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# A novel liposomal adjuvant system, CAF01, promotes long-lived *Mycobacterium tuberculosis*-specific T-cell responses in human

Jaap T. van Dissel[a,*,1], Simone A. Joosten[a,1], Søren T. Hoff[c], Darius Soonawala[a], Corine Prins[a], David A. Hokey[d], Dawn M. O'Dee[d], Andrew Graves[d], Birgit Thierry-Carstensen[b], Lars V. Andreasen[b], Morten Ruhwald[c], Adriëtte W. de Visser[a], Else Marie Agger[c], Tom H.M. Ottenhoff[a], Ingrid Kromann[b], Peter Andersen[c,**]

[a] *Leiden University Medical Center (LUMC), Department of Infectious Diseases, Leiden, The Netherlands*
[b] *Statens Serum Institut, Department of Vaccine Development, Artillerivej 5, Copenhagen 2300s, Denmark*
[c] *Statens Serum Institut, Department of Infectious Disease Immunology, Artillerivej 5, Copenhagen 2300s, Denmark*
[d] *Aeras, Rockville, MD, USA*

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ABSTRACT

Here, we report on a first-in-man trial where the tuberculosis (TB) vaccine Ag85B-ESAT-6 (H1) was adjuvanted with escalating doses of a novel liposome adjuvant CAF01. On their own, protein antigens cannot sufficiently induce immune responses in humans, and require the addition of an adjuvant system to ensure appropriate delivery and concomitant immune activation. To date no approved adjuvants are available for induction of cellular immunity, which seems essential for a number of vaccines, including vaccines against TB. We vaccinated four groups of human volunteers: a non-adjuvanted H1 group, followed by three groups with escalating doses of CAF01-adjuvanted H1 vaccine. All subjects were vaccinated at 0 and 8 weeks and followed up for 150 weeks. Vaccination did not cause local or systemic adverse effects besides transient soreness at the injection site. Two vaccinations elicited strong antigen-specific T-cell responses which persisted after 150 weeks follow-up, indicating the induction of a long-lasting memory response in the vaccine recipients. These results show that CAF01 is a safe and tolerable, Th1-inducing adjuvant for human TB vaccination trials and for vaccination studies in general where cellular immunity is required.



## 1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB). A third of the world's population is infected with MTB, in 2013 there was a global estimated 8.6 million cases of TB and 1.3 million deaths caused by this pathogen [1]. Currently, the only available vaccine against TB is bacillus Calmette-Guérin (BCG), a live attenuated vaccine derived from *Mycobacterium bovis*. BCG protects against severe forms of childhood TB but its efficacy against pulmonary TB in adults is highly variable. Therefore, there is an urgent need for second generation TB vaccines [2,3].

Several novel vaccines are being explored, among which a prime-boost strategy using new TB vaccine candidates to boost BCG is considered a promising strategy [4]. In a recent phase IIb trial, an experimental vaccine MVA85A (modified vaccinia virus Ankara expressing antigen 85A) was given to infants who had previously been BCG-vaccinated, however the MVA85A vaccine failed to demonstrate efficacy against TB infection as well as TB disease emphasizing that there is a continued need for developing and testing novel vaccination strategies against TB [5]. We have published two human clinical trials investigating the Hybrid 1(H1) subunit vaccine; based on the hybrid protein of Early Secretory Antigenic Target (ESAT-6) and Antigen 85B (Ag85B) adjuvanted with IC31® (H1:IC31) [6,7]. These reports demonstrated that the H1:IC31 vaccine was safe and generated long-lasting antigen-specific Th1 T-cell responses against the hybrid protein [6,7].

Here we report on an independent H1 TB vaccine trial in which the adjuvant IC31® is replaced by the CAF01 adjuvant. CAF01 is a novel two-component liposomal adjuvant system composed of a cationic liposome vehicle (dimethyldioctadecyl-ammonium

* Corresponding author at: Leiden University Medical Center (LUMC), Department of Infectious Diseases, C5P, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Tel.: +31 71 526 26 20; fax: +31 71 526 67 58.
** Corresponding author at: Statens Serum Institut, Department of Infectious Disease Immunology, Artillerivej 5, Copenhagen 2300s, Denmark. Tel.: +45 3268 3462; fax: +45 3268 3035.
*E-mail addresses:* j.t.van_dissel@lumc.nl (J.T. van Dissel), PA@ssi.dk (P. Andersen).
[1] These authors contributed equally to this work.

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(DDA)) stabilized with a glycolipid immunomodulator (trehalose 6,6-dibehenate (TDB)) which is a synthetic variant of cord factor located in the mycobacterial cell wall. In addition to acting as an immunomodulator, TDB also ensures long-term stability of the DDA liposomes. Based on immunological data as well as physicochemical stability data the optimal weight ratio of DDA to TDB was found to be 5:1 [8]. In animal models, CAF01 promotes a broad and complex immune response characterized by multifunctional T-cells with a Th1 profile and possesses the same ability to induce long-lived immune responses as IC31® presumably through the establishment of a vaccine depot [8–13]. In preclinical studies, CAF01 also induced a Th17 response due to TDB signaling through the C-type lectin receptor Mincle [14]. CAF01 adjuvanted H1 vaccine was protective in animal models of TB [11–13,15], but safety and immunogenicity of a CAF01-adjuvanted vaccine has not yet been assessed in humans.

We report herein the first phase I clinical trial in human volunteers employing a CAF01-adjuvanted subunit TB vaccine (H1:CAF01), with safety as primary endpoint. The secondary objective of the trial was to evaluate the immunogenicity of H1:CAF01 in humans.

## 2. Materials and methods

An elaborated description of materials and methods can be found in the online supplement.

### 2.1. Ethics statement

All subjects volunteered to participate in the clinical trial and gave informed consent after verbal and written information was provided. The trial protocol (EUDRACT No.: 2008-006003-23, ClinicalTrials.gov Identifier: NCT00922363, LUMC protocol: P09.111), the Investigator's Brochure and the Investigational Medicinal Product Dossier were following good clinical practice (GCP) and the declaration of Helsinki and were approved by the accredited Ethical Review Board of LUMC and the relevant national authorities.

### 2.2. The investigational vaccine

CAF01 is a two-component liposomal adjuvant system developed by SSI [16,8,9,10]. One component, DDA, is a cationic quaternary ammonium salt and the other component, TDB, is a glycolipid. Both components are synthetically manufactured. When these two components are mixed and rehydrated in the specific weight ratio 5:1, DDA to TDB, the liposomal adjuvant is named CAF01. The H1 recombinant fusion protein of Ag85B and ESAT-6, is developed and manufactured by Statens Serum Institut (SSI, Copenhagen, Denmark). H1 sterile solution and CAF01 sterile suspension were manufactured by SSI, in an accredited GMP facility and supplied to the LUMC pharmacy in separate vials of relevant strengths. The vaccine was reconstituted by addition of the specified volume of adjuvant to the antigen concentrate, and injected into the deltoid muscle with a 25 mm 22–25 Gauge needle in a volume of 0.5 ml.

### 2.3. Trial design

The trial was an open label, single-center, non-randomized phase I exploratory trial in mycobacteria-naïve individuals defined by a negative TST (<10 mm, 2 units RT-23 PPD (SSI, Denmark)) and a negative Quantiferon®-TB Gold In-Tube test (QFT; Qiagen, Venlo, The Netherlands). All individuals were HIV negative. The trial comprised four vaccination groups. Subjects in group 1 received 50 μg H1 with no adjuvant, whereas groups 2–4 received the same amount of antigen with 125/25 μg, 313/63 μg and 625/125 μg CAF01, respectively. In all vaccination groups, the subjects were vaccinated on trial days 0 and 56. After the original trial was completed, a protocol amendment was approved (CCMO 12.1306/MA/26270, NL26270.000.09) and all trial participants were invited to attend a long-term visit 150 weeks after initial enrolment. Long-term visits were successfully conducted for 31 out of the original 34 volunteers that received 2 vaccinations within the appropriate time window. Timing of the long-term visit was on average 150.7 weeks (median 152.1 weeks; range 123–167 weeks) post primary vaccination and is referred to as '150 weeks' throughout the manuscript.

### 2.4. Trial subjects

The trial population consisted of 38 volunteers, healthy adult females or males between 18 and 55 years of age who had not been BCG vaccinated and who did not have active, chronic or past TB disease, and who had no MTB infection as confirmed by a negative QFT and a negative TST at screening. The general health of all participants was assessed by reviewing their recorded medical history, and performing a physical examination, and standard blood (including hepatitis B, hepatitis C and HIV testing) and urine tests.

The volunteers were financially compensated as approved by the Institutional Review Board for the number and amount of blood and urine samples, inconvenience with respect to the intramuscular administration and for the time spent on trial visits and transportation to the trial site.

### 2.5. Safety parameters

The subjects remained under medical observation for 3 h after each intramuscular vaccination, for possible immediate adverse reactions. During the first week after each vaccination, symptoms and evening armpit temperature were recorded on a daily basis, thereafter on a weekly basis. A medical examination of local adverse reactions and temperature was performed on days 0, 1, 7 and 42 after both vaccinations.

Adverse events were coded into system organ class (SOC) and preferred terms (PT) according to MedDRA version 10.0 and were classified into local (loco-regional) and systemic adverse events. The intensity of adverse events was graded as mild (grade 1/easily tolerated), moderate (grade 2/sufficient to interfere with daily activities) or severe (grade 3/preventing normal activity).

The relatedness of adverse events to the vaccination was graded as not related, possibly related, probably related or certainly related. Abnormal laboratory findings were scored for severity into severity grades 1–4 (based on "Toxicity grading scale for healthy adults and adolescent volunteers enrolled in preventive vaccine clinical trials" – FDA 2007 guidelines).

### 2.6. Quantiferon®-TB Gold In-Tube test

QFT testing was done according to the manufacturer's instructions and categorized as positive when the result was $\geq$0.35 IU/ml at baseline, and at 32 and 150 weeks after the primary vaccination.

### 2.7. PBMC isolation and immunogenicity parameters

Blood samples for cellular immunity and antibody determinations were collected at baseline and at 1 and 6 weeks after both vaccinations, and at weeks 32, 52 and 150 post the primary vaccination. Briefly, 40 ml heparinized blood was centrifuged on Leucosep tubes (Greiner-bio-one, Austria) containing 15 ml Ficoll (LUMC pharmacy #902861) (20 min/800 g), after centrifugation plasma was removed for storage at −70 °C and PBMCs were removed and washed three times with sterile PBS (LUMC pharmacy). PBMCs were aliquoted and stored in liquid nitrogen in RPMI (Invitrogen

#22409-015) containing 20% fetal calf serum (PAA Laboratories #A15-043, Netherlands)/10% DMSO (Sigma #41650). After defrosting a minimum PBMC viability of 80% was considered acceptable for assay purposes.

### 2.8. Flow cytometry

PBMCs were stimulated with pools from Ag85B or ESAT-6 peptides for 6 h or left unstimulated before staining for CD3, CD4, CD14, CD19, CD45RO, IFN-γ, IL-2, TNF-α, IL-22, IL-17A and CD154 (see online supplement) [18].

### 2.9. Quantifying IFN-γ producing cells

IFN-γ was determined using ELISpot from frozen samples to enable batch processing of longitudinally collected samples [19,20]. In this protocol, cells were thawed and pre-stimulated for 16–18 h, followed by 24 h incubation in the ELISpot plate [10] (see online supplement).

### 2.10. Multiplex analysis of cytokine release

PBMCs were stimulated 6 days with H1 fusion protein and a panel comprising cytokines (IFN-γ, IL-2, IL-4, IL-10, IL-13, IL-17A, IL-22, TNF-α), chemokines (IP-10, MIG, MCP-1, MIP-1b) and growth factors (VEGF and GM-CSF) were measured in undiluted cell culture supernatant samples using a Milliplex multiplex bead assay (see online supplement).

### 2.11. Statistical analysis

Clinical data were collected in CRFs, subject diaries and laboratory records. The statistical analysis of the data was performed by JG Consult, an independent Contract Research Organization in accordance with a statistical analysis plan and GCP and ICH-guidelines and documented in the clinical trial report. Here we report safety results and safety analysis based on the statistical trial report which was performed using SAS software (SAS®, Cary, NC 27513, USA, version 9.2 TS Level 1M0) on Platform Windows XP PRO Version 5.1.2600. Adverse events were evaluated descriptively.

Immunogenicity results shown here were analyzed at SSI and LUMC using Prism 6.04 for Windows (GraphPad Software, Inc., La Jolla, CA 92037, USA). Change from baseline to each observed visit within groups and comparisons between groups were compared using Kruskal–Wallis test with Dunn's correction. No formal sample size calculation was performed in this trial. An alpha <0.05 was considered significant throughout the trial.

## 3. Results

### 3.1. Trial subjects

Of 49 screened subjects 38 were included in the clinical trial. The safety population consisted of all included subjects. Mean ages were 20.7, 22.2, 30.5, and 24.6 years in vaccination groups 1, 2, 3 and 4, respectively, overall mean age of 24.9 years, ranging from 18–51 years. Seven subjects (7 females) were vaccinated with 50 μg H1 (no adjuvant), 10 subjects (2 male, 8 female) with 50 μg H1 + 125/25 μg CAF01 (low adjuvant group), 11 subjects (2 male, 9 female) with 50 μg H1 + 313/63 μg CAF01 (intermediate adjuvant group) and finally, 10 subjects (1 male, 9 female) with 50 μg H1 + 625/125 μg CAF01 (high adjuvant group). A total of 34 subjects were included in the per-protocol population and 7, 9, 10 and 8 from groups 1, 2, 3 and 4, respectively, were included in the immunogenicity analysis (Fig. 1). Long-term visits, 150 weeks after initial enrolment, were successfully conducted for 31 out of the original 34 per protocol trial subjects; 7, 9, 9 and 6 from groups 1–4, respectively.

### 3.2. Safety results

All 38 subjects with at least one vaccination were included in the safety analysis. No vaccine related serious or severe adverse reactions occurred during the trial. Loco-regional injection site reactions occurred more frequently in those given the CAF01-adjuvanted antigen, and mainly included stiffness (defined as injection site movement impairment) and pain at the injection site one day after the vaccinations (Table 1). Of note, these reactions were not more frequent after the second vaccination and there was no significant difference between the three adjuvant doses. In total, any local adverse reactions were distributed with 6 events in 2 (29%) subjects in the non-adjuvanted group 1, 26 events in 10 (100%) subjects in group 2, 24 events in 9 (82%) subjects in group 3 and 26 events in 9 (90%) subjects in group 4. None of the subjects required analgesics and all experienced full recovery within a maximum of 4 days. A small, cold nodule at the injection site was noted in 1 subject in the intermediate CAF01 dose group 3. No signs of attendant inflammation or local vesiculation, axillary lymphadenitis or fistula did occur, and the nodule had disappeared within one week. One subject in group 4 (in concomitant treatment with tramadol) did not receive the second vaccination due to rash and itch on knees, hips and elbows, as a relation to the trial vaccine could not be ruled out. Three individuals observed reactivity at the TST injection site after vaccination, two were in the mid- and high-dose adjuvant groups (reporting erythema and induration) and one was in the group receiving H1 without adjuvant (reporting erythema and swelling). A fourth individual observed erythema and induration at the site of the first vaccination after the 2nd vaccination (Table 1).

Systemic adverse reactions included headache, fatigue, malaise and fever in one subject given antigen only. Extensive follow-up of blood and urine parameters did not reveal any obvious trends within or differences between the three vaccination groups, or laboratory abnormalities with respect to change from baseline that could be related to the vaccinations. In the two subjects who developed a transient fever the day after vaccination, a small rise in C-reactive protein occurred that had subsided within a week.

### 3.3. Immunogenicity results

#### 3.3.1. IFN-γ ELISpot

Stimulation with H1, Ag85B and ESAT-6 gave rise to an increased number of spot forming units (SFU) in all adjuvant groups (Fig. 2A and B). The highest proportion of responders to vaccination was seen in the low CAF01 group at week 32 and in the intermediate CAF01 group at week 32 and 52 (Fig. 2C). At this time point median responses were 301 SFU/per million PBMC (inter quartile range (IQR) 111–668 SFU) for H1; 308 SFU (IQR 108–558 SFU) for Ag85B and 39 SFU (IQR 9.5–136 SFU) for ESAT-6, $p < 0.05$ (Fig. 2B). No changes from baseline were seen in the non-adjuvant group at any time points. Overall, there was a clear trend in the adjuvant groups that responses increased after the first vaccination and that a second vaccination further increased the magnitude of responses (Fig. 2A).

#### 3.3.2. Multiplex analysis of secreted biomarkers

To assess the breadth of the vaccine-induced immune memory, we performed an exploratory multiplex analysis of 14 cytokines and chemokines in supernatants of 24 h H1 stimulated PBMCs. We observed a broad induction of multiple cytokines and chemokines at both weeks 14 and 32 for the three groups vaccinated with

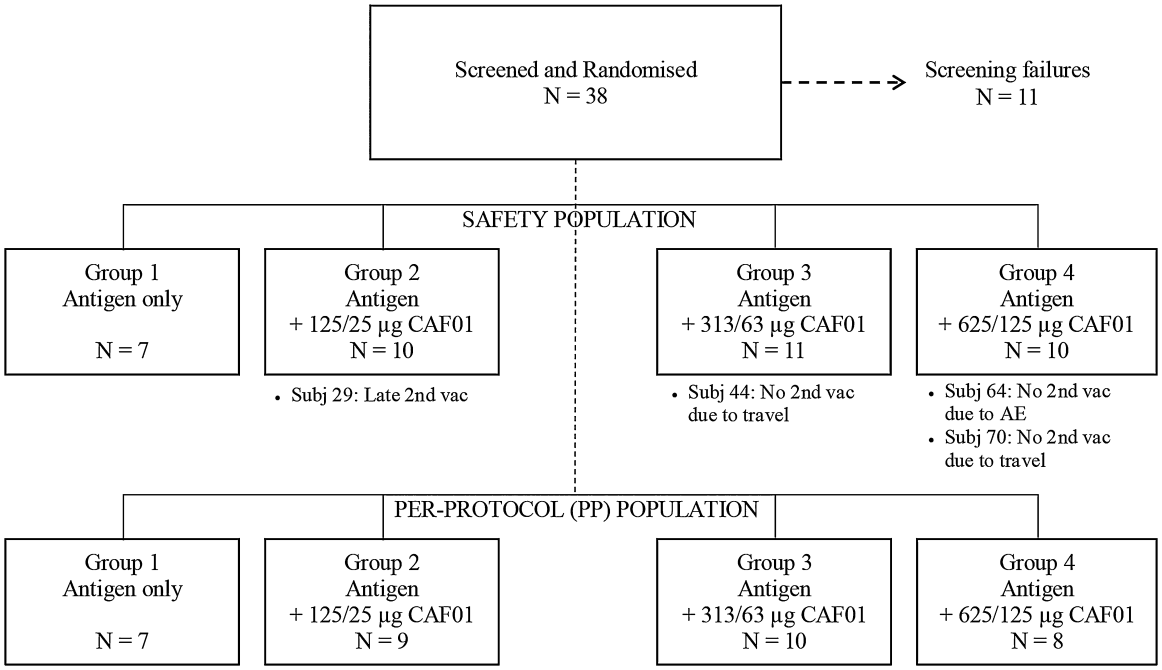


**Fig. 1.** Study overview and flow chart. Overview of volunteers screened and included into the clinical trial. All volunteers vaccinated were included in the safety population, whereas only those that received both vaccinations were included in the per-protocol population used for immunological analyses.

adjuvanted H1, responses in the intermediate CAF01 group are presented in Fig. 3 (all groups in supplementary Figure 1). The dominating markers were Th1 associated (IFN-γ, TNF-α, IP-10, MIG, MIP-1b and GM-CSF), but we also observed a substantial release of IL-13, but not IL-4. IL-2, IL-10 and IL-17 followed the same kinetic pattern, but levels were very low (<20 pg/ml) and failed to reach significance (Fig. 3 and data not shown). No clear pattern emerged for VEGF, IL-22 and MCP-1 (supplementary Figure 1).

**Table 1**

| | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| | H1 only | H1 + 125/25 µg CAF01 | H1 + 313/63 µg CAF01 | H1 + 625/125 ug CAF01 |
| Safety analysis set, *n* | 7 | 10 | 11 | 10 |
| Local adverse reactions; number of subjects (%), total number of reactions | | | | |
| *Reactions at site of injection* | | | | |
| Injection site stiffness | 1 (14) 1 | 3 (30) 3 | 9 (82) 13 | 7 (70) 8 |
| Injection site pain | 0 (0) 0 | 10 (100) 15 | 2 (18) 2 | 5 (50) 6 |
| Injection site swelling | 0 (0) 0 | 2 (20) 2 | 2 (18) 2 | 4 (40) 5 |
| Injection site erythema | 0 (0) 0 | 2 (20) 2 | 1 (9) 1 | 3 (30) 3 |
| Injection site induration | 0 (0) 0 | 1 (10) 1 | 1 (9) 1 | 0 (0) 0 |
| Injection site pruritus | 0 (0) 0 | 1 (10) 1 | 0 (0) 0 | 1 (10) 2 |
| Injection site bruising | 0 (0) 0 | 1 (10) 1 | 0 (0) 0 | 0 (0) 0 |
| *Other local reactions* | | | | |
| Lymphadenopathy | 0 (0) 0 | 1 (10) 1 | 1 (9) 1 | 0 (0) 0 |
| *Reactions at site of tuberculin skin testing* | | | | |
| Erythema | 1 (14) 1[b] | 0 (0) 0 | 1 (9) 2[b,a] | 1 (10) 1[b] |
| Induration | 0 (0) 0 | 0 (0) 0 | 1 (9) 2[b,a] | 1 (10) 1[b] |
| Swelling | 1 (14) 2[b] | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| *Reactions at site of first vaccination* | | | | |
| Erythema | 1 (14) 1 | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| Induration | 1 (14) 1 | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| Any local adverse reaction | 2 (29) 6 | 10 (100) 26 | 9 (82) 24 | 9 (90) 26 |
| Systemic adverse reactions; *n* (%), total number of reactions | | | | |
| Fatigue | 2 (29) 3 | 1 (10) 1 | 0 (0) 0 | 3 (30) 3 |
| Malaise | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 | 2 (20) 3 |
| Pyrexia | 1 (14) 1 | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| Rhinitis | 1 (14) 1 | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| Musculoskeletal stiffness | 0 (0) 0 | 0 (0) 0 | 1 (9) 1 | 0 (0) 0 |
| Myalgia | 1 (14) 1 | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 |
| Headache | 1 (14) 1 | 1 (10) 1 | 1 (9) 1 | 2 (20) 3 |
| Pruritus | 0 (0) 0 | 0 (0) 0 | 1 (9) 1 | 1 (10) 1 |
| Rash pruritic | 0 (0) 0 | 0 (0) 0 | 0 (0) 0 | 1 (10) 1 |
| Any systemic adverse reaction | 3 (43) 7 | 2 (20) 2 | 3 (27) 4 | 4 (40) 11 |

Injection site stiffness reactions (MedDRA PT term Injection site movement impairment) were of mild intensity (easily tolerated) except for one of moderate intensity.

[a] One subject had erythema and induration at the 1st injection site after the 2nd vaccination.

[b] Three subjects had erythema, induration and or swelling at the Tuberculin injection site (of original PPD skin testing) after the 1st and/or 2nd vaccination.

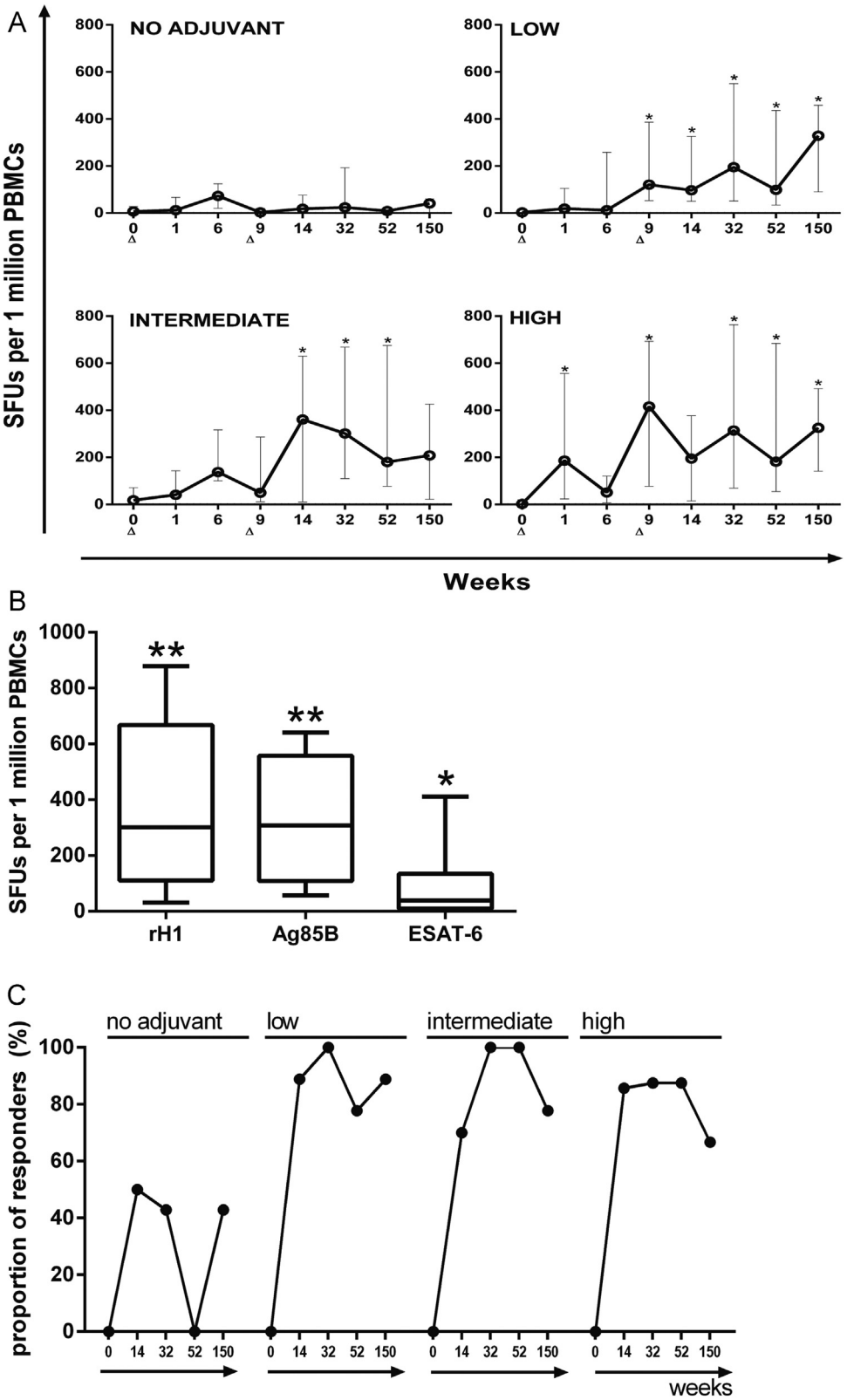


**Fig. 2.** IFN-γ spot forming units (SFUs) per 1 million PBMCs measured by ELISpot. (A) Healthy volunteers were given two doses of intramuscular injection of 50 μg of H1 antigen without CAF01 adjuvant ($n = 7$), low dose CAF01 adjuvant ($n = 9$), intermediate dose CAF01 adjuvant ($n = 10$) or high dose CAF01 adjuvant ($n = 8$). Vaccinations were done at week 0 and week 8 and PBMC samples were collected at weeks 0, 1, 6, 9, 14, 32, 52 and 150 and stimulated with H1 protein. Interconnected dots represent median, error bars interquartile range. Δ indicates vaccination, * indicates $p < 0.05$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test). (B) Healthy volunteers were given two doses of intramuscular injection of 50 μg of H1 antigen in intermediate dose CAF01 adjuvant ($n = 10$). Vaccinations were done at week 0 and week 8 and PBMC samples were collected at week 32. PBMCs were stimulated with H1 protein or Ag85B or ESAT-6 peptide pools and IFN-γ producing spots quantified using ELISpot. Responses are presented as median error bars interquartile range. * indicates $p < 0.05$, ** $p < 0.005$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test). (C) The proportion of responders to H1 stimulation was defined as the number of H1 induced IFN-γ-producing spots greater than the mean + 2.5 SD of unstimulated cells and was expressed as percentage of the group size.

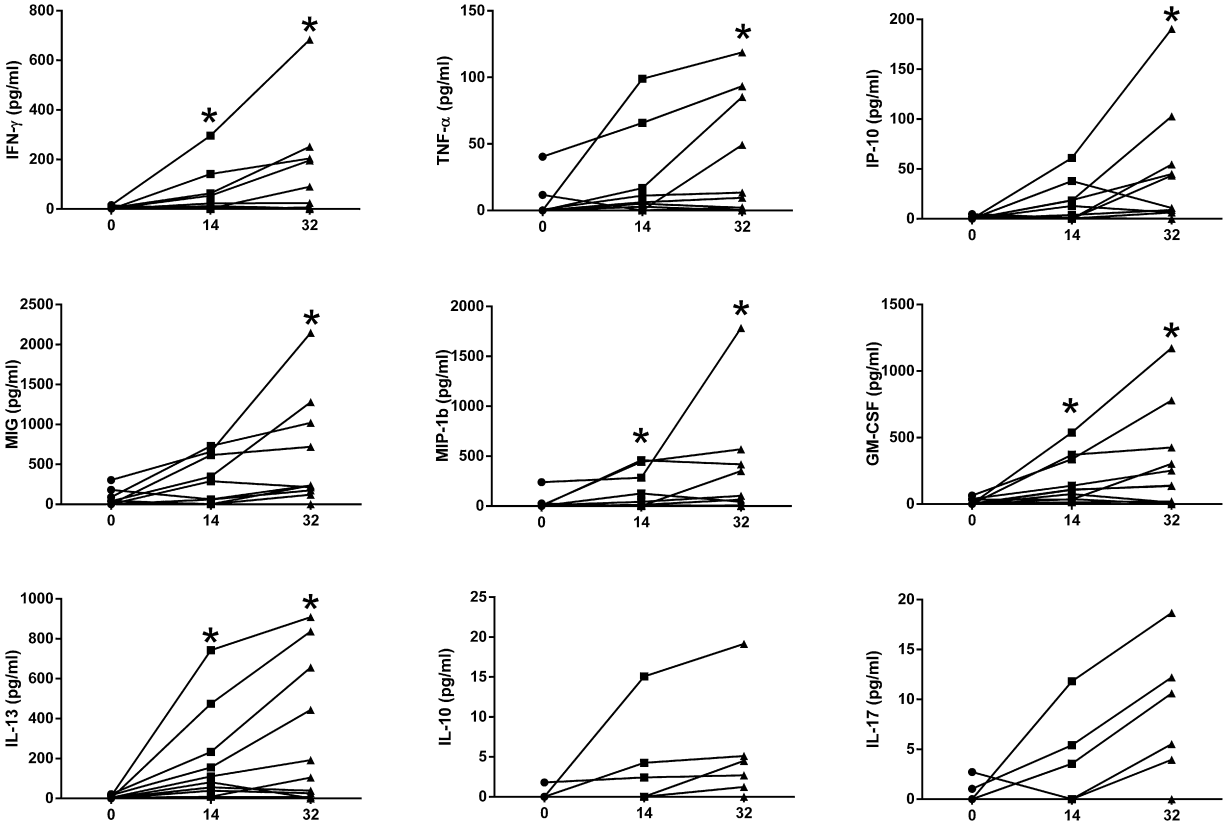


**Fig. 3.** Cytokine secretion in supernatants of 6 day H1 stimulated PBMCs. PBMCs were collected at baseline and at weeks 14 and 32 after two doses of intramuscular injection of 50 μg of H1 antigen. PBMCs were stimulated with H1 antigen for 6 days, supernatants were collected and cytokines and chemokines were measured using multiplex bead arrays. Only individuals from the intermediate dose CAF01 adjuvant are shown ($n = 10$). Responses are presented as individual measurements, * indicates $p < 0.05$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test).

### 3.3.3. *Flow cytometry*

To further assess the long-term immunogenicity of H1:CAF01, PBMC samples at week 150 were analyzed by Intracellular flow cytometry. Compared to the non-adjuvant group, intermediate and high dose CAF01 groups had increased frequencies of Ag85B-specific CD4 T-cells producing IFN-γ and/or IL-2 and/or TNF-α (Fig. 4A). Moreover, intermediate and high dose CAF01 groups induced significant TNF-α production, but only the intermediate CAF01 group reached significant levels of IL-2 (Fig. 4B) ESAT-6 specific CD4 T-cells were seen in the adjuvant groups, but responses were not significantly different from those in the non-adjuvant group. The most prevalent subset was IL-2/TNF-α double producing CD4 T-cells, and significantly increased frequencies of these cells were seen in the intermediate and high adjuvant groups compared to the non-adjuvant group (Fig. 4C). Responses were also detected in the triple positive subset and TNF-α single positive subset, but neither reached significance. No significant IL-17 responses to antigenic stimulation were detected (data not shown). No CD8 T-cell responses were observed following Ag85B or ESAT-6 stimulation (data not shown).

### 3.3.4. *Antibody responses*

No statistically significant changes from baseline were seen in any of the vaccination groups in IgG anti-Ag85B-ESAT-6 specific antibody titer (data not shown, methods in online supplement).

### 3.3.5. *Quantiferon®-TB Gold conversion*

QFT was performed at baseline at week 32, and 150 weeks after the last vaccination. All subjects were negative before vaccination (as per the inclusion criteria) and none in the non-adjuvanted group became QFT positive. However introducing CAF01 adjuvant in the vaccine caused 3 out of 8 (38%) individuals in the low CAF01 group to convert to a positive test, 6 out of 10 (60%) in the intermediate CAF01 group and 3 out of 8 (38%) in the high adjuvant group (Fig. 5). All but two of the QFT converters had reverted to negative at week 150. One QFT converter was lost to the extended follow up.

## 4. Discussion

This report describes the first clinical trial in humans investigating the TB vaccine H1:CAF01, combining a new liposomal adjuvant CAF01 with a well-defined TB subunit vaccine antigen H1. In this study, the vaccine was safe, well tolerated and generated long-lasting (3 years) T-cell responses, as monitored by IFN-γ ELISpot, intracellular cytokine staining and multiplex analysis of 14 secreted cytokines and chemokines.

Two vaccinations with H1:CAF01 did not lead to any serious adverse reactions. All adverse events that were assessed as related to the vaccination were mild or moderate and disappeared within days. The main H1:CAF01-related adverse event was stiffness and pain at the injection site, of mild to moderate severity, mostly the day after administration of the vaccine. A mild to moderate transient local reactogenicity of H1:CAF01 was anticipated based on the findings in nonclinical GLP toxicity studies and was also observed in previous vaccination studies in humans with the H1 antigen [6,7,21]. The vaccine did not consistently affect hematological or biochemical measurements. In conclusion, this clinical trial found no safety concerns associated with the administration of the CAF01-adjuvanted vaccine to healthy adults. As this was a phase I trial, the limitation to this conclusion is the limited number of

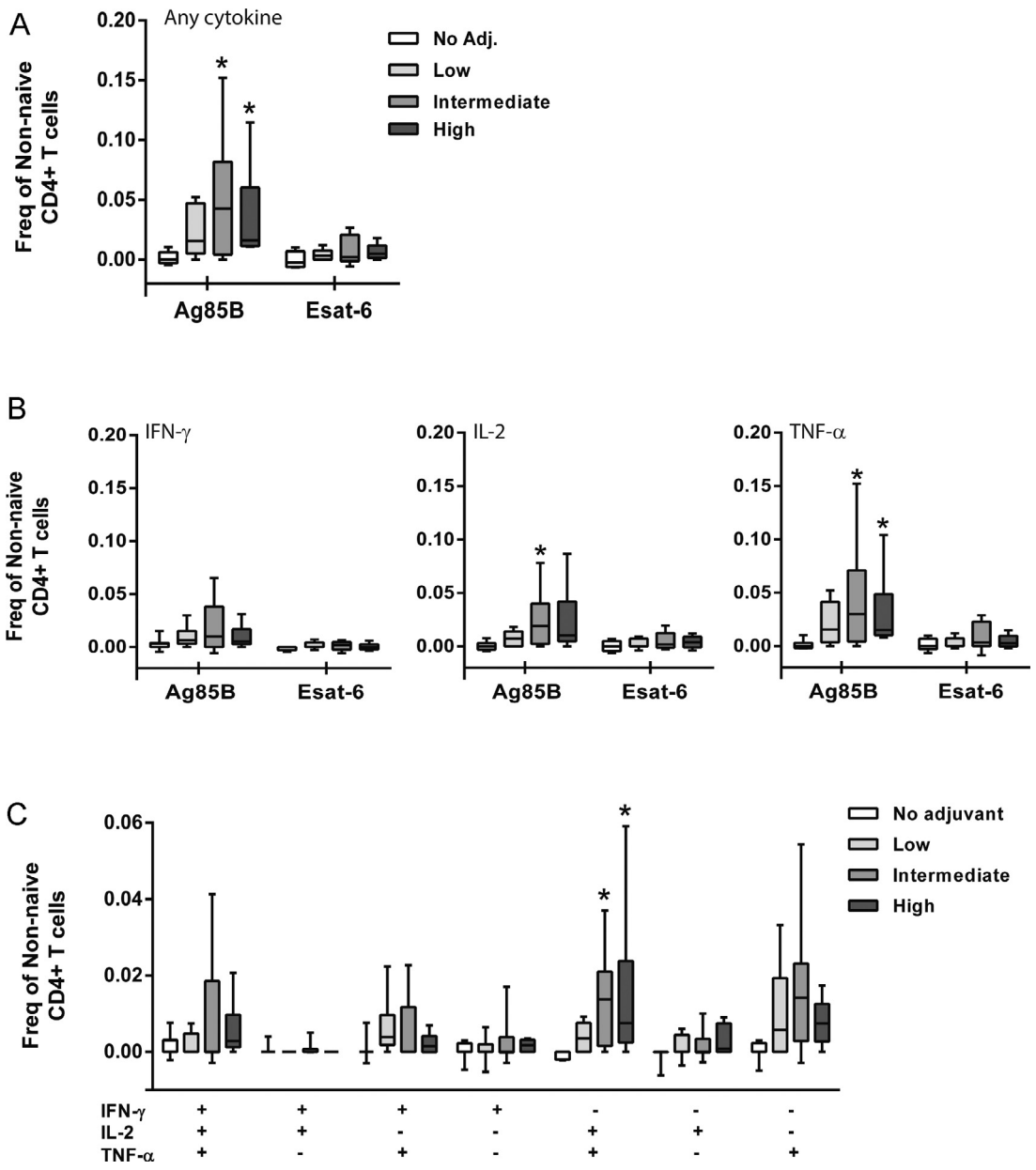


**Fig. 4.** Ag85B- and ESAT-6-specific CD4 responses in PBMCs collected at week 150 measured by flow cytometry. (A) Long-term visits at week 150 after vaccinations were successfully conducted for 31 out of the original 34 per protocol trial subjects; 7, 9, 9 and 6 from groups 1–4, respectively. PBMCs were isolated and frequencies of IFN-γ, IL-2 or TNF-α producing $CD4^+CD154^+$T-cells were measured after stimulation with Ag85B or ESAT-6 peptide pools. Responses are presented as box and whiskers, wherein the box represents median and interquartile range, whiskers range. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group. (B) Frequency of IFN-γ (left), IL-2 (middle) or TNF-α (right) producing $CD4^+CD154^+$T-cells were measured after stimulation of week 150 PBMCs with Ag85B or ESAT-6 peptide pools. Responses are presented as box and whiskers, wherein the box represents median and interquartile range, whiskers range. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group. (C) Patterns of single or combined production of IFN-γ, IL-2 or TNF-α by $CD154^+CD4^+$T-cells after stimulation of with Ag85B peptide pool are shown. The median frequency for each cytokine-producing cell subset is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group.

subjects, and we can exclude with certainty only frequently occurring adverse reactions. On the other hand, the lack of any significant reactogenicity despite the immunogenicity of the CAF01-adjuvanted vaccine is important, given the paucity of effective human adjuvants for the induction of cell-mediated immune responses [22].

Successful vaccination against TB disease would be a major step to diminish TB disease burden and spread, however an important challenge remains to determine vaccine efficacy. Despite significant investments in the search for an accurate surrogate endpoint for protection against TB disease, no such biomarker has been identified. However, there is general consensus that an effective TB vaccine needs to be able to elicit at least a Th1 cell response which is essential for bacterial containment [23]. Importantly, due to the nature of the pathogen, a novel vaccine will need to induce long-lived protection, most likely through the induction of central memory T ($T_{CM}$) cells. Whereas IFN-γ production is the classical hallmark of Th1 cell responses and for many years has been used as the primary measurement in TB vaccine clinical testing, CD4 T-cells with a regenerative potential are typically IL-2

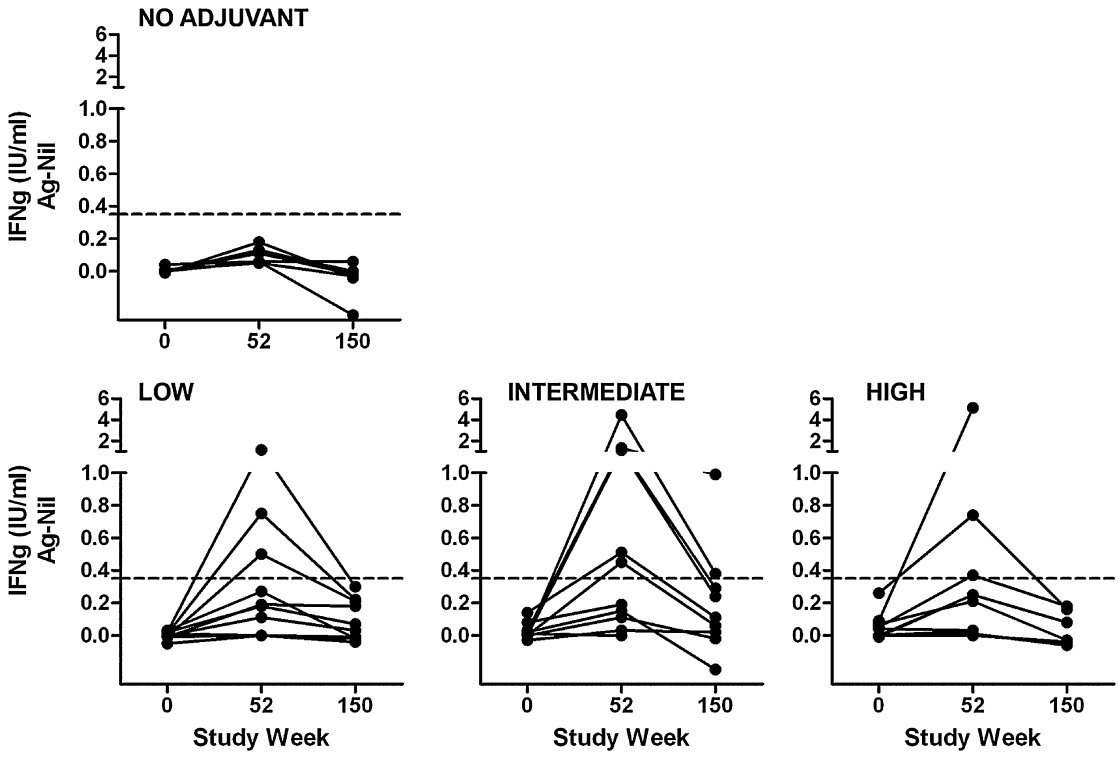


**Fig. 5.** Quantiferon TB Gold in tube (QFT) IFN-γ responses after vaccination with H1 alone and adjuvanted with low, intermediate and high CAF01 dose. Whole blood was collected in QFT blood collection tubes at baseline, week 52 and week 150 and incubated 16–24 h. Plasma was isolated by centrifugation and IFN-γ was determined using ELISA. Responses were classified as positive if the antigen-specific level (Ag-Nil) was ≥0.35 IU/ml.

positive and $T_{CM}$ are usually functionally defined by the expression of IL-2 and CCR7/CD62L. Two vaccinations of H1:CAF01 induced a strong long-lasting cellular immune response to H1 and its two antigen components ESAT-6 and Ag85B. Responses were strongest to the Ag85B antigen, as observed previously also for H1:IC31 [6,7]. Measured by IFN-γ ELISpot, the vaccine led to increased responses at subsequent visits which were sustained also after 150 weeks, demonstrating a clear and long-term vaccine take in all three adjuvanted vaccine groups, but not in the non-adjuvanted group, as observed previously also for H1:IC31 [6,7]. This pattern was confirmed by the broad induction of mainly Th1 associated cytokines (IFN-γ, IL-2, TNF-α, GM-CSF) and chemokines (MIG, IP-10 and MIP-1β). Three years after vaccination, the intermediate and high H1:CAF01 dose groups showed significant numbers of antigen-specific CD4 T-cells secreting IL-2 and TNF-α, consistent with a central memory differentiation state, ready to become effector T-cells if required [24]. These results are in line with two recent and closely related TB vaccine trials investigating H1:IC31 in HIV-infected individuals, and H56:IC31 in healthy individuals with or without latent TB (Klaus Reiter, Gavin Churchyard, Thomas Scriba, personal communication), and recent results from a phase I/II trial of the subunit vaccine M72 adjuvanted in the liposome based $AS01_E$ [25]. These results underpin that estimates of vaccine immunogenicity based on IFN-γ detection alone will miss other relevant vaccine-induced immune responses. The prolonged maintenance of immune competence elicited by the CAF01-adjuvanted subunit vaccine is in good agreement with observations from mouse studies [11,12], and suggests that the adjuvant, likely through establishment of an antigen depot and subsequent slow release and targeting of dendritic cells [16], may have particular abilities to maintain immune memory [26]. In this regard, it is interesting to note that the development of immune profiles differ markedly between viral vectored vaccines and adjuvanted subunit vaccines with the latter having a slowly developing response dominated by IL-2/TNF-α double positive T-cells and with no tendency of a waned response over the three years observation time. Although MVA85A induces highly durable Th1 responses, peak responses were observed already 7 days post-vaccination [27] and with triple and double positive TNF-α/IFN-γ T-cells resembling a more effector-memory profile [28]. Whether this difference has any influence on the overall protective capability remains to be seen.

Significant amounts of IL-13 were also found in the intermediate and high dose CAF01 groups. IL-13 is traditionally associated with Th2-type immune responses and together with IL-4 involved in inflammatory disorders, however, a number of recent findings suggest a more complex lineation. Gallo and Katzman identified IL-13 producing CD4 T-cells in mice co-expressing IFN-γ and IL-17 generated both during autoimmune diseases but also upon immunization [29]. Although the induction of IL-13 in human vaccine trials is a relatively unexplored field, IL-13 responses has also been observed in volunteers receiving the Th1-promoting adjuvant MPL® [30] and synthetic HIV-1 peptides coupled to a palmytoil tail was found to induce both IFN-γ and IL-13 in a phase II trial [31]. These novel data show that IL-13 is an integrated component of a vaccine-induced Th1/Th17 response and an important role of IL-13 could be to down-regulate the vigorous inflammatory response induced by these novel generation adjuvants. We recently identified IL-13 secretion after vaccination with CAF01-based subunit vaccines in mice and the cellular origin and the regulatory role in balancing Th1/Th17 responses is currently under exploration (Dietrich, unpublished).

This trial demonstrated promising immunogenicity results, a good safety profile and no dose dependent adverse events. Immunogenicity data suggests that the intermediate and high dose of adjuvant induced superior $T_{CM}$ profile, however this phase 1

safety trial was not designed for firm conclusion on dose selection. If these characteristics of CAF01 are confirmed for other disease targets, this adjuvant would be among the first candidates capable of inducing long-term memory cellular immune response in humans. This property is unique and not shared with currently approved adjuvants like aluminum salts and MF59, both of which primarily promote a Th2 or humoral immune response [22,32–34]. Based on results from animal models we expected CAF01 adjuvanted vaccines to also induce antibody responses to the vaccine antigen, however herein two vaccinations with H1:CAF01 did not induce significant IgG responses. Similarly, H1 in IC31® also failed to induce significant H1-specific IgG levels after two injections. We recently found specific IgG after a third administration of H56:IC31 (Hoff, Andersen, unpublished observation), suggesting that a third dose is required to induce IgG responses to this particular vaccine antigen in humans.

ESAT-6 is included in Interferon gamma release assay (IGRA) diagnostic test kits. In the present trial, similar to previous H1:IC31® trials, vaccination was associated with a transient conversion of the QFT in about half of the vaccinated subjects. Induction of ESAT-6 specific immune responses by vaccination with an ESAT-6-containing vaccine may very well interfere with current ESAT-6 based diagnostics. However, this may not pose a major diagnostic problem, as IGRAs are indicated in low endemic settings and TB vaccines will mainly be used in high endemic settings [35].

In conclusion, we report the first in man studies of the CAF01 adjuvant and demonstrate its safety in a phase I trial. Vaccination with CAF01 together with the H1 fusion protein resulted in long lasting T-cell immunity characterized by mainly IL-2 and TNF-α producing T-cells indicating that CAF01 is of relevance for future human vaccination studies.

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*Conflict of interest statement*: PA is co-inventor on a patent application claiming H1 as a vaccine and CAF01 as vaccine adjuvant. All rights have been assigned to Statens Serum Institut, a Danish not-for-profit governmental institute. BTC, EMA, IK, MR, SH and LVA are employed by Statens Serum Institut. The other authors involved in this study have no conflict of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.10.036.

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