B) and TB10.4, and an immunological adjuvant called IC31®. The rationale of the H4:IC31 vaccine is that presentation of Mtb-specific antigens in this setting could augment T cell immunity induced by BCG and thus improve protection against TB. Ag85B is a 30 kDa mycolyl transferase protein [3,4] that has previously been demonstrated to induce substantial protective immunity against aerosol challenge with the highly virulent Mtb Erdman strain in guinea pigs [5]. TB10.4 is one of three members of the very similar early secretory antigenic target (ESAT)-6 group of proteins found in Mtb culture supernatants [6]. TB10.4 has been shown to induce larger and broader immune responses in T cells isolated from TB patients compared to BCG-vaccinated and non-vaccinated donors [7]. Immunization of mice with a fusion protein of TB10.4 and Ag85B has been shown to induce a significant synergistic protective effect against subsequent aerosol challenge with Mtb [8]. Similarly, Ag85B and ESAT-6 (H1 antigen) induced potent and long-lived effector T cell responses in naïve volunteers when administrated in the presence of the IC31 adjuvant [9,10]. The proprietary IC31 adjuvant (Valneva, Vienna, Austria) is a combination of a leucine-rich peptide, KLK, and a synthetic oligonucleotide, ODN1a. KLK enhances the uptake of antigens into antigen-presenting cells and therefore improves the immune response to peptide antigens. ODN1a is a synthetic bacterial DNA analogue that resembles a CpG motif that will promote a Th1 response and the production of IFN-γ and IL-2, which are considered to play essential roles in protection against TB [11]. In addition, the IC31 adjuvant enhances the production of both IFN-γ and humoral responses via the TLR9 signaling pathway [12,13].

It has been demonstrated in animal studies that H4:IC31 boosts BCG immunity and provides greater protection against development of TB disease than BCG alone [14–16]. Therefore we aimed to test the safety and immunogenicity of the H4:IC31 vaccine in a prime-boost vaccination strategy in healthy, previously BCG-vaccinated individuals who received different antigen-adjuvant dose combinations of the study vaccine, in two phase I clinical trials performed in Sweden (Aeras protocol C-005-404) and Finland (Aeras protocol C-006-404), respectively. We designed the first-in-human C-005-404 study to optimize the dose of the IC31 adjuvant with a fixed dose of H4 antigen, and the subsequent C-006-404 study to optimize the dose of H4 antigen with a fixed dose of IC31 adjuvant.

2. Materials and methods

2.1. Subjects and study design

We conducted two phase I randomized, double-blind, placebo-controlled studies at the Department of Infectious Diseases, Karolinska University Hospital Huddinge in Stockholm, Sweden (Aeras protocol C-005-404; ClinicalTrials.gov ID NCT02066428) and at the Vaccine Research Center, University of Tampere in Finland (Aeras protocol C-006-404; ClinicalTrials.gov ID NCT02074956). Inclusion criteria were: previously BCG-vaccinated (⩾5 years), males or females (females required to be sterile for C-005-404), age 18-50 years, HIV-uninfected, good health based on medical history, normal BMI (19–33), no evidence of an ongoing TB infection and written informed consent completed. Both studies employed dose-escalation, with increasing amounts of the H4 antigen (5, 15, 50 or 150 μg) administered in the presence of increasing amounts of the IC31 adjuvant (0, 100 or 500 nmol) (Table 1). Statens Serum Institut (SSI) in Copenhagen, Denmark manufactured the H4 fusion protein and the IC31 adjuvant. Formulation buffer was used as placebo control. Subjects received vaccinations via intramuscular injection with H4:IC31 or placebo on study days 0 and 56. Treatment assignments were based on a randomly-generated sequence of subject identification numbers on a randomization schedule, provided by an unblinded statistician to the study pharmacist in a sealed tamper-evident envelope.

Protocol C-005-404 was approved by the Regional Ethical Review Board (Stockholm) and the Medicinal Product Agency in Sweden, while protocol C-006-404 was approved by the Hospital District of Pirkanmaa Ethics Committee (Tampere) and the National Agency for Medicines in Finland (Finnish Medicines Agency). The studies were conducted in accordance with the Declaration of Helsinki and applicable local regulations for conducting clinical trials on medicinal products in humans.

2.2. Adverse events

Safety of the H4:IC31 vaccine treatment regimens were based on the induction of adverse events (AEs) that represented both clinical and laboratory evaluations (see Supplementary materials). AE severity was graded according to the US Food and Drug Administration (FDA) toxicity tables for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials [17] using criteria that were pre-specified in the study protocols provided by the sponsor i.e. Areas. We recorded solicited and unsolicited AEs during the first 28 days after each vaccination i.e. study days 0–28 and 56–84, and serious AEs (SAEs) during the 6 month study period.

2.3. Immunogenicity testing

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected on study days 0, 7, 14, 28, 56, 63, 70, 84, and 182 for intracellular cytokine staining (ICS) performed at Aeras [18] and on study days 0, 56, 84, and 182 for assessment of Mtb-specific IFN-γ production using ELISpot performed at Leiden University Medical Center according to Protocol S2 previously described by Dr. Steven Smith et al. [19] (see Supplementary

Table 1
A dose matrix of the H4 and IC31 dose combinations administered to the study subjects in the C-005-404 and C-006-404 trials.[a]

	One vaccination Day 0		Two vaccinations Day 0 and 56				
H4 dose	50 μg	150 μg	5 μg	15 μg	50 μg	150 μg	Total (n = 124)
No adjuvant	8	8	–	–	8	–	24
100 nmol IC31	8	–	9	9	8	–	34
500 nmol IC31	8	–	8	8	16[b]	8	48
Placebo	–		18[c]				18

[a] A total of 124 participants were included in the two trials.
[b] Two doses of 50 μg H4 in 500 nmol IC31 were administered to 8 participants in each of the C-005-404 and C-006-404 trials.
[c] A total of 18 participants received two placebo vaccinations in the two trials.

materials). In study C-005-404, blood was also collected on study days 0, 7, 28, 63, 84, and 182 for Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood assay (FASCIA performed at the Public Health Agency of Sweden) [20,21] (see Supplementary materials). Subjects were screened for ongoing TB infection using QuantiFERON and the tuberculin skin test (TST) (see Supplementary materials).

2.4. Data analysis

The sample size for each trial was selected as adequate for an initial review of the safety profile of H4:IC31. Basic descriptive analysis was performed for each treatment regimen to examine AEs and immune responses measured by ICS, IFN-γ ELISpot and FASCIA. Comparisons between treatment regimens for ICS and IFN-γ ELISpot immune responses were conducted by area under the curve (AUC) analyses. The trapezoidal rule was used to calculate the AUC for each subject, with negative and positive peaks relative to each subject's baseline data included to calculate net peak area. Overall p-values for median AUC among treatment regimens were obtained using the Kruskal-Wallis test. Pairwise comparisons of median AUC between treatment regimens were conducted using a Mann-Whitney exact test. The Holm method was used to correct for multiple comparisons.

3. Results

3.1. Enrollment and demography

We screened a total of 206 healthy BCG-vaccinated individuals for eligibility, enrolled 125 individuals and randomized them into the different intervention groups as described in Fig. 1. Demographic characteristics were generally similar across treatment regimens within each trial (Supplementary Tables 1A and 1B). In the C-005-404 study, all 64 randomized subjects received the study day 0 vaccination and all subjects completed the study. In the C-006-404 study, 60 of 61 randomized subjects received the study day 0 vaccination and all subjects except two completed the study.

3.2. Adverse events (AEs)

The majority (83%) of subjects across both studies had AEs graded as mild or moderate (Tables 2a and 2b), and three (2%) subjects had no AEs. n = 18 (15%) subjects had at least one severe AE (Tables 2a and 2b) of which four were considered related to the study vaccine: fever (150/0 one dose regimen), increased protein in urine (15/100 two dose regimen), TST site reaction (50/100 two dose regimen, discussed below), and increased international normalized ratio (INR) (150/500 two dose regimen). The overall incidence and severity of the AEs were not different comparing H4:IC31 vaccinations with placebo (Tables 2a and 2b). Four SAEs were reported, none of which was considered related to study vaccination: mental status changes (50/0 one dose regimen); mesenteric lymphadenitis (50/500 two dose regimen); ileus (50/500 two dose regimen); and subdural hemorrhage (placebo).

Most subjects had at least one solicited or unsolicited AE recorded (Tables 3a and 3b). Among solicited AEs, myalgia, arthralgia, and fever (pyrexia) occurred at a higher frequency in subjects who received the H4:IC31 vaccine compared to placebo in the C-005-404 study (Table 3a). All cases of fever occurred within 1–2 days of the first vaccination, resolved within 2–3 days and did not recur after the second study vaccination. There was a trend towards an increased frequency of some systemic solicited AEs at the 150 μg H4 dose level, particularly when combined with 500 nmol IC31 (Tables 3a and 3b). Pain at the injection site was increased in subjects who received H4 together with the IC31 adjuvant, as compared to placebo (Tables 3a and 3b) or H4 alone (Table 3a). For other solicited AEs and all unsolicited AEs (except TST site reaction, discussed below), the AE profiles were similar across the H4:IC31 and placebo treatment regimens (Tables 3a and 3b). For each regimen, the AE profiles identified after the second vaccination were similar to those detected after the first vaccination (data not shown). See Supplementary Tables 2A and 2B, for a complete list of AEs in the trials.

In the C-005-404 study, some degree of post-study vaccination inflammation occurred at the screening TST injection site in 14 of the first 21 (66.7%) TST-negative subjects who received the H4:IC31 vaccine. Among these 14 subjects, TST site reactions were elicited by the H4 antigen alone (11/14, 78.6%) or H4 combined with a low dose (100 nm) of IC31 adjuvant (3/14, 21.4%) (Table 3a). One subject (50/100 two dose regimen) experienced a severe reaction at the TST site with onset the day of the first study vaccine administration. The subject had no screening TST reactivity or a reaction at the vaccine site (Supplementary materials).

3.3. Immunogenicity

We considered detection of antigen-specific T cell proliferation and cytokine production (single- or co-expression of IFN-γ, TNF-α and/or IL-2) in PBMC samples in response to vaccine-antigens as potentially important correlates of immune protection. T cell expansion in each of the H4:IC31 one dose regimens was limited to the $CD4^+$ T cell subset that responded to the vaccine antigen Ag85B in some subjects, although these responses were not sustained (Fig. 2A, Supplementary Table 3A). In each of the H4:IC31 two dose regimens, the study vaccine induced Ag85B-specific but also TB10.4-specific $CD4^+$ T cell responses, with a boosting effect seen after administration of the second dose (Fig. 2B and Fig. 2C, Supplementary Tables 3A and 3B). These responses peaked at 2 and 4 weeks after the second vaccination and were sustained up to 18 weeks after the last vaccination (Fig. 2B and Fig. 2C). The most strong and long-lived median $CD4^+$ T cell responses compared to placebo were seen after stimulation with Ag85B in the 5/500 ($p < 0.01$), 15/500, and 50/500 ($p < 0.01$) two dose regimens, while the proportion of antigen-specific $CD4^+$ T cells was lower using the higher H4 dose 150/500 (Fig. 2B and Fig. 2C, Supplementary Tables 3A, 3B and 4B). These results were supported by the whole blood FASCIA assay, where the two dose 50/500 treatment was shown to be the superior regimen that induced significant expansion of Ag85B-specific $CD4^+$ T cells ($p = 0.02$) (Supplementary Fig. 1A) and $CD8\alpha\alpha^+$ T cells ($p < 0.001$) (Supplementary Fig. 1B) compared to the two dose placebo regimen.

IFN-γ ELISpot responses were elevated in response to both Ag85B and TB10.4, but only for the two dose regimens together with the IC31 adjuvant (Fig. 3A and Fig. 3B). A boosting effect was seen after the second H4:IC31 dose, particularly in the presence of the higher IC31 adjuvant dose, and these responses were sustained up to 18 weeks after the last vaccination (Fig. 3B and Fig. 3C). Similar to antigen-specific $CD4^+$ T cell expansion (Fig. 2B and Fig. 2C), the most potent IFN-γ ELISpot responses were seen in the 5/500 ($p < 0.01$), 15/500 ($p = 0.02$) and the 50/500 ($p = 0.02$) two dose regimens compared to placebo (Fig. 3B and Fig. 3C, Supplementary Tables 4C and 4D). ICS analysis of Ag85B-specific $CD4^+$ T cells at 4 weeks after the second vaccination (study day 84) confirmed that only the two dose treatment regimens efficiently induced cytokine producing cells (Fig. 4A–C) These primarily included bi-functional IL-2/TNF-α producing and multifunctional IFN-γ/IL-2/TNF-α producing T cells and, to a lesser extent, mono-functional T cells (Fig. 4B and Fig. 4C). See Supplementary Tables 4A–4D, for complete analyses on the statistical differences between the vaccine groups.

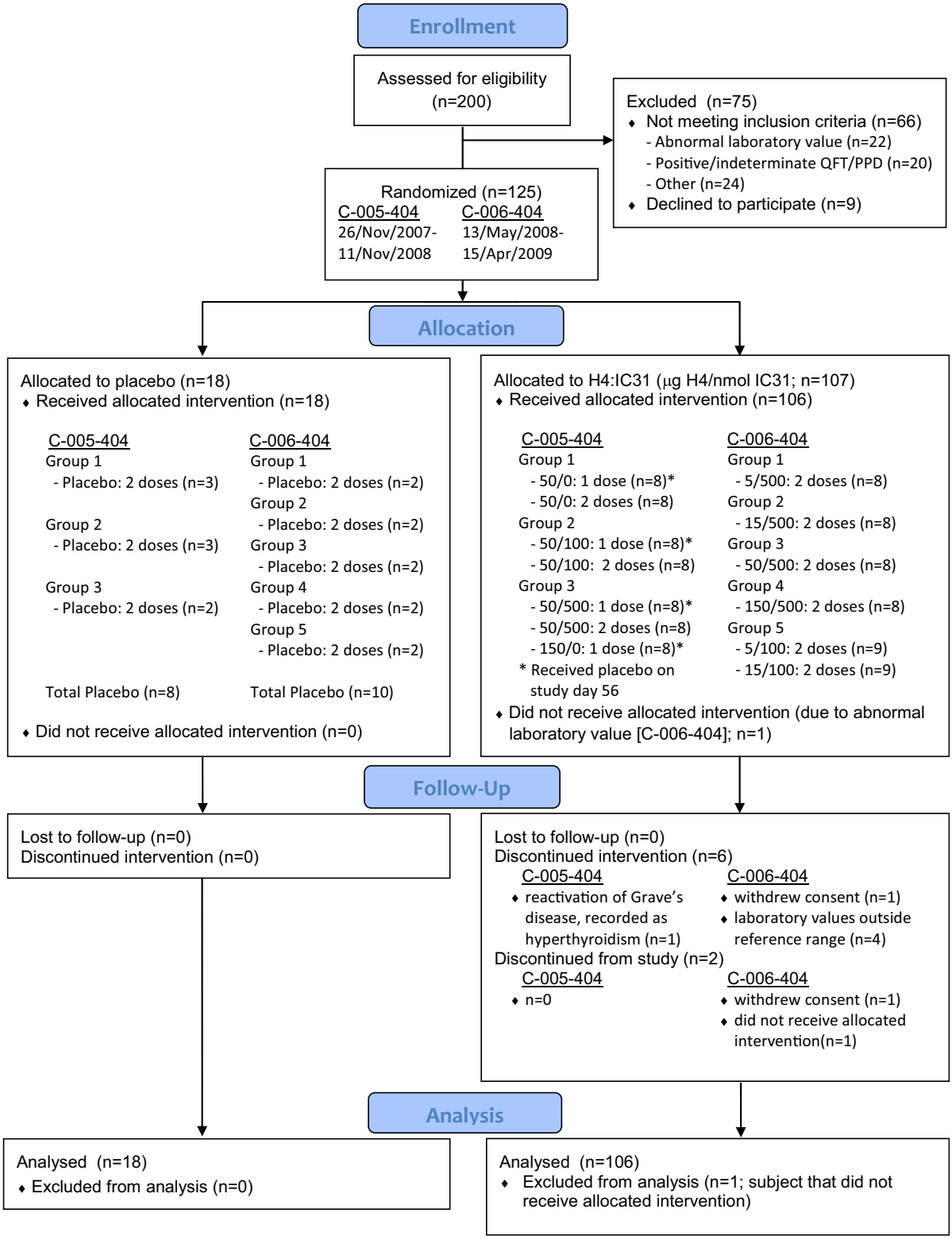

Fig. 1. Consort diagram of healthy previously BCG-vaccinated individuals, from screening to analysis. All subjects in C-005-404 completed the study. One C-005-404 subject (150/0 one dose treatment regimen) with a history of Grave's disease did not receive the study vaccine on study day 56 due to onset of hyperthyroidism. In the C-006-404 study, all subjects except two completed the study, one who retracted consent, and one who was not vaccinated and who was excluded from all analyses. Five C-006-404 subjects did not receive the study day 56 vaccination; one subject (150/500 two dose treatment regimen) withdrew consent and 4 subjects (one in each of the 5/100, 15/500, 50/500, and 150/500 two dose treatment regimens) had laboratory values outside the reference ranges of the local laboratory.

4. Discussion

These phase I vaccine trials were the first to explore a safe and immunogenic dose and dosage range and to identify potential side effects of the H4:IC31 vaccine candidate in humans. Our principal findings demonstrated that all vaccine doses and dosage combinations tested were well tolerated in previously BCG-vaccinated study subjects and most of the reported local and systemic AEs

Table 2a
Adverse events by highest severity for each subject: C-005-404.

		H4:IC31 (μg H4/nmol IC31)						
		50/0		50/100		50/500		150/0
Severity	Placebo (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)
Mild	3 (37.5)	4 (50.0)	2 (25.0)	4 (50.0)	2 (25.0)	1 (12.5)	2 (25.0)	1 (12.5)
Moderate	4 (50.0)	2 (25.0)	5 (62.5)	2 (25.0)	4 (50.0)	6 (75.0)	4 (50.0)	4 (50.0)
Severe	1 (12.5)	1 (12.5)	1 (12.5)	2 (25.0)	2 (25.0)	1 (12.5)	2 (25.0)	3 (37.5)

Table 2b
Adverse events by highest severity for each subject: C-006-404.

		H4:IC31 (μg H4/nmol IC31)					
Severity	Placebo 2 Doses (n = 10) n (%)	5/100 2 Doses (n = 9) n (%)	5/500 2 Doses (n = 8) n (%)	15/100 2 Doses (n = 9) n (%)	15/500 2 Doses (n = 8) n (%)	50/500 2 Doses (n = 8) n (%)	150/500 2 Doses (n = 8) n (%)
Mild	4 (40.0)	3 (33.3)	4 (50.0)	3 (33.3)	2 (25.5)	1 (12.5)	–
Moderate	5 (50.0)	6 (66.7)	3 (37.5)	5 (55.6)	5 (62.5)	6 (75.0)	6 (75.0)
Severe	–	–	1 (12.5)	1 (11.1)	1 (12.5)	–	2 (25.0)

Table 3a
Adverse events: C-005-404.

		H4:IC31 (μg H4/nmol IC31)						
		50/0		50/100		50/500		150/0
MedDRA Preferred term	Placebo (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)	2 Doses (n = 8) n (%)	1 Dose (n = 8) n (%)
Subjects with at least one solicited AE	**6 (75.5)**	**6 (75.5)**	**5 (62.5)**	**7 (87.5)**	**6 (75.0)**	**7 (87.5)**	**7 (87.5)**	**8 (100.0)**
Arthralgia	–	1 (12.5)	2 (25.0)	3 (37.5)	2 (25.0)	4 (50.0)	1 (12.5)	4 (50.0)
Diarrhoea	1 (12.5)	–	–	1 (12.5)	1 (12.5)	–	–	–
Fatigue	5 (62.5)	4 (50.0)	3 (37.5)	5 (62.5)	3 (37.5)	6 (75.0)	4 (50.0)	6 (75.0)
Headache	5 (62.5)	3 (37.5)	4 (50.0)	4 (50.0)	5 (62.5)	4 (50.0)	6 (75.0)	7 (87.5)
Injection site erythema	1 (12.5)	1 (12.5)	–	–	–	–	1 (12.5)	–
Injection site pain	1 (12.5)	–	–	2 (25.0)	3 (37.5)	1 (12.5)	5 (62.5)	2 (25.0)
Injection site swelling	1 (12.5)	–	–	–	1 (12.5)	1 (12.5)	3 (37.5)	–
Myalgia	1 (12.5)	4 (50.0)	4 (50.0)	6 (75.0)	3 (37.5)	3 (37.5)	2 (25.0)	5 (62.5)
Pyrexia	–	–	2 (25.0)	2 (25.0)	1 (12.5)	4 (50.0)	–	4 (50.0)
Subjects with at least one unsolicited AE	**7 (87.5)**	**7 (87.5)**	**8 (100.0)**	**8 (100.0)**	**7 (87.5)**	**7 (87.5)**	**8 (100.0)**	**8 (100.0)**
Aspartate aminotransferase increased	–	–	1 (12.5)	–	1 (12.5)	3 (37.5)	–	3 (37.5)
Blood creatine phosphokinase increased	–	–	2 (25.0)	1 (12.5)	1 (12.5)	5 (62.5)	–	1 (12.5)
Blood pressure systolic increased	1 (12.5)	–	–	2 (25.0)	2 (25.0)	–	2 (25.0)	–
Haemoglobin decreased	–	1 (12.5)	–	1 (12.5)	1 (12.5)	1 (12.5)	3 (37.5)	1 (12.5)
Heart rate decreased	3 (37.5)	–	–	–	6 (75.0)	3 (37.5)	5 (62.5)	2 (25.0)
Nasopharyngitis	2 (25.0)	1 (12.5)	2 (25.0)	1 (12.5)	1 (12.5)	–	2 (25.0)	2 (25.0)
Nausea	–	1 (12.5)	1 (12.5)	2 (25.0)	1 (12.5)	1 (12.5)	–	1 (12.5)
Neutrophil count decreased	2 (25.0)	–	2 (25.0)	3 (37.5)	2 (25.0)	1 (12.5)	2 (25.0)	3 (37.5)
Red blood cells urine	–	2 (25.0)	1 (12.5)	2 (25.0)	1 (12.5)	3 (37.5)	1 (12.5)	1 (12.5)
TST site reaction[a]	–	5 (62.5)	5 (62.5)	1 (12.5)	2 (25.0)	–	–	1 (12.5)

Note: Individual unsolicited AEs are shown for AEs reported in ⩾10% of subjects across the combined H4:IC31 regimens. A full list of AEs is presented in Supplementary Table 2A.

[a] Application site hypersensitivity or inflammatory reactions at the TST application site. Note that only the first 26 subjects randomized received a TST at screening.

were mild to moderate. The H4:IC31 vaccine was able to elicit persistent antigen-specific $CD4^+$ T cell responses in the peripheral circulation including enhanced T cell proliferation and IFN-γ production, and also induction of multifunctional Th1 cells. Two vaccinations with the H4 antigen using the lower doses ranging from 5 to 50 μg (i.e. 5, 15, or 50 μg) in combination with the higher dose (500 nmol) of the IC31 adjuvant induced the strongest T cell responses to Ag85B, while the H4 antigen alone resulted in very low T cell responses. This supports the fact that adjuvant properties are required for the induction of a strong and sustained immune response [22]. Importantly, a second vaccination with H4:IC31 did not increase the frequency or severity of any reported AEs as compared to a single vaccination.

The frequency and severity of AEs appeared to be independent of the number (one or two) or dose (5, 15 or 50 μg) of H4 antigen in the lower dose range, while an increased frequency of some systemic solicited AEs was seen at the 150 μg H4 dose level. The AE profiles among subjects who received the H4 antigen in the presence or absence of the IC31 adjuvant were similar, except for injection site pain, which suggested that the adjuvant did not contribute

Table 3b
Adverse events: C-006-404.

MedDRA Preferred term	Placebo 2 Doses (n = 10) n (%)	H4:IC31 (μg H4/nmol IC31) 5/100 2 Doses (n = 9) n (%)	5/500 2 Doses (n = 8) n (%)	15/100 2 Doses (n = 9) n (%)	15/500 2 Doses (n = 8) n (%)	50/500 2 Doses (n = 8) n (%)	150/500 2 Doses (n = 8) n (%)
Subjects with at least one solicited AE	**8 (80.0)**	**9 (100.0)**	**6 (75.0)**	**8 (88.9)**	**7 (87.5)**	**6 (75.0)**	**8 (100.0)**
Arthralgia	–	1 (11.1)	1 (12.5)	1 (11.1)	–	–	2 (25.0)
Diarrhoea	1 (10.0)	3 (33.3)	1 (12.5)	1 (11.1)	2 (25.0)	–	–
Fatigue	6 (60.0)	6 (66.7)	2 (25.0)	6 (66.7)	5 (62.5)	4 (50.0)	7 (87.5)
Headache	6 (60.0)	5 (55.6)	2 (25.0)	4 (44.4)	5 (62.5)	5 (62.5)	6 (75.0)
Injection site erythema	–	2 (22.2)	–	–	–	–	1 (12.5)
Injection site swelling	–	1 (11.1)	–	–	1 (12.5)	–	2 (25.0)
Injection site pain	2 (20.0)	8 (88.9)	3 (37.5)	5 (55.6)	6 (75.0)	4 (50.0)	5 (62.5)
Myalgia	4 (40.0)	6 (66.7)	1 (12.5)	5 (55.6)	3 (37.5)	4 (50.0)	5 (62.5)
Pyrexia	2 (20.0)	–	2 (25.0)	1 (11.1)	3 (37.5)	1 (12.5)	2 (25.0)
Subjects with at least one unsolicited AE	**8 (80.0)**	**9 (100.0)**	**7 (87.5)**	**8 (88.9)**	**8 (100.0)**	**6 (75.0)**	**8 (100.0)**
Aspartate aminotransferase increased	–	1 (11.1)	1 (12.5)	1 (11.1)	1 (12.5)	2 (25.0)	–
Bradycardia	–	–	2 (25.0)	–	3 (37.5)	–	–
Haemoglobin decreased	2 (20.0)	1 (11.1)	–	1 (11.1)	3 (37.5)	1 (12.5)	2 (25.0)
Heart rate decreased	1 (10.0)	1 (11.1)	–	3 (33.3)	–	2 (25.0)	2 (25.0)
Nausea	–	1 (11.1)	–	–	–	1 (12.5)	3 (37.5)
Neutrophil count decreased	2 (20.0)	3 (33.3)	1 (12.5)	3 (33.3)	4 (50.0)	1 (12.5)	3 (37.5)
Pharyngolaryngeal pain	–	1 (11.1)	–	–	–	–	4 (50.0)
Protein in urine	1 (10.0)	1 (11.1)	1 (12.5)	2 (22.2)	1 (12.5)	–	2 (25.0)
Respiratory tract infection	4 (40.0)	2 (22.2)	4 (50.0)	4 (44.4)	4 (50.0)	2 (25.0)	1 (12.5)

Note: Individual unsolicited AEs are shown for AEs reported in ⩾10% of subjects across the combined H4:IC31 regimens. A full list of AEs is presented in Supplementary Table 2B.

significantly to the observed AEs. Among the unsolicited AEs, a post-vaccination inflammatory reaction was observed at the TST injection site in some of the subjects who were screened with the TSTs at the time of inclusion. Most of these TST reactions were mild or moderate, although one subject developed a more severe TST site reaction. Thus, TST screening was removed from the study protocol and should be omitted from subsequent studies of H4:IC31. Accordingly, TST was not performed during screening in another trial testing safety and immunogenicity of H4:IC31 in BCG-vaccinated adults enrolled in a high-endemic setting in South Africa (Aeras protocol C-011-404) [18]. The TST reaction mostly occurred upon vaccination with the H4 antigen alone, which suggested that this local inflammation was caused by the Mtb-specific antigens and not by the adjuvant. A similar post-vaccination reaction at the site of skin testing was reported in a clinical trial with another recombinant protein adjuvant TB vaccine candidate, M72F/AS02 [23]. This delayed-type hypersensitivity is likely caused by PPD-specific T cells that will rapidly migrate to the TST injection site in response to vaccine-antigens that are also present in PPD. Thus, the risk for post-vaccination TST site reactions needs to be considered upon administration of similar TB vaccine constructs.

There are no well-defined correlates or biomarkers of protective immunity in TB, although $CD4^+$ Th1 cells that express IFN-γ, IL-2 and TNF-α are considered necessary [24,25]. Ag85B-TB10.4 responses may provide the most consistent protection in populations with a high TB burden [26]. The TB10.4 antigen is recognized by T cells from both BCG-vaccinated and Mtb-infected individuals [7], and TB10.4-specific $CD4^+$ T cells correlated with protection against TB in mice [27]. Adoptive transfer of Ag85B/TB10.4-specific memory $CD4^+$ T cells from immunized mice also conferred protection against *M. bovis* BCG challenge in recipient mice [28]. Likewise, the H4:IC31 vaccine was able to elicit expansion of and cytokine-production in Mtb-specific $CD4^+$ T cells in BCG-vaccinated adults, similar to the low vaccine-specific $CD4^+$ T cell responses demonstrated in a related randomized trial conducted in South Africa (C-011-404) [18]. Overall, the magnitude and quality of the $CD4^+$ T cell responses induced by H4:IC31 seemed very similar between the South Africa trial and the dose-escalation trials conducted in a low-TB-endemic setting in Sweden and Finland. Both IFN-γ ELISpot and ICS results confirmed that after two doses of H4:IC31, cytokine producing T cells were detected primarily in response to the Ag85B component of H4 [18]. In mice, comparable T cell responses have been detected against both Ag85B and TB10.4 after vaccination with an Ag85B/TB10.4 subunit vaccine [8]. Despite the dominance of Ag85B-specific T cell responses, it is possible that even very low responses to TB10.4 in H4:IC31 vaccinated individuals could be expanded upon exposure to Mtb, especially since TB10.4 epitopes are presented during active TB infection [7].

Multifunctional T cells, co-expressing several cytokines including IFN-γ, IL-2, and TNF-α, have been associated with immune control of parasites [28], and viral [29] and bacterial infections [30]. It has been suggested that the IFN-γ/IL-2/TNF-α triple positive cells represent effector memory T cells, the IL-2/TNF-α double positive cells central memory cells, and the IFN-γ single positive cells terminally differentiated effector T cells [24]. In the experimental mouse model, the TB subunit vaccine Ag85B-ESAT-6/CAF01 was shown to induce high levels of multifunctional memory $CD4^+$ T cells with protective efficacy that accumulated at the site of infection after Mtb challenge, while fewer multifunctional T cells were evident upon BCG vaccination [31]. Importantly, two doses of the Ag85B-ESAT-6/CAF01 subunit vaccine induced long-term memory responses [22]. The H4:IC31 vaccine induced primarily IFN-γ/IL-2/TNF-α triple positive and IL-2/TNF-α double positive $CD4^+$ Th1 subsets that correlated with protection against subsequent challenge with Mtb in mice [14,16]. An increased proportion of TB10.4-specific multifunctional $CD4^+$ T cells were also found at the site of infection when mice received H4:IC31 as a boost to the BCG vaccine compared to vaccination with BCG alone [14]. Our observations suggest that the H4:IC31 vaccine induced similar multifunctional $CD4^+$ T cell profiles in BCG-vaccinated human subjects, which is also consistent with the H4:IC31 trial in South Africa [18]. Such multifunctional $CD4^+$ T cells were also observed in

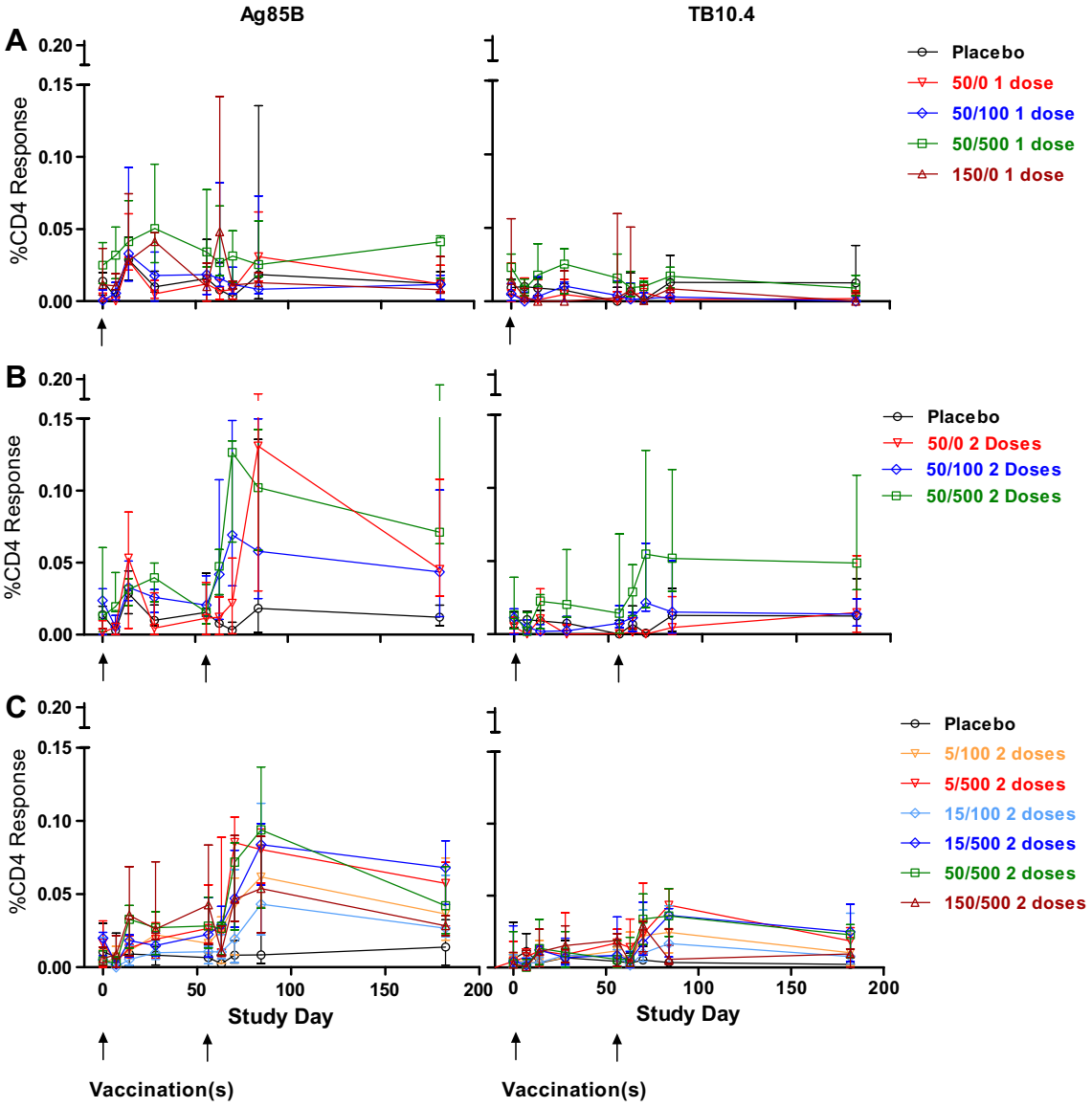

Fig. 2. H4:IC31 vaccination induces elevated levels of antigen-specific CD4$^+$ T cells in the peripheral circulation of BCG-vaccinated study subjects. Frequencies of Ag85B- and TB10.4-specific CD4$^+$ T cells induced by the H4:IC31 vaccine were measured by 7-color ICS assay and flow cytometry after stimulation of PBMCs with Mtb antigen-peptide pools. DMSO-subtracted antigen-specific expansion of the CD4$^+$ T cell subset in response to Ag85B or TB10.4 is presented for the different treatment regimens in study C-005-404 one dose (A) and two dose (B) regimens and study C-006-404 (C). Samples for the ICS assay were collected at study days 0, 7, 14, 28, 56, 63, 70, 84, and 182. Data are presented in linear graph plots showing the median and interquartile range for each group.

PPD-negative human subjects after vaccination with the M72/AS01 vaccine [32]. The induction of memory cells by the H4:IC31 vaccine can be a source of new effector cells that emerge after immune contraction, which could be particularly important for control of chronic infections.

These trials clearly show stronger and more persistent antigen-specific CD4$^+$ T cell responses upon vaccination with the H4 antigen together with the IC31 adjuvant compared to H4 alone. The IC31 adjuvant has previously been demonstrated to augment sustained Ag85B-ESAT-6 specific IFN-γ responses in healthy human subjects [9]. We also found a clear advantage of two vaccinations compared to only one dose. In the South Africa trial, the H4:IC31 vaccine containing the 15 μg dose showed a stronger boosting effect after the second vaccination compared to the 5 and 50 μg dose levels [18], while a similar discrepancy between the 5, 15 and 50 μg H4 doses was not apparent in our trials. In addition, one dose was shown to induce post-vaccination IFN-γ responses above pre-vaccination responses in most of 8 participants in the South African trial [18], whereas participants receiving only one dose in our Nordic trials maintained low T cell responses. The explanation for these differences is unclear, but it is difficult to make conclusions from these smaller studies (around 8 subjects/per vaccine regimen), although the results may reflect on the different study populations i.e. Africans from a high-TB-endemic country who may also become exposed to non-tuberculous mycobacteria versus northern Europeans from low-TB-endemic countries who may be more naïve to Mtb as well as environmental mycobacteria.

Interestingly, an increased H4 antigen dose from 50 μg to 150 μg induced weaker CD4$^+$ T cell responses, which is consistent with other studies showing that induction of Mtb-specific multifunctional T cells by Ag85B-TB10.4/IC31 was highly depended on the H4 antigen dose [16,18]. In contrast to higher antigen doses, a lower antigen dose selectively increased the number of

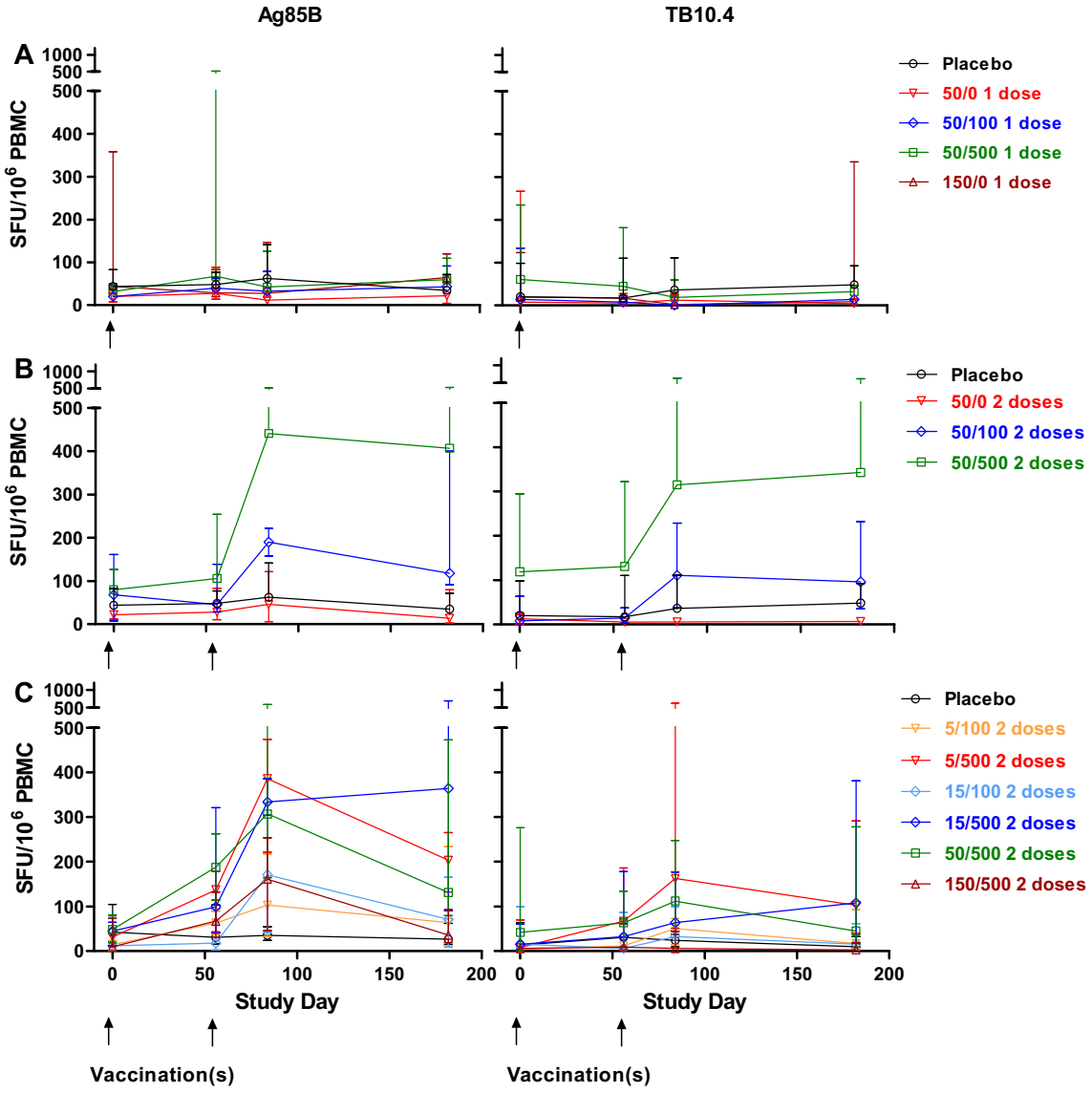

Fig. 3. H4:IC31 vaccination induces antigen-specific IFN-γ production in peripheral T cells from BCG-vaccinated study subjects. Frequencies of Ag85B-specific IFN-γ producing T cells induced by the H4:IC31 vaccine were measured using the IFN-γ ELISpot assay after stimulation of PBMCs with Mtb antigen-peptide pools. Expression of IFN-γ in Ag85B-specific T cells is shown for the different treatment regimens at study days 0, 56, 84 and 182 in study C-005-404 one dose (A) and two dose (B) regimens and C-006-404 (C). The data illustrate medium-subtracted spot-forming cells per 10^6 PBMCs for all vaccinated subjects. Data are presented in linear graph plots showing the median and interquartile range for each group.

multifunctional T cells, and was associated with a considerably stronger protection against subsequent Mtb challenge in mice [16]. Therefore, finding the appropriate antigen and adjuvant doses is a crucial factor when testing new vaccines. Our data suggest that low range H4 antigen doses in combination with the higher dose of the IC31 adjuvant would be the optimal vaccine regimen to use in a larger scale.

Protective immunity in TB is also dependent on the induction of $CD8^+$ cytolytic T cell (CTL) responses [33]. Using a whole blood assay (FASCIA), we demonstrated that similar to $CD4^+$ T cell responses, significant proliferation of Ag85B-stimulated $CD8\alpha\alpha^+$ T cells were seen with the two dose 50/500 H4:IC31 vaccine compared to the two dose placebo regimen. These results were consistent with previous data demonstrating an increased in vitro proliferation of $CD4^+$ T cells and $CD8\alpha\alpha^+$ T cells in response to Ag85B, after immunization with the TB vaccine candidate rBCG AFRO-1 in non-human primates [21]. It is believed that $CD8\alpha\alpha^+$ T cells comprise effector memory cells and terminally differentiated memory cells, and persistence of these cells may reflect the presence of continuous antigen stimulation [34]. IC31 is mostly considered a $CD4^+$ T cell priming adjuvant that induces activation of major histocompatibility complex (MHC) class II^+ dendritic cells (DCs) including an up-regulated expression of co-stimulatory molecules [35]. It has been suggested that adjuvant-activated DCs may also trigger $CD8^+$ T cells via TLR9-dependent production of IFNβ and enhanced MHC class I antigen presentation [36]. However, few studies have investigated the effects of the IC31 adjuvant on the induction of $CD8^+$ T cell responses in vivo and/or in humans [37], which may be relatively lower compared to $CD4^+$ T cells [14]. To enhance $CD8^+$ T cell activation more efficiently, a pore-forming protein such as a bacterial cytolysin, e.g. perfringolysin [21,38] or listeriolysin [39], can be introduced in the vaccine construct to permit leakage of antigens from the phagosome to the cytosolic MHC class I pathway.

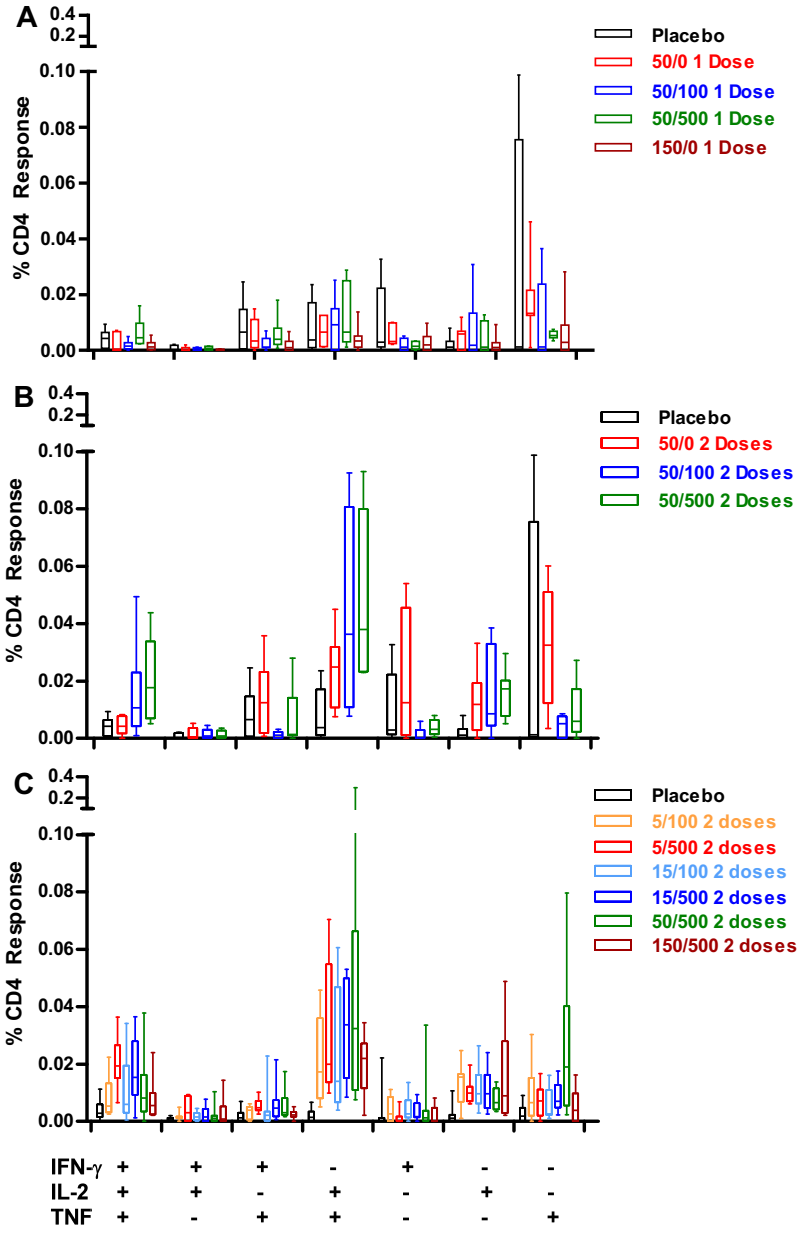

Fig. 4. H4:IC31 vaccination induces antigen-specific multifunctional CD4$^+$ Th1 responses in the peripheral circulation of BCG-vaccinated study subjects. Frequencies of cytokine-expressing Ag85B-specific CD4$^+$ T cells expressing IFN-γ, IL-2 and/or TNF-α induced by the H4:IC31 vaccine, were measured by 7-color ICS assay and flow cytometry after stimulation of PBMCs with Mtb antigen-peptide pools. DMSO-subtracted cytokine responses in Ag85B-specific CD4$^+$ T cells are shown for the different treatment regimens at study day 84 in study C-005-404 one dose (A) and two dose (B) regimens and C-006-404 (C). Data are presented in box and whiskers plots showing the median, interquartile range, minimum and maximum for each group.

Encouraging results from these trials support further clinical evaluation and development of the H4:IC31 vaccine candidate to boost protective immunity against TB. In view of that, H4:IC31 is currently being evaluated for the ability to prevent Mtb infection in QuantiFERON-negative healthy adults compared to placebo and BCG revaccination in a randomized controlled trial conducted in South Africa (Aeras protocol C-040-404 and Clinicaltrial.gov ID: NCT02075203).

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Conflict of interest statement

The authors declare no conflict of interest.

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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.2017.01.055.

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