AJRCCM Articles in Press. Published on February 18, 2010 as doi:10.1164/rccm.200910-1484OC
Media embargo until 2 weeks after above posting date; see thoracic.org/go/embargo

The Novel TB Vaccine, AERAS-402, Induces Robust and Polyfunctional CD4 and CD8 T Cells in Adults[1]

Running Title: AERAS-402 induces robust T cell immunity in adults

Brian Abel[1$], Michele Tameris[1$], Nazma Mansoor[1], Sebastian Gelderbloem[2], Jane Hughes[1] Deborah Abrahams[1], Lebohang Makhethe[1], Mzwandile Erasmus[1], Marwou de Kock[1], Linda van der Merwe[1], Anthony Hawkridge[2], Ashley Veldsman[1], Mark Hatherill[1], Giulia Schirru[3], Maria Grazia Pau[3], Jenny Hendriks[3], Gerrit Jan Weverling[3], Jaap Goudsmit[3], Donata Sizemore[4], J. Bruce McClain[4], Margaret Goetz[4], Jacqueline Gearhart[4], Hassan Mahomed[1], Gregory D. Hussey[1], Jerald C. Sadoff[4*], Willem A. Hanekom[1*@]

[1]South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine and School of Child and Adolescent Health, University of Cape Town, South Africa, [2]Aeras Global TB Vaccine Foundation, Rondebosch, Cape Town, South Africa, [3]Crucell N.V., 2301 CA Leiden, The Netherlands, and [4]Aeras Global TB Vaccine Foundation, Rockville, Maryland, USA

[$]BA and [$]MT; and *JCS and *WAH; contributed equally to the manuscript.

Competing interests: None declared.

[1] **Funding:** This study was funded by the Aeras Global Tuberculosis Vaccine Foundation and by Crucell N.V. BA is supported by an NRF Innovation Postdoctoral Fellowship, and WAH is supported by the NIH (RO1-AI065653 and NO1-AI70022).

Copyright (C) 2010 by the American Thoracic Society.

[@]Send correspondence to Willem Hanekom: willem.hanekom@uct.ac.za

Tel: +27 21 4066080. Fax: +27 21 4066693

Descriptor number: 11.4

Word count: 3160

At a Glance Commentary:

Scientific Knowledge on the subject

Effective tuberculosis vaccines are urgently needed to boost BCG-induced immunity, especially in TB endemic countries. This is the first clinical report of an Ad35 vectored vaccine given to humans in a heterologous prime-boost strategy. The AERAS-402 vaccine comprises a recombinant, replication deficient Ad35, which expresses the mycobacterial antigens Ag85A, Ag85B, and TB10.4. The findings in this study strongly support further clinical trials assessing the efficacy of AERAS-402 as a boosting vaccine.

What this study adds to the field

AERAS-402 vaccination was safe and immunogenic in healthy *Mycobacterium tuberculosis* uninfected BCG vaccinated adults, and induced a robust polyfunctional CD4 T cell response. It also induced a robust and durable CD8 T cell response.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale: AERAS-402 is a novel TB vaccine designed to boost immunity primed by BCG, the only licensed vaccine.

Objectives: We investigated the safety and immunogenicity of AERAS-402 in healthy *Mycobacterium tuberculosis* uninfected BCG vaccinated adults from a TB endemic region of South Africa.

Methods: Escalating doses of AERAS-402 vaccine were administered intramuscularly to each of 3 groups of healthy South African BCG vaccinated adults, while a 4th group received 2 injections of the maximum dose. Participants were followed up for 6 months with all adverse effects documented. Vaccine-induced CD4 and CD8 T cell immunity was characterized by an intracellular cytokine staining assay of whole blood and peripheral blood mononuclear cells (PBMCs).

Measurements and Main Results: AERAS-402 was well tolerated, and no vaccine-related serious adverse events were recorded. The vaccine induced a robust CD4 T cell response dominated by cells co-expressing IFN-γ, TNF-α, and IL-2 ("polyfunctional" cells). AERAS-402 also induced a potent CD8 T cell response, characterized by cells expressing IFN-γ and/or TNF-α, which persisted for the duration of the study.

Conclusions: Vaccination with AERAS-402 is safe and immunogenic in healthy adults. The immunity induced by the vaccine appears promising: polyfunctional T cells are thought to be important for protection against intracellular pathogens like *M. tuberculosis*, while evidence is accumulating that CD8 T cells are also important. AERAS-402 induced a robust and durable CD8 T cell response, which appears extremely promising.

Word count: 230

Key words: TB, vaccine, immunity, CD4, CD8

Clinical Trials Registry Information: NHREC no. 1381 registered at www.sanctr.gov.za

Introduction

A third of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*), and every year 1.8 million people die from tuberculosis (TB) disease(1). Effective vaccination strategies may constitute the most sustainable interventions. The only current TB vaccine, bacille Calmette Guerin (BCG) reliably protects infants against miliary disease and meningitis(2, 3). However, the vaccine's efficacy in protecting against lung TB is highly variable(4). A concerted effort has been made toward strategies where a heterologous vaccine would boost immunity primed by BCG or a recombinant BCG, in an effort to ultimately better protect against pulmonary disease(5-14). Here, we investigated one such candidate boost vaccine, AERAS-402.

The AERAS-402 vaccine comprises a recombinant, replication deficient Adenovirus, serotype 35 (Ad35), which expresses a fusion protein created from the sequences of the mycobacterial antigens Ag85A, Ag85B, and TB10.4. The antigens are fused contiguously as a one-piece fusion polyprotein that should be expressed upon immunization with the Ad35 vaccine (AERAS-402) (15). In animal models, recombinant adenoviral vectors have been used to deliver vaccine antigens in combination with BCG, poxvirus-vectored vaccines and DNA-based vaccines(8, 16-20). In these studies, heterologous prime-boost strategies have demonstrated enhanced immunogenicity and protective immunity against malaria(18, 21), and *Mtb*(22). Recombinant human Adenovirus serotype 5 (Ad5) vaccines have been well tolerated, and have shown good safety profiles, in Phase I trials. However, prevalence of neutralizing antibody titers against Ad5 of up to 90% in sub-Saharan Africa(23), with associated limitations of the usefulness of this vector(16, 20, 24), has

prompted exploration of alternate adenovirus vectors such as Ad35. The seroprevalence, and levels of neutralizing antibody titers, to Ad35 are lower than those of Ad5 worldwide including sub-Saharan Africa (20% vs. 90%, respectively), with significant levels of neutralizing titers (>200) in <5% of persons in sub-Saharan Africa(23).

Protective immunity against TB disease has yet to be fully elucidated. T cell immunity, comprising CD4 and CD8 cells, are thought to be important for effective prevention of disease following *Mtb* infection(25). Induction of a durable *Mtb*-specific T cell response is therefore an objective of novel vaccine strategies. Several T cell effector molecules may play critical roles in *Mtb* control, including T-helper type 1 (Th1) cytokines interferon-γ (IFN-γ)(26-28), tumour necrosis factor-α (TNF-α)(29-31) and interleukin-2 (IL-2)(32). IL-2 promotes secondary expansion of memory T cells, and maintenance of a stable pool of these cells(33). Moreover, vaccination-induced "polyfunctional" T cells, which co-express IFN-γ, TNF-α and IL-2, have been associated with efficient control of murine *Leishmania major*(34) and *Mtb* infection(35) upon virulent challenge.

In this Phase I study we evaluated the safety and immunogenicity of AERAS-402 in healthy *Mtb* uninfected, BCG-vaccinated South African adults. Escalating doses of AERAS-402 were administered intramuscularly to each of 3 groups, while a 4th group received 2 injections of the maximum dose. The vaccine was safe and immunogenic. This is the first clinical report of an Ad35 vectored vaccine given to humans in a heterologous prime-boost strategy.

Materials and Methods

Study design

This study was a Phase I double-blind, randomized, placebo-controlled dose escalation study in 4 groups of healthy, *Mtb* uninfected adults, previously vaccinated with BCG. The Medicines Control Council of South Africa and the Research Ethics Committees of the University of Cape Town approved the protocol and subsequent amendments. Written, informed consent was obtained from all participants. The trial was conducted according to International Conference on Harmonization/Good Clinical Practice (ICH-GCP) guidelines and Guidelines for Good Clinical Practice in the Conduct of Clinical Trials in Human Participants in South Africa.

Enrollment and vaccination

The aim was to enroll 40 participants, who would be assigned to 1 of 4 study groups. Healthy adult volunteers aged 21-45 years, were recruited from Worcester region of the Western Cape province of South Africa. Inclusion and exclusion are defined in an online data supplement. In each of Groups 1-3, 7 participants would be assigned to receive a single dose of 1mL of the vaccine, AERAS-402. The 1mL contained 3 X 10^8 viral particles (vp) for study Group 1, 3 X 10^9 vp for Group 2 and 3 X 10^{10} vp for Group 3. In each of these groups, 3 participants would receive 1mL of placebo, which was the AERAS-402 vaccine diluents, consisting of sterile buffer containing Tris, $MgCl_2$, NaCl, sucrose polysorbate 80, and water. Vaccine or placebo would be administered in a double blind fashion (shown in Table 1). Participants in Group 4 would receive two doses of vaccine or placebo, at study days 0 and 56. In this group, 8 participants would be assigned to receive study vaccine (3 X 10^{10} vp), and 2 to

receive placebo (shown in Table 1). In every case, vaccine or placebo was administered into the deltoid muscle on the contralateral side to the BCG vaccine, and the 2nd dose (Group 4) was administered in the opposite arm, i.e., ipsilateral to the BCG vaccine.

Follow-up and safety evaluation

Participants in Groups 1-3 were evaluated on days 0, 2, 7, 14, 28, 42, 84 and 182, whereas participants from Group 4 were evaluated on days 0, 7, 14, 28, 56, 58, 63, 70, 84 and 182, after vaccination. Blood for safety evaluation, which included biochemistry and hematology tests, was collected pre-vaccination and on days 0, 7, and 28. In Group 4, these tests were also done on days 56, 63, and 84. Adverse events were recorded during the first 28 days after vaccination in all groups, and additionally from days 56–84 in Group 4. Participants received a daily diary card to record adverse events themselves for the first 7 days after vaccination. Assessment and classification of adverse events are provided in an online data supplement.

PBMC-based intracellular cytokine staining assay

The frequency of antigen-specific cytokine responses in PBMC-derived T cells was determined as previously described (8), and the antibody panel is described in the online data supplement.

Whole blood intracellular cytokine staining assay

The frequency and pattern of antigen-specific cytokine producing T cells in the whole blood were determined as previously described (36), and the antibody panel is described in the online data supplement.

Neutralizing antibody titers against Ad35 viruses

Serum was isolated from blood obtained from the 20 study subjects in Groups 3 and 4 prior to initial injection of AERAS-402 or placebo on Study Day 0, and again 6 months after initial injection on day 182. These serum specimens were analyzed for the presence of neutralizing activity against Ad35, using a validated assay at Crucell(37).

Data analysis

Basic descriptive analysis was performed to examine adverse events. Comparisons of immunogenicity results between different time points were performed with Mann Whitney U tests, using Prism 4.03 (GraphPad). Analysis of data is described in an online data supplement.

Results

Participants, vaccination and follow-up

Three-hundred-and-ninety-six adults were screened between April and October 2007 in order to enroll 40 healthy adults into this trial – a screening to enrollment ratio of approximately 10:1. The main reason for the high screening failure rate was latent infection with *Mtb*, as demonstrated by a positive QuantiFERON®-TB Gold In-Tube (QFT™) test and/or TST ≥ 15mm (Table 2, which also lists other reasons for screening failures).

The groups enrolled and vaccinated are described in Table 1. In Group 4, 2 of 8 participants did not meet eligibility criteria for revaccination with the study vaccine, 1 due to abnormal urine analysis, and 1 due to an elevated white cell count. Since these 2 participants only received a single dose, for purposes of analyzing immune responses, they were therefore moved into Group 3 for analysis purposes only. All vaccine recipients, including the latter 2 participants, completed all study procedures and attended all visits.

AERAS-402 displayed an acceptable safety profile in healthy *Mtb* uninfected adults

A total of 158 adverse events were recorded across the study groups; 129 in vaccine recipients and 29 in placebo recipients. Eighty (50.6%) adverse events were related to vaccination, of which, sixty-nine (43.7%) were considered related to the study vaccine (Table 3a). The adverse events related to placebo administration include the following: injection site pain (n=2), malaise (n=3), myalgia (n=1), sore throat (n=1), upper respiratory tract infection (n=1), headache (n=2), nausea (n=1).

The majority of events were considered mild (74%) or moderate (19%) in severity (Table 3a). Three serious adverse events were recorded during the study. A vaccine recipient reported pain in the ipsilateral arm 51 days after vaccination, which required hospitalization and extensive investigations, but no specific diagnosis was made. The other two SAEs were an attempted suicide and HIV seroconversion, none of which were considered to be vaccine related events.

Of the adverse events recorded, 11 were graded severe according to the parameters of the FDA Toxicity Tables or the investigator's evaluation of their impact on normal daily activities. Three of these were solicited adverse events as recorded on the diary cards, namely fever and malaise (presumed to be related to the study vaccine), and sore throat (thought not to be related). Two cases of fever were reported during the study; one participant had a maximum temperature of 39.2^{o}C on the day of vaccination, which returned to 37^{o}C on Day 2 post-vaccination, while the second case of fever was classified as mild (38^{o}C to 38.4^{o}C).

Among the 8 unsolicited severe adverse events, 2 were recorded in the placebo group and 6 were recorded in the AERAS-402 groups. Of these eight, three were serious adverse events (SAEs) considered not related to the study vaccine and detailed elsewhere, and the remaining five were laboratory test abnormalities where the deviation from normal met the criteria for a grade 3 or 4 event according to the Toxicity Table. Two of the abnormal blood tests were considered possibly related to study vaccine, namely an increased creatinine phosphokinase (CPK) and a decrease in hemoglobin. The remaining six in this group of severe, unsolicited adverse events was considered unlikely or not related to the vaccine.

There appeared to be a relationship between the incidence of injection site pain and dosage level (Table 3b), albeit not statistically significantly. There did not appear to be an increase in incidence of solicited or unsolicited AEs after the second dose of vaccine, compared with administration of a single dose only (Tables 3a and b).

When vaccine and placebo recipients were compared, the overall incidence of adverse events was similar regardless of dose of AERAS-402 (Table 3a).

Proportion and kinetics of vaccine responses, measured by PBMC-based and whole blood-based flow cytometric assays

PBMC, cryopreserved from each time point, were later thawed and incubated with peptide pools, to measure T cell-specific expression of IFN-γ, TNF-α and IL-2 by flow cytometry. In the PBMC assay, all 3 cytokines were measured in a single flow cytometric channel. Whole blood from vaccine recipients was also incubated with the peptide pools of the vaccine antigens, to detect expression of 4 cytokines and 3 surface markers individually, with multiparameter flow cytometry. The gating strategy for the latter analysis is shown in Supplementary Figure 1. The whole blood analysis was completed in Groups 3 and 4, for participants who received the highest dose of the vaccine. The whole blood assay was more sensitive than the PBMC-based assay when comparing Groups 3 and 4: for example, 56% and 33% of vaccine recipients in groups 3 and 4 showed responses to Ag85A/b detectable with the PBMC assay, whereas 100% and 83% showed responses detectable with the whole blood assay (defined here as any cytokine response), respectively (Supplementary Table 1). Of note, PBMC were stimulated for 6 hours, whereas whole blood was stimulated for 12 hours. It is possible that the extended period of stimulation of the WBA could account for the increased sensitivity observed.

Both the PBMC-based assay (Figure 1 and Supplementary Figure 3) and the whole blood assay (Figure 2A and 3A) showed that the Ag85-specific CD4 and CD8 T cell response peaked at 28 days post-vaccination. The TB10.4-specific CD4 T cell response often peaked earlier (Supplementary Figure 2 and 4, and Figure 2B), whereas the CD8 T cell response to this antigen peaked at day 28 (Supplementary Figure 2 and 4, and Figure 3B). The Ag85-specific CD8 T cell response persisted significantly over baseline for the duration of the study (Figure 3A). Overall, placebo recipients did not show an increase in specific CD4 and CD8 T cells above baseline (Figures 1-3 and Supplementary Figure 2-4).

Double vaccination (Group 4) did not result in significant boosting of T cell responses (Figures 1-3 and Supplementary Figure 2-4).

AERAS-402 induced polyfunctional CD4 T cells that co-expressed Th1 cytokines, but did not induce IL-17-expressing CD4 T cells

To assess the potential functional characteristics of CD4 T cells induced by AERAS-402 more comprehensively, we analysed co-expression patterns of cytokines, as detected with the whole blood assay. We were particularly interested in co-expression of cytokines in so-called multifunctional or polyfunctional cells, which may be associated with more optimal protection (see Introduction).

Seven subsets of vaccine-induced CD4 T cells could be delineated, based on expression of IFN-γ, TNF-α and IL-2, alone or in combination (Figure 4A and B). The peak Ag85-specific and TB10.4-specific CD4 T cell response was dominated by a polyfunctional IFN-γ^+IL-2^+TNF-α^+ subset (Figure 4A and B). The only Ag85-specific

CD4 T cell subset frequency that was significantly higher at day 182, compared with baseline, expressed IFN-γ and IL-2 together (Figure 4A).

At day 7 post-vaccination, about 40-50% of specific CD4 T cells expressed 2 or 3 cytokines, while at day 28 post-vaccination, >50% of specific CD4 T cells expressed 2 or 3 cytokines simultaneously (Figure 4C). At 84 days, single-cytokine expressing cells predominated (Figure 4C).

AERAS-402 did not induce specific expression of the pro-inflammatory cytokine IL-17 (Figures 4D). In contrast, mycobacteria-specific IL-17+ CD4 T cell subsets were detectable upon BCG stimulation, as we have shown previously(38) (Figure 4D and E).

The vaccine-specific CD8 response to AERAS-402 was dominated by IFN-γ-expressing cells

The Ag85-specific CD8 T cell response was dominated by a subset expressing only IFN-γ. This response was long-lived (Figure 4F). Smaller subsets, co-expressing IFN-γ and IL-2 or IFN-γ and TNF-α, were also induced, and the former population persisted for the duration of the study (Figure 4F). The TB10.4-specific CD8 T cell response was also dominated by IFN-γ expression; however, significant increases over baseline could not be detected (Figure 4G). This may be due to the small number of participants, and high pre-vaccination responses to TB10.4 (Figure 4G). A very small number of polyfunctional (IFN-γ^+TNF-α^+IL-2^+) TB10.4-specific CD8 T cells was induced by the vaccine, as detected 28 days after vaccination (Figure 4G); however, this population did not persist.

High neutralizing antibody levels against the Ad35 vector in participants who received 2 doses of vaccine

Although it is known that antibody levels against Ad35 are relatively low in African populations(23), neutralizing activity by pre-existing and by vaccination-induced antibodies was anticipated to impact the T cell response to vaccine antigens. Ad35-specific neutralizing activity was therefore determined in serum obtained at days 0, 28, 84, and 182 for both groups, as well as prior to the 2nd boost (day 56) for Group 4 participants. Among 20 subjects analyzed from Groups 3 and 4, only 1 (5%) had quantifiable levels of Ad35 neutralizing activity at day 0 (Figure 5A-C). Of 9 subjects who were vaccinated with AERAS-402 in Group 3, 3 subjects (33.3%) had an increase in Ad35 titer from day 0 to 28 (Figure 5A), whereas 5 out of 6 subjects in Group 4 had an increase (83.3%; Figure 5B, C). In group 4 participants prior to revaccination (day 56), 4 out of 6 subjects had measureable titers (66.7%), which further increased in 3 of these participants (50%) post-boosting (Figure 5B). At the end of the study (day 182), only 1/9 participant in Group 3 (11.1%; Figure 5A, C) had a significant titer, compared with 5/6 participants (83.3%) in Group 4 (Figure 5B, C). No increase in antibody levels was shown in placebo recipients (Figure 5D).

Discussion

In this study we demonstrated that AERAS-402 displayed an acceptable safety profile and was immunogenic in healthy *Mycobacterium tuberculosis*-uninfected adults, previously vaccinated with BCG. This is the first reported clinical trial in humans in which adenovirus serotype 35 has been used as a vaccine vector in a heterologous prime-boost strategy. There were no serious adverse events that were related to the vaccine, and increasing vaccine dose did not result in an increase in adverse events.

We regard the vaccine candidate assessed in this study as safe since the AEs were mostly mild to moderate, of short duration and resolved without sequelae. As a comparison, almost 100% of recipients of BCG demonstrate local AEs, some even ulceration at the site of the vaccine administration; however, BCG is regarded as one of the safest vaccines ever used.

Since there was a protracted period between priming with BCG and boosting with AERAS-402, it cannot be excluded that further priming of the immune response could have occurred via exposure to environmental mycobacteria. Regardless of the priming mechanism, immunity induced by the vaccine was impressive. Interestingly, analysis of baseline responses revealed that the majority of individuals had pre-existing responses to the vaccine antigens, which may be a consequence either of memory responses to BCG vaccination at birth, or from exposure to environmental (non-tuberculous) mycobacteria later in life.

The most striking immunogenicity results were that the vaccine induced a robust CD8 T cell response, against both Ag85A/b and TB10.4. This response was persistent up to the last measurement of the induced immune response. IFN-γ producing T cells predominated, although other smaller subsets, expressing combinations of cytokines, were also detected. It may be important to note that our assays did not specifically assess cytotoxic activity, which is a major functional characteristic of CD8 T cells. Regardless, to date, most new TB vaccines have been reported to induce reasonable CD4 T cell responses, but relatively negligible CD8 T cell responses. CD8 T cell responses were induced and measureable directly *ex vivo* following administration of MVA85A only at high antigen dose, but not following any other vaccines(39). Whelan, et al. reported that vaccination of individuals with a lower dose of MVA85 lead to an Ag85-specific CD8 T cell response, which was detectable when dendritic cells were used as antigen-presenting cells in assays to expand specific CD8 T cells (40). Strong evidence is emerging that CD8 T cells mediate important roles in protective immunity against TB(41-45); we therefore hypothesize that the induction of robust vaccine-specific CD4 and CD8 T cell responses after AERAS-402 would correlate with a more efficacious outcome.

AERAS-402 induced a robust and highly complex vaccine-specific CD4 T cell response, which was dominated in both the Ag85A/b and TB10.4 responses by a polyfunctional IFN-γ^+TNF-α^+IL-2^+ subset, which did not persist beyond 28 days post-vaccination, and a prominent population expressing TNF-α and IL-2 together. The Ag85A/b response was further characterized by an IFN-γ^+IL-2^+ subset that persisted for the duration of the study. The induction of polyfunctional CD4 T cells may be important, as recent data suggests that stable long-lived populations of

polyfunctional T cells correlates well with protection against subsequent challenge with intracellular pathogens(34, 35). In animal models of vaccination against *Leishmania major*(34) and against *Mtb* (35), vaccination strategies that induce the highest frequency of polyfunctional antigen-specific CD4 T cells are associated with the best outcome, especially when detected in the primary site of the infection. Specifically, it was demonstrated that the magnitude and quality of the immune response measured in the lung, but not in the spleen or blood, correlated well with host protection after aerosol challenge with *Mtb* in the mouse (35).

In a study by Aagaard *et al.* it was revealed that vaccination of mice and guinea pigs with Ag85B and TB10.4 in IC31H adjuvant resulted in the induction of polyfunctional CD4 T cells that were associated with protection against subsequent challenge with *Mtb* (46). Interestingly, it was demonstrated that the magnitude and quality of the vaccine induced T cell response, as well as the protective efficacy, was highly dependent on the antigen dose (46).

Importantly, elite controllers of HIV infection have been shown to have high frequencies of polyfunctional HIV-specific T cells, whereas a rapid onset of disease has been associated with diminishing levels of polyfunctional T cells(47-49).

In contrast to MVA85A, Aeras-402 did not induce any IL-17-expressing CD4 T cells. Mycobacteria-specific IL-17-expressing T cells have been detected both in the mouse and the human(6, 38, 50). At this stage, we cannot say whether induction of Th17 cells, following novel vaccination, would result in more or lesser optimal immunity. On the one hand, IL-17 may be needed for an optimal Th1 response, although the persistence of the Th1 response shown in this study would argue to the

contrary. Also, too much inflammation, induced by IL-17, may not necessarily be optimal.

Preexisting neutralizing antibodies against Ad5 has been shown to inhibit the immunogenicity of rAd5 vaccines in both preclinical studies(16, 20, 24), and in clinical trials(51). This is a major concern, since up to 90% of individuals in sub-Saharan Africa have detectable anti-Ad5 antibodies(23). AERAS-402 incorporates Ad35, which has been shown to be prevalent in only 20% of individuals in sub-Saharan Africa(23) with neutralizing titers >200 in <5% of individuals; this vector is therefore much more attractive for antigen delivery. Anti-Ad35 neutralizing antibodies were present in only 5% of participants of this trial, before vaccination, which is lower than reported for this region. As shown in the results, AERAS-402 did indeed induce anti-Ad35 antibodies: 22% of participants who received a single dose, and 83.3% of participants who received two doses, had detectable anti-Ad35 titers at the end of the trial. Assessment of anti-Ad35 neutralizing titers immediately before the second dose of the vaccine in Group 4, revealed that two-thirds of individuals had significant titers, which likely lead to suppression of immunogenicity, and subsequently sub-optimal boosting of the T cell response induced by second immunization with AERAS-402. This is in agreement with a preclinical study by Thorner *et al*, which demonstrated reduced immunogenicity to Ad35 vaccination if high anti-Ad35 titers were already present(20). All Ad35 titers at day 182 were within the range of titers seen in a previous study of naturally occurring neutralizing activity to adenovirus(23).

Other known mechanisms of immune regulation such as the induction of regulatory T cells and immune-suppressive factors such as IL-10 and TGF-β, might further

explain the negligible boost observed in Group 4, however these were not assessed in the current study. The aim of this study was to measure vaccine take and not immune mechanisms; therefore, these possibilities were not assessed.

This is the first study showing safety and immunogenicity of AERAS-402 in a heterologous prime-boost strategy in human vaccinees. AERAS-402 administration was found to be safe and immunogenic in healthy *Mycobacterium tuberculosis* uninfected adults previously vaccinated with BCG. AERAS-402 induces a robust CD8 T cell response as well as a polyfunctional CD4 T cell response, and supports further clinical trials assessing the efficacy of AERAS-402 as a boosting vaccine.

Acknowledgements

We thank all the participants who participated in this trial, and K. Radosevic for critically reviewing the manuscript.

References

1. World Health Organization Report: Global Tuberculosis Control - Epidemiology, Strategy, Financing. 2009. WHO/htm/TB/2009.411; 2009.

2. Rodrigues LC, Diwan VK, Wheeler JG. Protective Affect of BCG Against Tuberculous Meningitis and Miliary Tuberculosis: A Meta-Analysis. *Int J Epidemiol* 1993;22:1154-1158.

3. Trunz BB, Fine P, Dye C. Effect of BCG Vaccination on Childhood Tuberculous Meningitis and Miliary Tuberculosis Worldwide: A Meta-Analysis and Assessment of Cost-Effectiveness. *Lancet* 2006;367:1173-1180.

4. Fine PA, Milstein J, Clements C. Issues Relating to the Use of BCG in Immunization Programmes: A Discussion Document. 1999.

5. Brookes RH, Hill PC, Owiafe PK, Ibanga HB, Jeffries DJ, Donkor SA, Fletcher HA, Hammond AS, Lienhardt C, Adegbola RA, et al. Safety and Immunogenicity of the Candidate Tuberculosis Vaccine MVA85A in West Africa. *PLoS ONE* 2008;3:e2921.

6. Hawkridge T, Scriba TJ, Gelderbloem S, Smit E, Tameris M, Moyo S, Lang T, Veldsman A, Hatherill M, Merwe L, et al. Safety and Immunogenicity of a New Tuberculosis Vaccine, MVA85A, in Healthy Adults in South Africa. *J Infect Dis* 2008;198:544-552.

7. Hess J, Miko D, Catic A, Lehmensiek V, Russell DG, Kaufmann SH. Mycobacterium Bovis Bacille Calmette-Guerin Strains Secreting Listeriolysin of Listeria Monocytogenes. *Proc Natl Acad Sci U S A* 1998;95:5299-5304.

8. Magalhaes I, Sizemore DR, Ahmed RK, Mueller S, Wehlin L, Scanga C, Weichold F, Schirru G, Pau MG, Goudsmit J, et al. rBCG Induces Strong Antigen-

Specific T Cell Responses in Rhesus Macaques in a Prime-Boost Setting with an Adenovirus 35 Tuberculosis Vaccine Vector. *PLoS ONE* 2008;3:e3790.

9. McShane H, Brookes R, Gilbert SC, Hill AV. Enhanced Immunogenicity of CD4(+) T-Cell Responses and Protective Efficacy of a DNA-Modified Vaccinia Virus Ankara Prime-Boost Vaccination Regimen for Murine Tuberculosis. *Infect Immun* 2001;69:681-686.

10. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, Fletcher HA, Hill AV. Recombinant Modified Vaccinia Virus Ankara Expressing Antigen 85A Boosts BCG-Primed and Naturally Acquired Antimycobacterial Immunity in Humans. *Nat Med* 2004;10:1240-1244.

11. Radosevic K, Wieland CW, Rodriguez A, Weverling GJ, Mintardjo R, Gillissen G, Vogels R, Skeiky YA, Hone DM, Sadoff JC, et al. Protective Immune Responses to a Recombinant Adenovirus Type 35 Tuberculosis Vaccine in Two Mouse Strains: CD4 and CD8 T-cell Epitope Mapping and Role of Gamma Interferon. *Infect Immun* 2007;75:4105-4115.

12. Skeiky YA, Sadoff JC. Advances in Tuberculosis Vaccine Strategies. *Nat Rev Microbiol* 2006;4:469-476.

13. Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, Hitt M, Xing Z. Single Mucosal, but Not Parenteral, Immunization with Recombinant Adenoviral-Based Vaccine Provides Potent Protection from Pulmonary Tuberculosis. *J Immunol* 2004;173:6357-6365.

14. Xing Z, Santosuosso M, McCormick S, Yang TC, Millar J, Hitt M, Wan Y, Bramson J, Vordermeier HM. Recent Advances in the Development of Adenovirus- and Poxvirus-Vectored Tuberculosis Vaccines. *Curr Gene Ther* 2005;5:485-492.

15. Havenga M, Vogels R, Zuijdgeest D, Radosevic K, Mueller S, Sieuwerts M, Weichold F, Damen I, Kaspers J, Lemckert A, et al. Novel Replication-Incompetent Adenoviral B-Group Vectors: High Vector Stability and Yield in PER.C6 cells. *J Gen Virol* 2006;87:2135-2143.

16. Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM, Nanda A, Ewald BA, Gorgone DA, Lifton MA, et al. Immunogenicity of Heterologous Prime-Boost Regimens Involving Recombinant Adenovirus Serotype 11 (ad11) and Ad35 Vaccine Vectors in the Presence of Anti-Ad5 Immunity. *J Virol* 2005;79:9694-9701.

17. McCoy K, Tatsis N, Korioth-Schmitz B, Lasaro MO, Hensley SE, Lin SW, Li Y, Giles-Davis W, Cun A, Zhou D, et al. Effect of Preexisting Immunity to Adenovirus Human Serotype 5 Antigens on the Immune Responses of Nonhuman Primates to Vaccine Regimens Based on Human- or Chimpanzee-Derived Adenovirus Vectors. *J Virol* 2007;81:6594-6604.

18. Ophorst OJ, Radosevic K, Havenga MJ, Pau MG, Holterman L, Berkhout B, Goudsmit J, Tsuji M. Immunogenicity and Protection of a Recombinant Human Adenovirus Serotype 35-Based Malaria Vaccine Against Plasmodium Yoelii in Mice. *Infect Immun* 2006;74:313-320.

19. Shott JP, McGrath SM, Pau MG, Custers JH, Ophorst O, Demoitie MA, Dubois MC, Komisar J, Cobb M, Kester KE, et al. Adenovirus 5 and 35 Vectors Expressing Plasmodium Falciparum Circumsporozoite Surface Protein Elicit Potent Antigen-Specific Cellular IFN-Gamma and Antibody Responses in Mice. *Vaccine* 2008;26:2818-2823.

20. Thorner AR, Lemckert AA, Goudsmit J, Lynch DM, Ewald BA, Denholtz M, Havenga MJ, Barouch DH. Immunogenicity of Heterologous Recombinant

Adenovirus Prime-Boost Vaccine Regimens is Enhanced by Circumventing Vector Cross-Reactivity. *J Virol* 2006;80:12009-12016.

21. Stewart VA, McGrath SM, Dubois PM, Pau MG, Mettens P, Shott J, Cobb M, Burge JR, Larson D, Ware LA, et al. Priming with an Adenovirus 35-Circumsporozoite Protein (CS) Vaccine Followed by RTS,S/AS01B Boosting Significantly Improves Immunogenicity to Plasmodium Falciparum CS Compared to that with Either Malaria Vaccine Alone. *Infect Immun* 2007;75:2283-2290.

22. Radosevic K, Rodriguez A, Lemckert A, Goudsmit J. Heterologous Prime-Boost Vaccinations for Poverty-Related Diseases: Advantages and Future Prospects. *Expert Rev Vaccines* 2009;8:577-592.

23. Kostense S, Koudstaal W, Sprangers M, Weverling GJ, Penders G, Helmus N, Vogels R, Bakker M, Berkhout B, Havenga M, et al. Adenovirus Types 5 and 35 Seroprevalence in AIDS Risk Groups Supports Type 35 as a Vaccine Vector. *Aids* 2004;18:1213-1216.

24. Barouch DH, Pau MG, Custers JH, Koudstaal W, Kostense S, Havenga MJ, Truitt DM, Sumida SM, Kishko MG, Arthur JC, et al. Immunogenicity of Recombinant Adenovirus Serotype 35 Vaccine in the Presence of Pre-existing Anti-Ad5 Immunity. *J Immunol* 2004;172:6290-6297.

25. Winslow GM, Cooper A, Reiley W, Chatterjee M, Woodland DL. Early T-Cell Responses in Tuberculosis Immunity. *Immunol Rev* 2008;225:284-299.

26. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An Essential Role for Interferon Gamma in Resistance to Mycobacterium Tuberculosis Infection. *J Exp Med* 1993;178:2249-2254.

27. Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine Secretion by CD4 T Lymphocytes Acquired in Response to Mycobacterium Tuberculosis Infection. *J Immunol* 1993;151:518-525.

28. Ottenhoff TH, Kumararatne D, Casanova JL. Novel Human Immunodeficiencies Reveal the Essential Role of Type-I Cytokines in Immunity to Intracellular Bacteria. *Immunol Today* 1998;19:491-494.

29. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ. Structural Deficiencies in Granuloma Formation in TNF Gene-Targeted Mice Underlie the Heightened Susceptibility to Aerosol Mycobacterium Tuberculosis Infection, which is Not Compensated for by Lymphotoxin. *J Immunol* 1999;162:3504-3511.

30. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor Necrosis Factor-Alpha is Required in The Protective Immune Response Against Mycobacterium Tuberculosis in Mice. *Immunity* 1995;2:561-572.

31. Jacobs M, Togbe D, Fremond C, Samarina A, Allie N, Botha T, Carlos D, Parida SK, Grivennikov S, Nedospasov S, et al. Tumor Necrosis Factor is Critical to Control Tuberculosis Infection. *Microbes Infect* 2007;9:623-628.

32. Johnson BJ, Bekker LG, Rickman R, Brown S, Lesser M, Ress S, Willcox P, Steyn L, Kaplan G. rHuIL-2 Adjunctive Therapy in Multidrug Resistant Tuberculosis: A Comparison of Two Treatment Regimens and Placebo. *Tuber Lung Dis* 1997;78:195-203.

33. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 Signals During Priming are Required for Secondary Expansion of CD8+ Memory T Cells. *Nature* 2006;441:890-893.

34. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, Hoff ST, Andersen P, Reed SG, Morris SL, et al. Multifunctional Th1 Cells Define a Correlate of Vaccine-Mediated Protection Against Leishmania Major. *Nat Med* 2007;13:843-850.

35. Forbes EK, Sander C, Ronan EO, McShane H, Hill AV, Beverley PC, Tchilian EZ. Multifunctional, High-Level Cytokine-Producing Th1 Cells in the Lung, but Not Spleen, Correlate with Protection Against Mycobacterium Tuberculosis Aerosol Challenge in Mice. *J Immunol* 2008;181:4955-4964.

36. Hanekom WA, Hughes J, Mavinkurve M, Mendillo M, Watkins M, Gamieldien H, Gelderbloem SJ, Sidibana M, Mansoor N, Davids V, et al. Novel Application of A Whole Blood Intracellular Cytokine Detection Assay to Quantitate Specific T-Cell Frequency in Field Studies. *J Immunol Methods* 2004;291:185-195.

37. Sprangers MC, Lakhai W, Koudstaal W, Verhoeven M, Koel BF, Vogels R, Goudsmit J, Havenga MJ, Kostense S. Quantifying Adenovirus-Neutralizing Antibodies by Luciferase Transgene Detection: Addressing Preexisting Immunity to Vaccine and Gene Therapy Vectors. *J Clin Microbiol* 2003;41:5046-5052.

38. Scriba TJ, Kalsdorf B, Abrahams DA, Isaacs F, Hofmeister J, Black G, Hassan HY, Wilkinson RJ, Walzl G, Gelderbloem SJ, et al. Distinct, Specific IL-17- and IL-22-Producing CD4+ T Cell Subsets Contribute to the Human Anti-Mycobacterial Immune Response. *J Immunol* 2008;180:1962-1970.

39. Beveridge NE, Fletcher HA, Hughes J, Pathan AA, Scriba TJ, Minassian A, Sander CR, Whelan KT, Dockrell HM, Hill AV, et al. A Comparison of IFNgamma Detection Methods Used in Tuberculosis Vaccine Trials. *Tuberculosis (Edinb)* 2008;88:631-640.

40. Whelan KT, Pathan AA, Sander CR, Fletcher HA, Poulton I, Alder NC, Hill AV, McShane H. Safety and Immunogenicity of Boosting BCG Vaccinated Subjects with BCG: Comparison with Boosting with A New TB Vaccine, MVA85A. *PLoS One* 2009;4:e5934.

41. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, Mex P, Miekley D, Kaufmann SH. Poor Correlation Between BCG Vaccination-Induced T Cell Responses and Protection Against Tuberculosis. *Proc Natl Acad Sci U S A* 2007;104:12434-12439.

42. van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM, Andersen P. Control of Latent Mycobacterium Tuberculosis Infection is Dependent on CD8 T Cells. *Eur J Immunol* 2000;30:3689-3698.

43. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, Sandhoff K, Brinkmann V, Kaufmann SH, Schaible UE. Apoptotic Vesicles Crossprime CD8 T Cells and Protect Against Tuberculosis. *Immunity* 2006;24:105-117.

44. Woodworth JS, Behar SM. Mycobacterium Tuberculosis-Specific CD8+ T Cells and Their Role in Immunity. *Crit Rev Immunol* 2006;26:317-352.

45. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, Shen Y, Halliday L, Fortman J, McAllister M, et al. A Critical Role for CD8 T cells in A Nonhuman Primate Model of Tuberculosis. *Plos Pathogens* 2009;5:e1000392.

46. Aagaard C, Hoang TT, Izzo A, Billeskov R, Troudt J, Arnett K, Keyser A, Elvang T, Andersen P, Dietrich J. Protection and Polyfunctional T Cells Induced by Ag85B-TB10.4/IC31 Against Mycobacterium Tuberculosis is Highly Dependent on The Antigen Dose. *PLoS One* 2009;4:e5930.

47. Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, Bornstein E, Asher TE, Samri A, Schnuriger A, Theodorou I, et al. Superior Control of HIV-1 Replication

by CD8+ T cells is Reflected by Their Avidity, Polyfunctionality, and Clonal Turnover. *J Exp Med* 2007;204:2473-2485.

48. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, et al. HIV Nonprogressors Preferentially Maintain Highly Functional HIV-Specific CD8+ T cells. *Blood* 2006;107:4781-4789.

49. Ferre AL, Hunt PW, Critchfield JW, Young DH, Morris MM, Garcia JC, Pollard RB, Yee HF, Jr., Martin JN, Deeks SG, et al. Mucosal Immune Responses to HIV-1 in Elite Controllers: A Potential Correlate of Immune Control. *Blood* 2008.

50. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y, et al. IL-17-Mediated Regulation of Innate and Acquired Immune Response Against Pulmonary Mycobacterium Bovis Bacille Calmette-Guerin Infection. *J Immunol* 2007;178:3786-3796.

51. Harro CD, Robertson MN, Lally MA, O'Neill LD, Edupuganti S, Goepfert PA, Mulligan MJ, Priddy FH, Dubey SA, Kierstead LS, et al. Safety and Immunogenicity of Adenovirus-Vectored Near-Consensus HIV Type 1 Clade B gag Vaccines in Healthy Adults. *AIDS Res Hum Retroviruses* 2009;25:103-114.

Supplementary Information

Assessment and classification of adverse events

Serious adverse events were recorded throughout the period of the trial, and all participants were enrolled into a registry protocol with long-term follow up for delayed adverse events. Further local and systemic solicited and unsolicited events were recorded by study staff at routine trial visits. Adverse events were assessed for causality (not related, unlikely to be related, possibly, probably and definitely related) and for severity (mild or grade 1, moderate or grade 2, and severe or grade 3), according to the FDA Guidance Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials (April 2005; http://www.fda.gov/cber/gdlns/toxvac.htm).

Inclusion and exclusion criteria

Inclusion criteria included receipt of BCG >5 years prior to enrollment, as documented through medical history or presence of a BCG scar, no history of past TB disease, a normal chest radiograph, no household exposure to an individual with TB during the year prior to enrollment, a body mass index between 18 and 30 kg/m^2, and no use of immunosuppressive medication within 45 days before entry into the study. Following baseline assessment, inclusion further required a negative QuantiFERON®-TB Gold In-Tube (QFT™) and a Mantoux skin test result of <15 mm (0.1mL of 2 Tuberculin Units PPD, RT 23, Statens Serum Institut), a serology that showed no active or chronic infection with HIV or Hepatitis B or C, and normal routine hematological and biochemical test results within 36 hours of randomization. Urine was tested for evidence of recreational drug use, and if positive, the participant

was excluded. Female participants were required to withhold from sexual intercourse, be infertile, or be routinely using a reliable form of contraception for the duration of the trial.

Flow cytometry antibodies

Antibodies for detecting cytokine responses by PBMC-derived CD4 and CD8 T cells were as follows: CD3-APC (clone SK7, BD Biosciences), CD4-PE (clone RPA-T4, BD Biosciences), CD8-PerCP (clone SK1, BD Biosciences), IFN-γ-Alexa Fluor 488 (clone B27, Caltag), IL-2- Alexa Fluor (clone MQ1-17H12, Caltag) and TNF-α-FITC (clone MP9-20A4, Caltag).

Antibodies for detecting cytokine responses by whole blood-derived CD4 and CD8 T cells were as follows: CD3-Pacific Blue (UCTH1), CD4-PerCPCy5.5 (SK3), CD8-APC (SK1), IFN-γ-AlexaFluor700 (K3), IL-2-FITC (5344.111) and TNF-α-PECy7 (MAb11); all from BD Biosciences, and IL-17-PE (eBio64CAP17, eBiosciences). Single stained mouse κ beads were used to calculate compensations for every run. Data analysis was performed with FlowJo software version 8.5.3 (TreeStar).

Data analysis

The sample size for this Phase I study was selected as adequate for an initial review of the safety profile of AERAS-402, rather than for statistical reasons, and to permit initial estimates of reactogenicity. If no serious adverse effects were observed among 29 subjects receiving AERAS-402, an approximation to the upper 95% confidence bound on the rate of serious adverse effect occurrence would be 10.3%. The sample size did not provide adequate power to detect other than large

differences between the dose levels in the incidence of local and general side effects.

In intracellular cytokine assays, background values (unstimulated) were subtracted for vaccine antigen-specific results. For both PBMC and whole blood assays, a positive response to a vaccine antigen was defined as an increase over the pre-vaccination level, and above the threshold of the assay after subtraction of background. This flow cytometer threshold of detection was determined to be 0.03% for the PBMC assay (Aeras flow cytometer), and 0.01% for the whole blood assay (SATVI flow cytometer). Furthermore, for the whole blood assay, statistical thresholds of detection were calculated using 3 median absolute deviations + 1 median from the unstimulated values, and are tabulated below.

Parameter	**Statistical threshold**
CD4 IFN-γ^+	0.014%
CD4 TNF-α^+	0.034%
CD4 IL-2^+	0.070%
CD8 IFN-γ^+	0.024%
CD8 TNF-α^+	0.028%
CD8 IL-2^+	0.016%

The threshold of detection for the neutralizing antibody titers against Ad35 is 16, therefore any titers below this are considered undetectable.

Basic descriptive analysis was performed to examine adverse events. Comparisons of immunogenicity results between different time points were performed with Mann Whitney U tests, using Prism 4.03 (GraphPad).

The boolean gate platform was used with individual cytokine gates to create all possible response pattern combinations. The data analysis programs PESTLE (version 1.5.4) and SPICE (Simplified Presentation of Incredibly Complex Evaluations; version 4.1.6) were used to analyze flow cytometry data and generate

graphical representations of T cell responses using background-deducted flow cytometric data (both kindly provided by Mario Roederer, Vaccine Research Center, NIAID, NIH).

Figure Legends

Figure 1. Frequency of Ag85A/b-specific T cells induced by AERAS-402, as measured by flow cytometry following incubation of PBMC with a peptide pool of the antigens. CD4 T cell (left panels) and CD8 T cell (right panels) responses, in AERAS-402 vaccinated (blue boxes) and placebo vaccinated (red boxes) participants are shown. Participants from groups 1 **(A and B)**, 2 **(C and D)** and 3 **(E and F)** received a single, escalating dose of AERAS-402 on day 0 (indicated by the black arrow under the x-axis). Group 4 participants **(G and H)** received two doses of AERAS-402 on days 0 and 56 (indicated by black arrow under the axis), and were bled additionally on days 56, 63, and 70. Total cytokine-positive frequencies denote any T cell that expresses IFN-γ, TNF-α, or IL-2 alone or in combination. Background values (unstimulated) were subtracted for each condition from each individual. For each plot, the median is represented by the horizontal line, the interquartile range by the box and the range by the whiskers. The open circles and accompanying numbers represent high responders that exceed the maximum value on the scale. The p values indicated were derived from comparing responses with those at baseline, using the Mann Whitney U test.

Figure 2. Frequency of Ag85A/b-specific **(A, C, E)** and TB10.4-specific **(B, D, F)** CD4 T cells induced by AERAS-402, as measured by flow cytometry following incubation of whole blood with a peptide pool of the antigens. Vaccinations for Group 3 and Group 4 are indicated with blue and red arrows, respectively, under the x-axis. Both total (any) cytokine-expressing **(A-D)** and polyfunctional IFN-γ^+IL-2^+TNF-α^+ **(E and F)** CD4 T cell responses, are shown, for Group 3 (single high dose,

administered on day 0, blue arrow) and Group 4 (2 doses of the vaccine, administered on days 0 and 56, red arrows). Each line displayed represents a vaccine participant. Background values (unstimulated) were subtracted for each condition from each individual. The p values indicated were derived from comparing responses with those at baseline, using the Mann Whitney U test.

Figure 3. Frequency of Ag85A/b-specific **(A and C)** and TB10.4-specific **(B and D)** CD8 T cells induced by AERAS-402, as measured by flow cytometry following incubation of whole blood with a peptide pool of the antigens. Vaccinations for Group 3 and Group 4 are indicated with blue and red arrows, respectively, under the x-axis. Total (any) cytokine-expressing CD8 T cell responses are shown, for Group 3 (single high dose, administered on day 0, blue arrow) and Group 4 (2 doses of the vaccine, administered on days 0 and 56, red arrows). Each line displayed represents a vaccine participant. Background values (unstimulated) were subtracted for each condition from each individual. The p values indicated were derived from comparing responses with those at baseline, using the Mann Whitney U test.

Figure 4. Detailed analysis of cytokine expression patterns of specific CD4 and CD8 T cells induced by AERAS-402, as measured by flow cytometry following incubation of whole blood with a peptide pool of the antigens. Patterns of single or combined expression of the Th1 cytokines in Ag85A/b-specific **(A)** and TB10.4-specific **(B)** CD4 T cells of participants vaccinated with a single high dose of AERAS-402 (Group 3) are shown, as frequencies of specific CD4 T cells. **(C)** Among these participants, pie charts representing the mean proportions of cells producing 3 cytokines (red), 2 cytokines (blue), and 1 cytokine only (green), out of the total cytokine CD4 T cell

response, on days 7, 28, and 84 post-vaccination. **(D-E)** Again among these participants, frequency of specific IL-17-expressing CD4 T cells, following AERAS-402 vaccination, among AERAS-402 vaccinated **(D)** and placebo-vaccinated participants **(E)**. BCG was used a positive control. **(F-G)** Patterns of single or combined expression of the cytokines in Ag85A/b-specific **(F)** and TB10.4-specific **(G)** CD8 T cells of participants vaccinated with a single high dose of AERAS-402 (Group 3) are shown, as frequencies of specific CD8 T cells. Background values (unstimulated) were subtracted for each condition from each individual. The open circles and accompanying numbers represent high responders that exceed the maximum value on the scale. For each plot, the median is represented by the horizontal line, the interquartile range by the box and the range by the whiskers. Differences between pre-vaccination and post-vaccination responses were evaluated with the Mann Whitney U test: p values <0.05 are shown.

Figure 5. Ad35 neutralizing antibody titers induced by AERAS-402 vaccination of Group 3 and 4 participants. Longitudinal analysis of Ad35 neutralizing antibody titers at days 0, 28, 84, and 182 post-vaccination in serum from Group 3 participants **(A)** (single high dose) and at days 0, 28, 56, 84, and 182 post-vaccination in serum from Group 4 participants **(B)** (2 high doses). Comparison of Ad35 neutralizing antibody titers at days 0, 28, and 182 post-vaccination in serum from Group 3 and 4 participants **(C)** and placebo recipients **(D)**.

Table 1. Treatment allocation by study group and demographic characteristics of participants enrolled

	Group 1	Group 2	Group 3	Group 4	Total (%)
AERAS-402 Treatment (n)	7	7	7	8*	**29 (72.5%)**
Vaccine Doses	1	1	1	2	
Vaccine Dose (viral particles)	3 X 10^8	3 X 10^9	3 X 10^{10}	3 X 10^{10}	
Placebo Treatment (n)	3	3	3	2	**11 (27.5%)**
Total Treatment (n)	10	10	10	10*	**40 (100%)**
Male (n)	4 (40%)	6 (60%)	4 (40%)	3 (30%)	**17 (42.5%)**
Age, median (min-max, years)	24.0 (22.0-38.0)	26.0 (22.0-38.0)	27.5 (22.0-39.0)	27.0 (21.0-38.0)	**26.5 (21.0-39.0)**
Ethnic Group (n)					
Black	0	1	2	4	**7 (17.5%)**
Coloured	7	9	7	3	**26 (65%)**
White	3	0	1	3	**7 (16.5%)**
BMI, mean (SD), kg/m^2	24.8±2.9	23.4±3.4	24.1±3.5	24.4±2.9	**24.2±3.1**

*2 of 8 participants did not meet eligibility criteria for revaccination.

Table 2. Reasons for exclusion of individuals who underwent screening.

Some subjects were excluded for multiple reasons

Total individuals screened	396 (100%)
Screening failures	356 (89.9%)
Breastfeeding	3 (0.8%)
Quantiferon positive	247 (62.4%)
TST ≥ 15mm	143 (36.1%)
Abnormal ECG	10 (2.5%)
Abnormal biochemistry	20 (5.1%)
Abnormal hematology	22 (5.6%)
Abnormal urine dipstix	2 (0.5%)
Abnormal chest radiograph	2 (0.5%)
Chronic illness: hypertensive (n=2); Chronic corneal herpes infection (n=1)	3 (0.8%)
Loss to follow up during screening process	3 (0.8%)
Pregnant	4 (1.0%)
Withdrew consent during screening process	9 (2.3%)
Age outside range	3 (0.8%)
BMI outside range of 18 – 30	5 (1.3%)
Smoker (> 3 days / week)	4 (1.0%)
Hepatitis B surface antigen positive	19 (4.8%)
Hepatitis C surface antigen positive	2 (0.5%)
HIV positive	22 (5.5%)

Table 3a. Summary of adverse events (AE)

Group	Placebo	Group 1	Group 2	Group 3	Group 4	Total
Severity						
Mild	23	29	16	26	23	**117 (74.0%)**
Moderate	4	4	10	11	1	**30 (19.0%)**
Severe	2	3	2	0	4	**11 (7.0%)**
Total AE	**29**	**36**	**28**	**37**	**28**	**158 (100%)**
AE related to the vaccine	**11 (37.9%)**	**20 (55.6%)**	**17 (60.7%)**	**14 (37.8%)**	**18 (64.3%)**	**80 (50.6%)**

Table 3b. Table of solicited and unsolicited events

	Placebo (n=11)	Group 1 (n=7)	Group 2 (n=7)	Group 3 (n=9)	Group 4 (n=6)	Total (n=40)
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Subjects with at least one Adverse Event	**9 (81.8)**	**7 (100%)**	**6 (85.7%)**	**8 (88.9%)**	**5 (83.3%)**	**35 (100%)**
SOLICITED EVENTS						
Arthralgia	-	2 (28.6)	-	1 (11.1)	-	3 (8.6)
Conjunctivitis	-	-	-	1 (11.1)	-	1 (2.9)
Diarrhoea	-	3 (42.9)	-	-	-	3 (8.6)
Dysuria	1 (9.1)	-	-	2 (22.2)	-	3 (8.6)
Injection site erythema	-	-	1 (14.3)	1 (11.1)	-	2 (5.7)
Injection site pain	2 (18.2)	1 (14.3)	1 (14.3)	5 (55.5)	4 (66.7)	13 (37.1)
Injection site swelling	-	-	2 (28.6)	-	-	2 (5.7)
Malaise	3 (27.3)	2 (28.6)	2 (28.6)	4 (44.4)	1 (16.7)	12 (34.3)
Myalgia	1 (9.1)	2 (28.6)	1 (14.3)	3 (33.3)	2 (33.4)	9 (25.7)
Fever	-	-	1 (14.3)	1 (11.1)	-	2 (5.7)
Rash	1 (9.1)	-	-	-	-	1 (2.9)
Sore throat	1 (9.1)	2 (28.6)	2 (28.6)	-	3 (50)	8 (22.9)
Upper respiratory tract infection	2 (18.2)	3 (42.9)	1 (14.3)	1 (11.1)	1 (16.7)	8 (22.9)
UNSOLICITED EVENTS						
Abdominal pain	-	-	1 (14.3)	-	-	1 (2.9)
Activated partial thromboplastin time	-	-	1 (14.3)	-	1 (16.7)	2 (5.7)
Acute HIV infection	-	-	1 (14.3)	-	-	1 (2.9)
Alanine aminotransferase increased	-	1 (14.3)	-	-	-	1 (2.9)
Aspartate aminotransferase increased	-	1 (14.3)	-	-	-	1 (2.9)
Blood creatine phosphokinase increased	4 (36.4)	2 (28.6)	3 (42.9)	2 (22.2)	2 (33.4)	13 (37.1)
Blood pressure systolic increased	1 (9.1)	-	-	1 (11.1)	1 (16.7)	3 (8.6)
Bradycardia	-	1 (14.3)	1 (14.3)	-	-	2 (5.7)
Cystitis	1 (9.1)	-	-	-	-	1 (2.9)
Dysmenorrhoea	1 (9.1)	1 (14.3)	-	-	-	2 (5.7)
Dyspepsia	-	-	-	1 (11.1)	-	1 (2.9)
Escherichia urinary tract infection	-	-	-	1 (11.1)	1 (16.7)	2 (5.7)
Gamma-glutamyltransferase increased	1 (9.1)	-	-	-	1 (16.7)	2 (5.7)
Haematuria	-	-	-	2 (22.2)	2 (33.4)	4 (11.4)
Haemoglobin decreased	3 (27.3)	4 (57.1)	2 (28.6)	3 (33.3)	4 (66.7)	16 (45.7)
Headache	2 (18.2)	4 (57.1)	1 (14.3)	2 (22.2)	1 (16.7)	10 (28.6)
Hypertension	1 (9.1)	-	-	-	-	1 (2.9)
Lymphocyte count decreased	1 (9.1)	1 (14.3)	-	-	-	2 (5.7)
Nausea	1 (9.1)	-	2 (28.6)	-	-	3 (8.6)
Neutrophil count decreased	-	-	-	2 (22.2)	-	2 (5.7)
Ocular hyperaemia	-	1 (14.3)	-	-	-	1 (2.9)
Pain in extremity	1 (9.1)	1 (14.3)	-	-	-	2 (5.7)
Proteinuria	-	-	1 (14.3)	3 (33.3)	1 (16.7)	5 (14.3)
Prothrombin time prolonged	-	1 (14.3)	1 (14.3)	-	-	2 (5.7)
Schistosomiasis	-	-	-	1 (11.1)	-	1 (2.9)
Sinusitis	-	1 (14.3)	-	-	-	1 (2.9)
Suicide attempt	-	-	1 (14.3)	-	-	1 (2.9)
Tachycardia	-	-	2 (28.6)	-	-	2 (5.7)
Tonsillitis	-	-	-	1 (11.1)	-	1 (2.9)
Toothache	-	1 (14.3)	-	-	-	1 (2.9)
Vaginal infection	1 (9.1)	-	-	-	-	1 (2.9)
White blood cell count increased	-	1 (14.3)	1 (14.3)	-	1 (16.7)	3 (8.6)

Figure 1

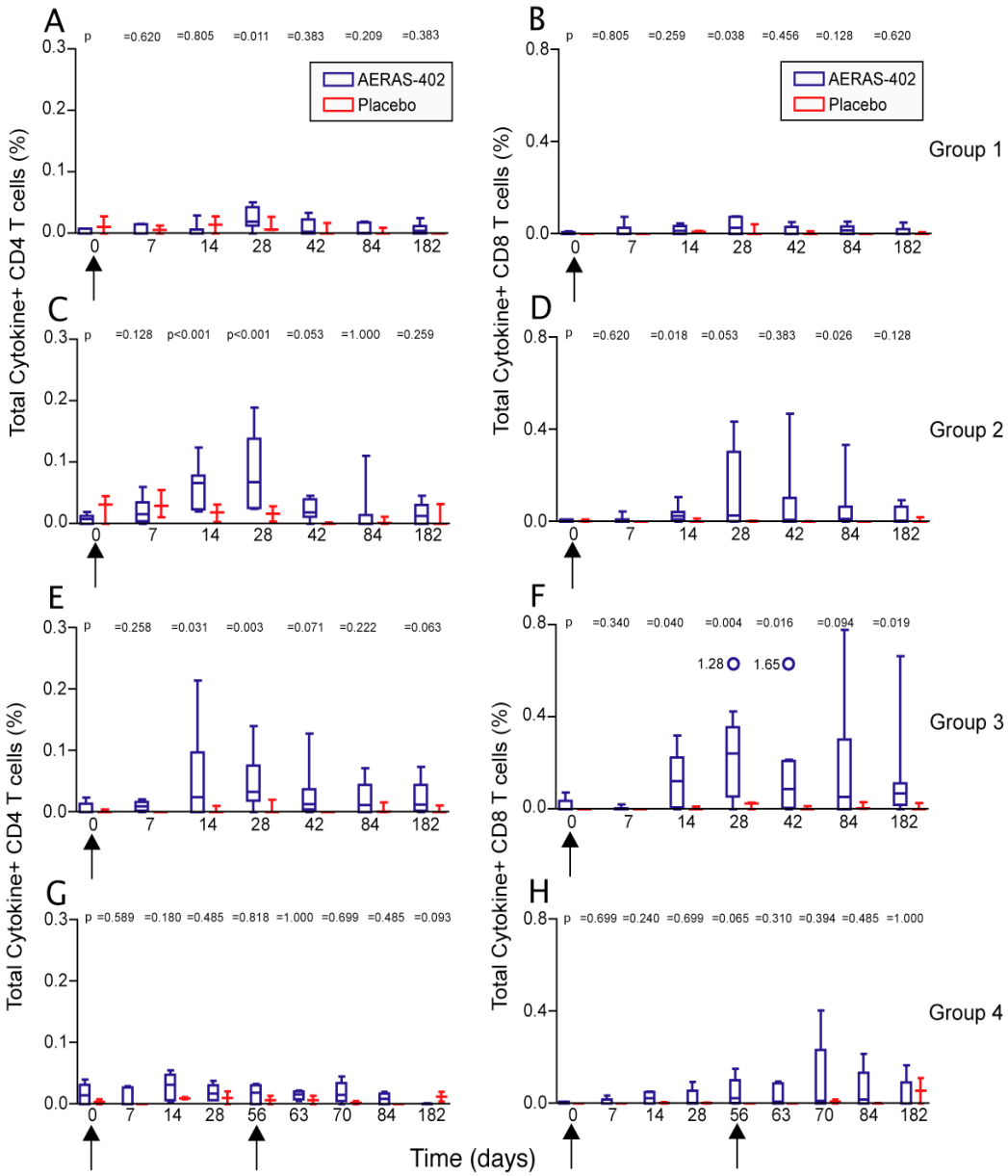

A
B
C
D
E
F
G
H
AERAS-402
Placebo
Group 1
Group 2
Group 3
Group 4
Total Cytokine+ CD4 T cells (%)
Total Cytokine+ CD8 T cells (%)
Time (days)
0
7
14
28
42
56
63
70
84
182
1.28
1.65

Figure 2

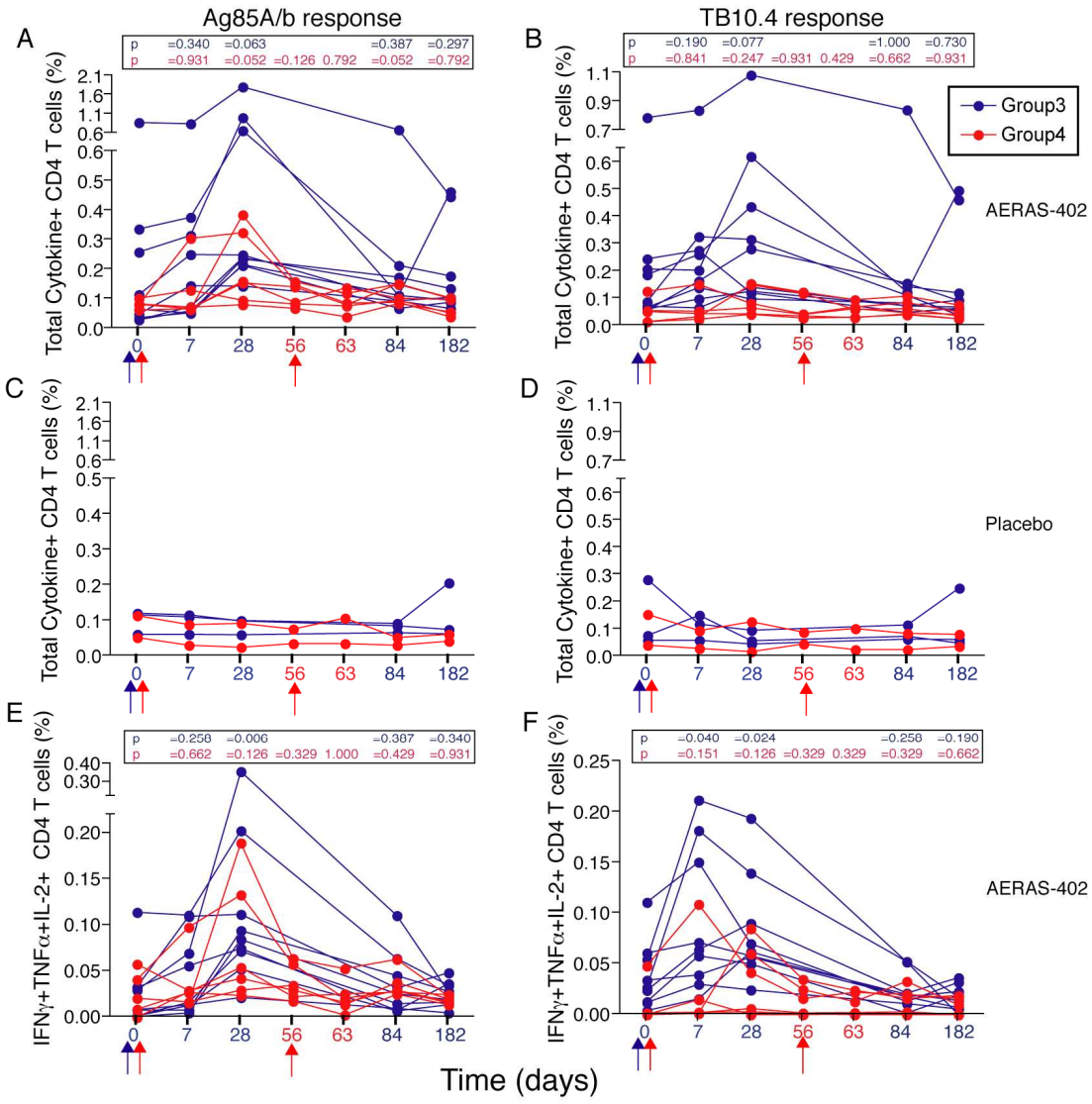

Ag85A/b response
TB10.4 response
A
B
C
D
E
F
Group3
Group4
AERAS-402
Placebo
AERAS-402
Total Cytokine+ CD4 T cells (%)
IFNγ+TNFα+IL-2+ CD4 T cells (%)
Time (days)
0
7
28
56
63
84
182

Figure 3

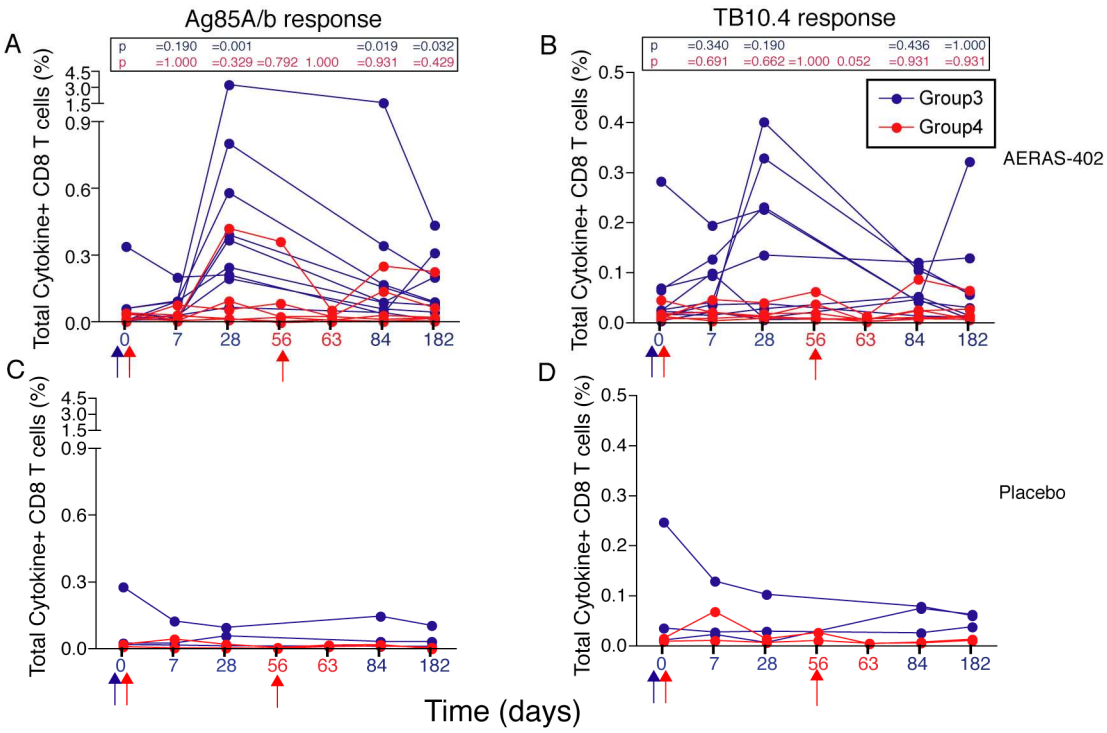

Ag85A/b response
TB10.4 response
A
B
C
D
p =0.190 =0.001 =0.019 =0.032
p =1.000 =0.329 =0.792 1.000 =0.931 =0.429
p =0.340 =0.190 =0.436 =1.000
p =0.691 =0.662 =1.000 0.052 =0.931 =0.931
Group3
Group4
AERAS-402
Placebo
Total Cytokine+ CD8 T cells (%)
Time (days)
0 7 28 56 63 84 182

Figure 4

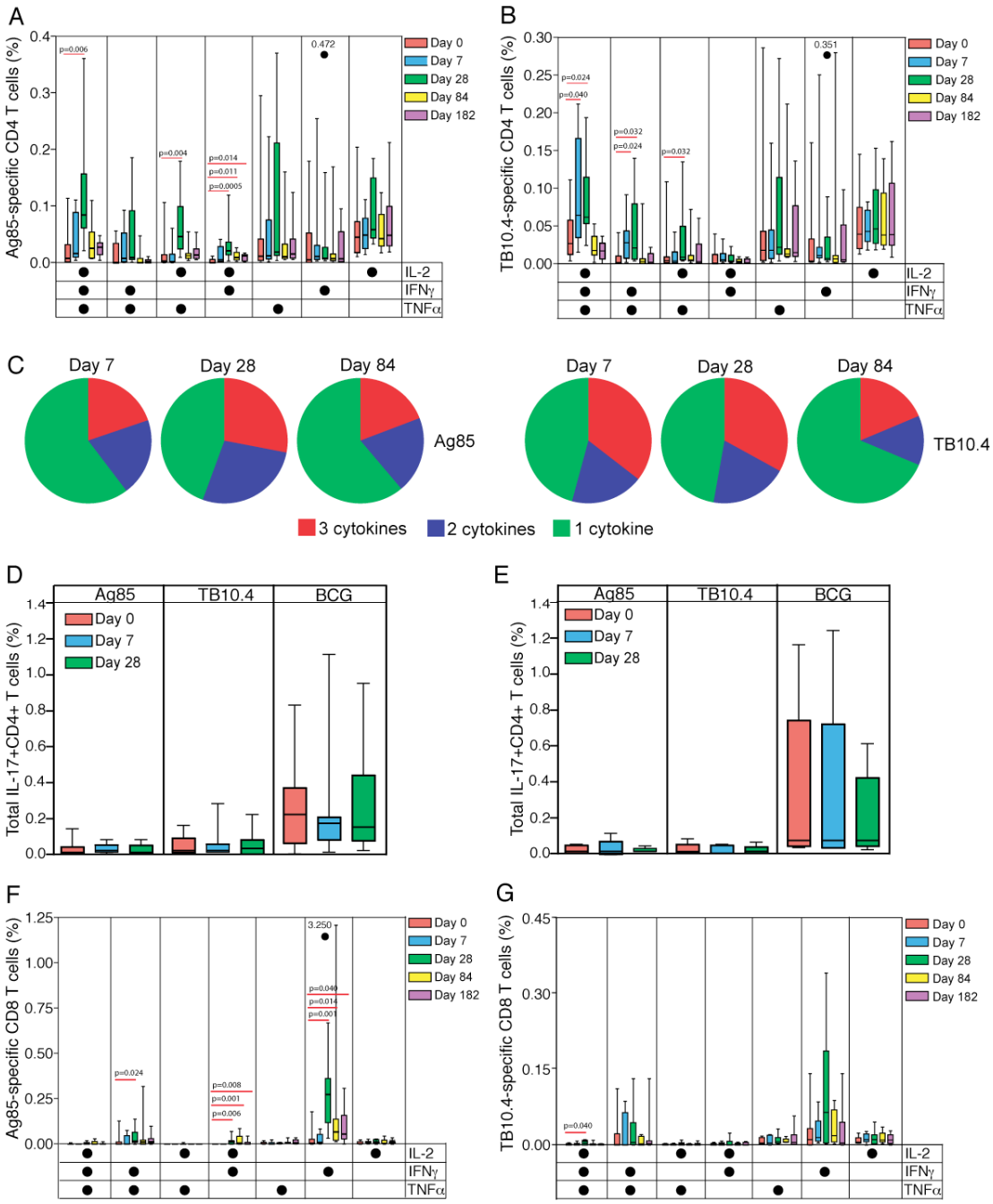
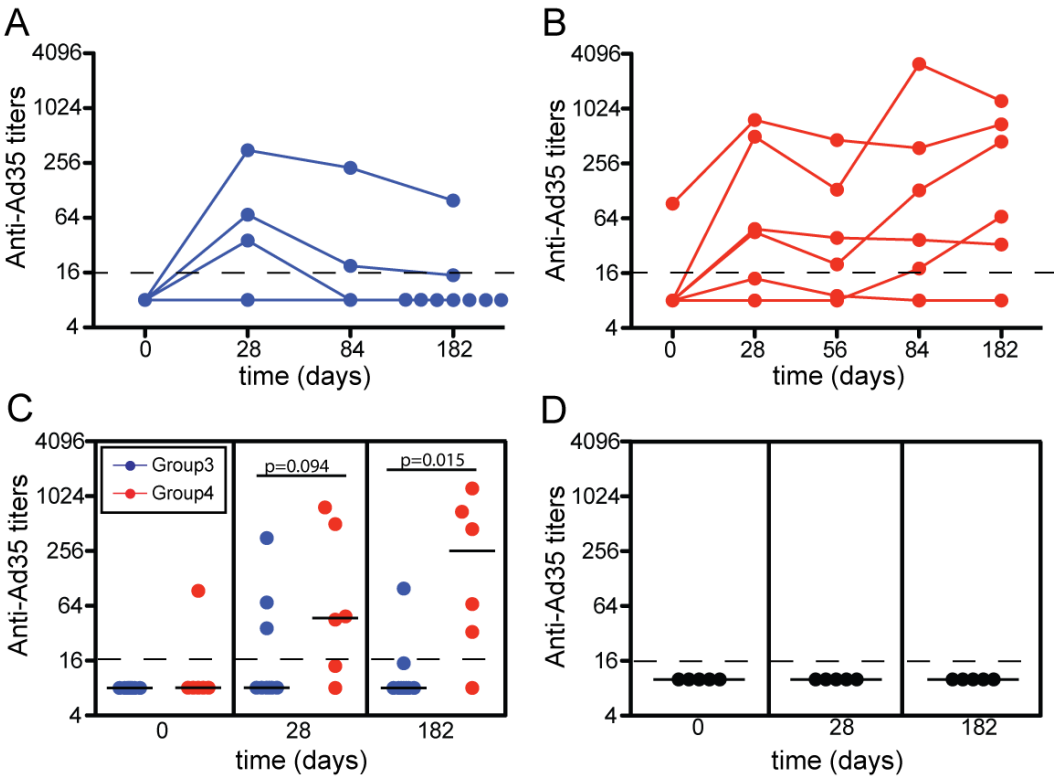

A
Ag85-specific CD4 T cells (%)
Day 0
Day 7
Day 28
Day 84
Day 182
IL-2
IFNγ
TNFα
B
TB10.4-specific CD4 T cells (%)
C
Day 7
Day 28
Day 84
Ag85
TB10.4
3 cytokines
2 cytokines
1 cytokine
D
Ag85
TB10.4
BCG
Total IL-17+CD4+ T cells (%)
E
F
Ag85-specific CD8 T cells (%)
G
TB10.4-specific CD8 T cells (%)

Figure 5


A
B
C
D
Anti-Ad35 titers
4096
1024
256
64
16
4
0
28
56
84
182
time (days)
Group3
Group4
p=0.094
p=0.015

The Novel TB Vaccine, AERAS-402, Induces Robust and Polyfunctional CD4 and CD8 T Cells in Adults

Online Data Supplement

Brian Abel, Michele Tameris, Nazma Mansoor, Sebastian Gelderbloem, Jane Hughes, Deborah Abrahams, Lebohang Makhethe, Mzwandile Erasmus, Marwou de Kock, Linda van der Merwe, Anthony Hawkridge, Ashley Veldsman, Mark Hatherill, Giulia Schirru, Maria Grazia Pau, Jenny Hendriks, Gerrit Jan Weverling, Jaap Goudsmit, Donata Sizemore, J. Bruce McClain, Margaret Goetz, Jacqueline Gearhart, Hassan Mahomed, Gregory D. Hussey, Jerald C. Sadoff, Willem A. Hanekom

Supplementary Figure 1. Flow cytometric analysis of AERAS-402-induced T cell cytokine production. Representative dotplots from a single participant are shown. (**A**) Gating strategy used to identify CD4 and CD8 T cells. From left to right, leukocytes from whole blood were acquired and cell doublets excluded using forward scatter-area versus -height parameters. Small lymphocytes were then selected from singlets and, following that, T cells by gating on CD3+ cells. Finally, CD4 and CD8 T cells were selected (extreme right plot). (**B**) Representative gating of cytokine-positive CD4 T cells in unstimulated (UNS) whole blood, or blood stimulated with Ag85A/b peptide pool, TB10.4 peptide pool, or with BCG.

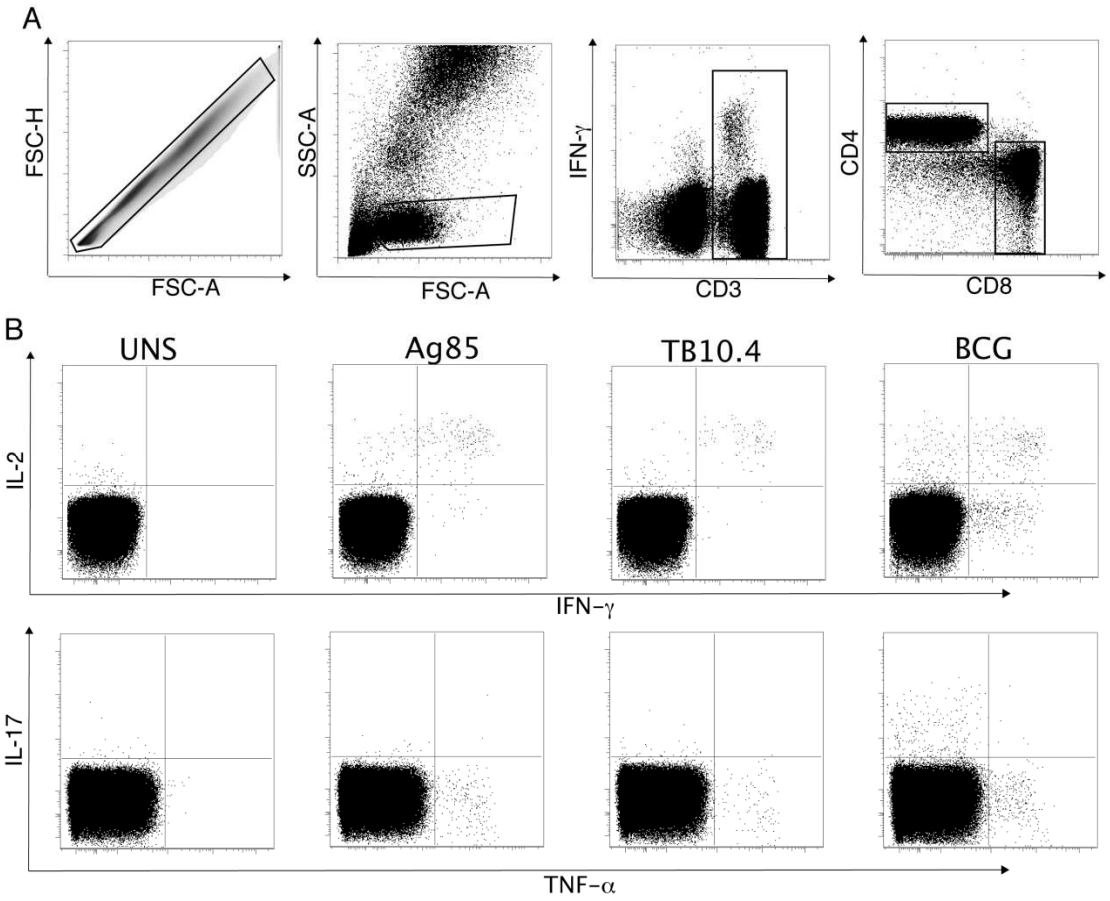
Supplementary Table 1. Proportion of Responders to AERAS-402

Supplementary Figure 2. Frequency of TB10.4-specific T cells induced by AERAS-402, as measured by flow cytometry following incubation of PBMC with a peptide pool of the antigens. CD4 T cell (left panels) and CD8 T cell (right panels) responses, in AERAS-402 vaccinated (blue boxes) and placebo vaccinated (red boxes) participants are shown. Participants from groups 1 (**A and B)**, 2 **(C and D)** and 3 **(E and F)** received a single, escalating dose of AERAS-402 on day 0 (indicated by the black arrow under the x-axis). Group 4 participants **(G and H)** received two doses of AERAS-402 on days 0 and 56 (indicated by black arrow under the axis). Background values (unstimulated) were subtracted for each condition from each individual. For each plot, the median is represented by the horizontal line, the interquartile range by the box and the range by the whiskers. The p values indicated were derived from comparing responses with those at baseline, using the Mann Whitney U test.

Supplementary Figure 3. Longitudinal analysis of Ag85-specific T cells induced by AERAS-402, as measured by flow cytometry following incubation of PBMC with a peptide pool of the antigens. CD4 T cell (left panels) and CD8 T cell (right panels) responses, in AERAS-402 vaccinated (A-H) and placebo vaccinated (I-J) participants are shown. Participants from groups 1 (**A and B)**, 2 **(C and D)** and 3 **(E and F)** received a single, escalating dose of AERAS-402 on day 0 (indicated by the red arrow under the x-axis). Group 4 participants **(G and H)** received two doses of AERAS-402 on days 0 and 56 (indicated by red arrows under the axis). Background values (unstimulated) were subtracted for each condition from each individual. Each line represents a single participant followed up longitudinally in the study.

Supplementary Figure 4. Longitudinal analysis of TB10.4-specific T cells induced by AERAS-402, as measured by flow cytometry following incubation of PBMC with a peptide pool of the antigens. CD4 T cell (left panels) and CD8 T cell (right panels) responses, in AERAS-402 vaccinated (A-H) and placebo vaccinated (I-J) participants are shown. Participants from groups 1 (**A and B)**, 2 **(C and D)** and 3 **(E and F)** received a single, escalating dose of AERAS-402 on day 0 (indicated by the red arrow under the x-axis). Group 4 participants **(G and H)** received two doses of AERAS-402 on days 0 and 56 (indicated by red arrows under the axis). Background values (unstimulated) were subtracted for each condition from each individual. Each line represents a single participant followed up longitudinally in the study.

Supplementary Figure 1. Gating strategy

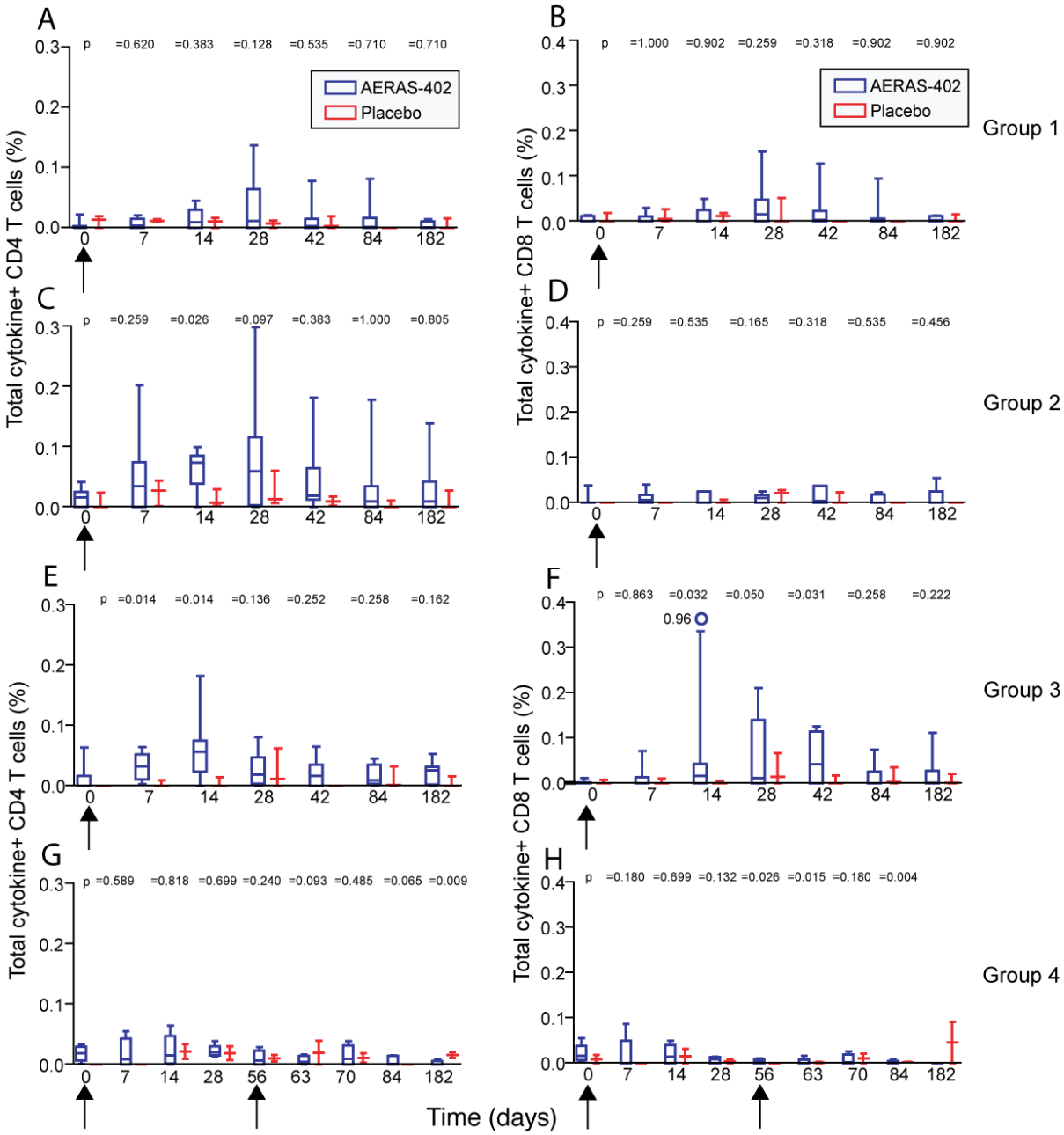


Supplementary Table 1. Proportion of responders to AERAS-402 vaccination

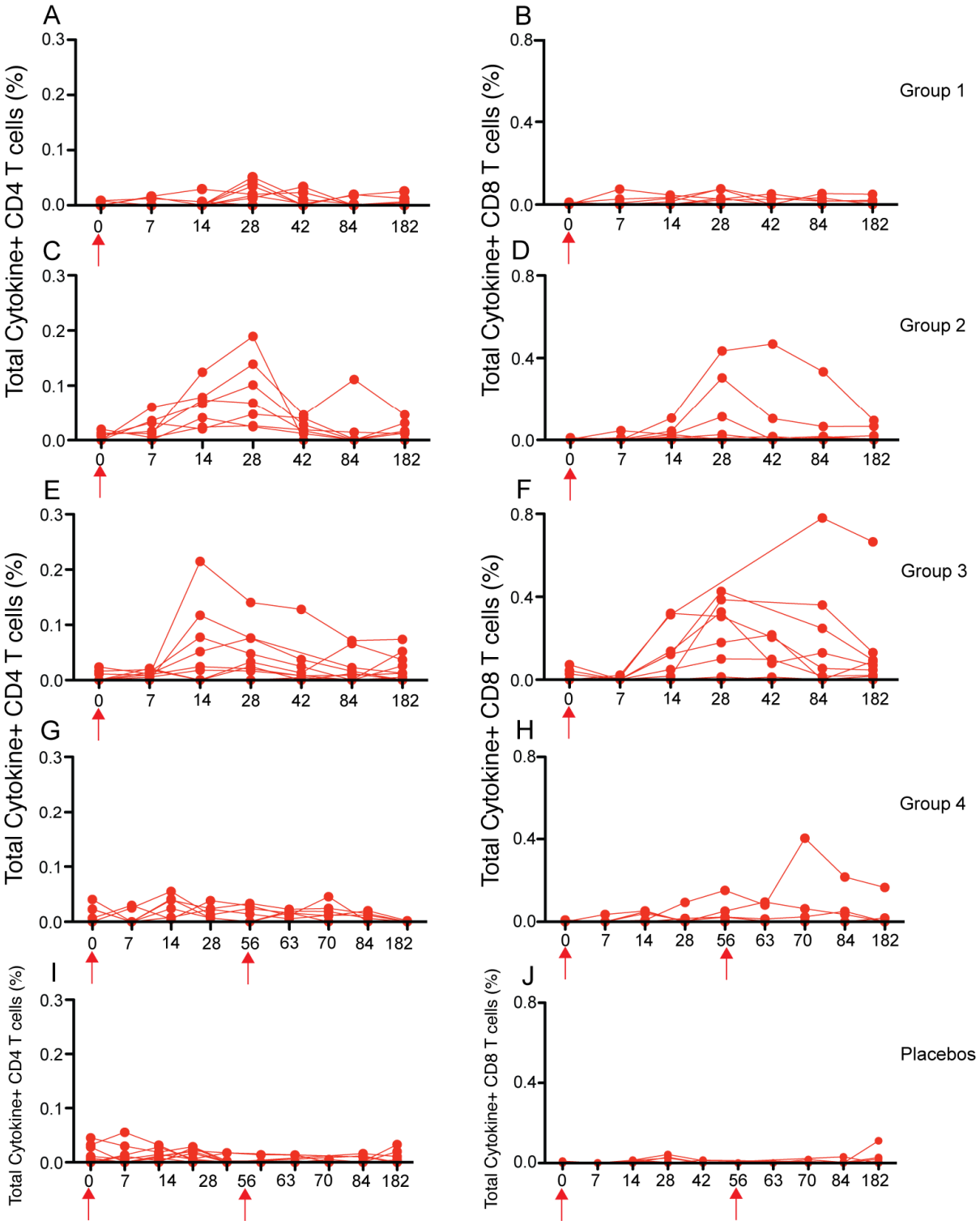
ND = not done

			Proportion of responders to AERAS-402 (%)			
Assay	**T cell**	**Antigen**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
PBMC	CD4	Ag85A/b	3/7 (43%)	6/7 (86%)	5/9 (56%)	2/6 (33%)
WBA	CD4	Ag85A/b	ND	ND	9/9 (100%)	5/6 (83%)
PBMC	CD8	Ag85A/b	4/7 (57%)	3/7 (43%)	6/9 (67%)	4/6 (67%)
WBA	CD8	Ag85A/b	ND	ND	8/9 (89%)	4/6 (67%)
PBMC	CD4	TB10.4	3/7 (43%)	6/7 (86%)	6/9 (67%)	2/6 (33%)
WBA	CD4	TB10.4	ND	ND	8/9 (89%)	4/6 (67%)
PBMC	CD8	TB10.4	2/7 (29%)	1/7 (14%)	3/9 (33%)	2/6 (33%)
WBA	CD8	TB10.4	ND	ND	8/9 (89%)	2/6 (33%)

Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.