

First Human Immunization with A Live-Attenuated *Mycobacterium tuberculosis*: a randomized, double-blind, controlled phase I trial

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SUMMARY

Background

Tuberculosis remains one of the world's deadliest transmissible diseases despite the widespread use of BCG. MTBVAC is a new live tuberculosis vaccine based on a genetically attenuated *phoP*-/*fadD26*-deletion mutant of *M. tuberculosis* that expresses most antigens present in human isolates in contrast to BCG.

Methods

We conducted this randomized, double-blind, controlled phase I study at CHUV, Lausanne, Switzerland, to compare MTBVAC to BCG in healthy, PPD-negative adults. Primary outcome was safety in all vaccinated participants. Secondary outcome included whole blood cell mediated immune response to live MTBVAC and BCG as well as interferon-gamma release assay (IGRA) on peripheral blood mononuclear cells. Volunteers fulfilling the inclusion criteria were randomly allocated (on a 3:1 basis) in a dose-escalation manner to three cohorts. Each cohort included 9 subjects who were injected with MTBVAC 5×10^3 , 5×10^4 , or 5×10^5 colony forming units (CFU) in 0.1 mL and 3 subjects with BCG (single dose of 5×10^5 CFU in 0.1 mL). Each subject received a single intradermal injection in the non-dominant arm starting with the lowest MTBVAC dose.

Findings

Thirty-six volunteers were recruited. Vaccination with MTBVAC (5×10^3 , 5×10^4 , 5×10^5 CFU/0.1mL) was as safe as with BCG, and did not induce serious adverse events. All individuals were IGRA negative at the end of follow-up (D210). After whole blood stimulation with live MTBVAC or BCG, MTBVAC was immunogenic in a dose-dependent manner. At the same dose level as BCG (5×10^5 CFU), although no

statistical significance could be achieved, there were more responders in the MTBVAC group with a greater frequency of polyfunctional CD4⁺ central memory T-cells.

Interpretation

For the first time in the history of human vaccinology, a live-attenuated *M. tuberculosis* vaccine has reached clinical evaluation showing comparable safety as BCG. The absence of ESAT-6 and CFP-10 *conversion at the end of the study* warrants use of IGRA in future efficacy trials to study not only disease prevention but also infection prevention. MTBVAC was at least as immunogenic as BCG. Combined with an excellent safety profile, these data support advanced clinical development in high-burden tuberculosis endemic countries.

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INTRODUCTION

Mycobacterium tuberculosis is responsible for more deaths than any other single infectious organism, with an annual disease rate of 9 million new cases and 1.5 million deaths reported in 2013¹. Pulmonary tuberculosis (TB), the respiratory form of the disease, fuels the current expanding epidemic². BCG is a live attenuated derivative of the bovine pathogen *Mycobacterium bovis*, originally isolated from cattle, and is currently the only vaccine available for the prevention of TB in human. Because of its efficacy in reducing the risk of severe complications in childhood (miliary TB and TB meningitis), BCG has been maintained as part of the routine immunization schedule in high-burden countries³ in contrast to high-income, low-burden countries, where BCG is generally not recommended for use⁴.

The members of *M. tuberculosis* complex including *M. tuberculosis* and *M. bovis* are characterized by 99.9% similarity at the nucleotide level but differ widely in their host tropisms, phenotypes, and pathogenicity⁵. Genomic comparison studies have shown that during its evolution *M. bovis* had lost more than 100 genes expressed in *M. tuberculosis*, and during *in vitro* attenuation of *M. bovis* BCG, additional genes were lost including the chromosomal region of difference 1 (RD1), which contains the major antigen and secreted virulence factor ESAT-6⁶. Approximately 23 % of the known *M. tuberculosis*-specific human T-cell epitopes are absent in BCG⁷.

MTBVAC, the first and only live-attenuated *M. tuberculosis* vaccine in the current global TB vaccine portfolio^{8,9}, is based on the rational attenuation of the clinical isolate *M. tuberculosis* MT103, which belongs to the most widespread lineage 4 (Europe-America-Africa)². Two genetic deletion of independent virulence factors, *phoP* and *fadD26* genes were generated in the absence of antibiotic resistance markers⁹ fulfilling the Geneva consensus requirements for progressing live mycobacterial vaccines to clinical trials¹⁰. The *phoP* gene is a virulence factor of *M. tuberculosis*¹¹, which encodes

a transcription factor regulator controlling more than 2 % of its genome, including genes essential for ESAT-6 secretion^{12,13}. Recent experiments have shown that the virulence of *M. bovis* B strain is due to overexpression of *phoP* gene as a result of an insertion sequence in its promoter¹⁴. The *fadD26* gene is involved in synthesis of major complex virulence cell-wall lipid PDIM¹⁵.

Extensive preclinical studies demonstrated adequate attenuation and safety profile of MTBVAC comparable to BCG, with superior immunogenicity and efficacy against pulmonary forms of TB⁹. Here we present the first-in-human safety and immunogenicity trial with an attenuated *M. tuberculosis* vaccine that expresses most antigens present in human isolates in contrast to BCG.

METHODS

Trial design

This randomized, double-blind, controlled, dose-escalation study was conducted to compare safety, reactogenicity and immunogenicity of MTBVAC with BCG in healthy adults. The study was approved by the local Ethical Review Board (Lausanne, Switzerland, #146/12) and the Swiss National Regulatory Authority (Swissmedic, Bern, #2012GT1002) and undertaken in accordance with the Helsinki Declaration and Good Clinical Practices. Written consent was obtained from all participants before entering the study.

Study participants and study settings

Adult males and females aged 18-45 years were eligible when clinically healthy, HIV (-1 & -2) negative, ELISPOT TB (ESAT-6/CFP-10) negative, with no history of active TB or of chemoprophylaxis for TB, no documented history of BCG vaccination. Provided they had clinical laboratory values considered acceptable by the investigator, and in the absence of pregnancy and lactation, subjects fulfilling the inclusion criteria were enrolled and randomly allocated (on a 3:1 basis) in a dose-escalation manner to three cohorts. Each cohort included 9 subjects who were intradermally injected with MTBVAC 5×10^3 , 5×10^4 , or 5×10^5 colony forming units (CFU) in 0.1 mL diluent (sterile water for injection) and 3 subjects with BCG (single dose of 5×10^5 CFU in 0.1 mL diluent). Each subject received a single intradermal injection in the non-dominant arm starting with the lowest MTBVAC dose (5×10^3 CFU, cohort 1). Each volunteer was followed for 12 months with a last visit at the vaccination centre at D210 and a phone call for safety at one year. Study was fully carried out at the Vaccination and Immunotherapy Center (VIC), Centre Hospitalier Universitaire Vaudois (CHUV), from Jan 2013 to Nov. 2014.

Randomisation and masking

Volunteers within each cohort were assigned to one of 2 vaccine arms (MTBVAC or BCG) in the order in which they were enrolled into the study in a 3:1 manner. The treatment allocation at the investigator site was performed by the CHUV Pharmacy. Upon providing a subject number, the randomization system applied the minimization algorithm to determine the treatment number to be used for the subject. As soon as the target number of subjects in a specific group was reached, the enrolment was frozen for this group. Data pertaining to MTBVAC or BCG arms were collected in an observer-blinded manner. MTBVAC and BCG were prepared and blinded by the pharmacist whereas the vaccination was done by the medical team. The principle investigator and /or co-investigator were responsible for assessing safety and post-vaccination side effects. Blinding, including laboratories, was maintained throughout the vaccination and follow up periods of the vaccination trial until freezing of safety and immunogenicity data banks.

Study vaccines and procedures

MTBVAC was produced and characterized by BIOFABRI, S.L. (Porriño, Spain) in compliance with Good Manufacturing Practices as a freeze-dried preparation following the European Pharmacopoeia monograph and the “WHO Recommendations To Assure The Quality, Safety And Efficacy Of BCG Vaccines” ¹⁶. BCG was a commercial formulation of Danish strain (BCG SSI) from Statens Serum Institute, Copenhagen, Denmark. Each subject received a single intradermal injection of BCG or MTBVAC in the non-dominant arm, starting with the lowest MTBVAC dose (5×10^3 CFU, cohort 1).

Primary outcome

Safety and reactogenicity: Safety was assessed based on a collection of solicited local (injection site pain, erythema, induration, pruritus and discharges) and systemic (fever,

headache, fatigue, musculoskeletal pains, gastrointestinal symptoms) adverse events (AEs) within 7 days of vaccination (diary card). AEs intensities were scored from 1 to 3. Grade 3 (severe) AEs were defined as those preventing normal activity, erythema or induration >50 mm in diameter, or an axillary temperature $\geq 39.0^{\circ}\text{C}$. Unsolicited local and systemic AEs as well as serious AEs (SAEs) were collected during the entire study period. After at least 35 days of follow-up within each cohort, an intermediate safety report of blinded data was reviewed by an independent Data Safety Monitoring Board prior to allowing to proceed to the next higher dose group of MTBVAC. Hematological and biochemical parameters (complete blood count, creatinine, urea, bilirubin, alkaline phosphatase, alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) as well as urine parameters were performed before and after vaccination, at days 0, 7, 28, 56, 90, 150 and 210.

Environmental control and microbiological follow-up: In agreement with Swissmedic requirements, the trial evaluated potential shedding of the vaccine from the site of injection and excretion in urine and stools as part of the environmental risk assessment. Urine and stool samples and cutaneous swabs from the injection site were collected and plated on selective solid agar medium for detection of mycobacteria until two consecutive negative cultures were obtained. Positive samples were typed by polymerase chain reaction (PCR) for differentiation between BCG *versus* *M. tuberculosis* based on the detection of RD9 or TBD1, respectively⁵.

Secondary outcome: immunogenicity

Ex vivo Interferon-gamma ELISPOT: Peripheral blood mononuclear cells (PBMCs) were isolated from blood-EDTA by centrifugation on Ficoll as previously described¹⁷. PBMC responses to *M. tuberculosis* purified protein derivative (PPD) (NIBSC; 300 IU/mL), ESAT-6 and CFP-10 (pools of 15-amino-acid peptides with 11 overlaps covering the protein sequences, minimum 75% purity by HPLC, JPT Peptide

Technologies Berlin, Germany; 1 µg/mL each peptide) were assessed on fresh cells. Staphylococcal enterotoxin B (SEB) (Sigma) was used as a positive control (200 ng/mL). Unstimulated PBMCs were used as a measure of background interferon-gamma (IFN γ) production. Results are reported as spot-forming unit (SFU) per million PBMC, calculated by subtracting the mean count of the unstimulated PBMCs from the mean count of quadruplicate antigen wells and correcting for the number of PBMCs per well (200'000). Assays with >50 SFU per 10⁶ cells for the negative control and <500 SFU per 10⁶ cells after SEB stimulation were considered non valid and were repeated on frozen cells. ELISPOT for ESAT-6 and CFP-10, according to CHUV routine laboratory standards, was defined as positive if the number of SFUs was ≥ 55 SFU per 10⁶ cells and more than fourfold higher than the negative control. For PPD ELISPOT, positivity was set at 100 SFU, based on the mean SFU + 3SD of a group of healthy volunteers who were negative for ESAT-6 and CFP-10 by ELISPOT, and had no history of BCG vaccination nor BCG scar, and no evidence of active TB.

Whole blood assay and intracellular cytokine staining (ICS): Blood was collected at day 0, 28, 90 and 210 in heparinized syringes and processed within 75 min of collection, as previously described¹⁸. The rationale for using D28 as peak response was based on previous preclinical experiments comparing immune responses to MTBVAC and BCG and on clinical data with BCG or rBCG^{9,13}. Briefly, 1 mL whole blood was incubated for 12 h at 37 °C (in a closed test tube in a water bath) with 1.2 \times 10⁶ CFU/mL of viable freshly reconstituted BCG (SSI) or MTBVAC (Biofabri, Porriño, Spain) and the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences, 0.5µg/mL each). 1 mL blood incubated with phytohemagglutinin B (PHA, Sigma, 10µg/mL) and 1 mL incubated with the co-stimulatory antibodies alone (unstimulated) served as positive and negative controls, respectively. Brefeldin A (Sigma, 10 µg/mL) was added for the last 5 h of incubation. Following incubation, red blood cells were lysed and white cells fixed with BD FACS Lysing Solution (BD Biosciences) and the cells cryopreserved in

nitrogen. Cryopreserved cells from the same volunteer at 4 time-points were thawed simultaneously in RPMI 8% AB serum and washed in phosphate buffered saline (PBS, Bishel). Cells were stained with a rat anti-CCR7 (3D12) then permeabilized using Perm/Wash Solution (BD Biosciences). Cells were then stained with the following antibodies: anti-CD3 PerCPCy5.5 (clone SK7), anti-CD4 Pacific Blue (RPA-T4), anti-IL-2 FITC (5344.111), anti-CD8 APC-H7 (SK1), anti-rat-IgG APC, anti-TNF- α PE (MAb11 all from BD Biosciences), anti-CD45RA ECD (2H4, Beckman Coulter) and anti-IFN γ AlexaFluor700 (B27, Biolegend). Cells were acquired (at least 200000 CD3+) on a LSR II flow cytometer (BD Biosciences), using FACS Diva 6.1.2, Flowjo 9.6.4 (Treestar), SPICE v5.1 (downloaded from <http://exon.niaid.nih.gov>) and GraphPad Prism v6.05 softwares were used to analyze data from boolean gating. Gating strategy is shown in Fig. S1. Memory response (in % of CD4 or CD8 subsets) included the total T cell subset with exclusion of CD45RA+ CCR7+ double positive cells. T central memory cells (T_{CM}) within this subset were defined as CD45RA- CCR7+ and T effector memory cells (T_{EM}) as CD45RA- CCR7-. For each immune readout, a volunteer was considered as a responder when its individual response was superior to the mean response of all volunteers at D0 + 3SD.

Statistical Analysis

Safety analysis was performed on all evaluable subjects (i.e. those meeting all eligibility criteria, complying with the procedures defined in the protocol, with no elimination criteria during the study). Immunogenicity analysis was performed on the According to Protocol (ATP) cohort, comprising all participants who did not meet any elimination criteria during the study and for whom immunogenicity data were available. No sample size calculation in relation to a specific objective was performed. Statistical analyses of safety were conducted using SASv9. Proportions of subjects reporting an unsolicited AE, classified by the Medical Dictionary for Regulatory Activities (MedDRA)-preferred term, were tabulated with 95% CI. Similar analyses were conducted for grade

3 AEs and for those with a causal relationship to vaccination. Any SAE) was to be described. Per protocol, all solicited local AEs were considered to be related to vaccination. Biochemistry and hematology values outside of predefined reference ranges were recorded and assessed for clinical significance.

Descriptive statistics of the CD4⁺ and CD8⁺ T-cell responses were performed at each time-point using GraphPad Prism v6.05. For the ICS results, non parametric Friedman test with Dunn's post test was used to compare responses at different time-points with day 0 (D0), and non parametric Kruskal-Wallis test with Dunn's post test was used to compare responses in BCG-vaccinated and MTBVAC-vaccinated participants. Results were considered to be significantly different if $p < 0.05$.

Role of the funding source

The study sponsor (Biofabri) was involved in the study design, in the interpretation of data, and in the writing of the report. The corresponding author (FS) had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Participant flow and clinical characteristics

One hundred subjects expressed interest to participate to the study. After pre-selection by phone, 44 volunteers were screened for eligibility, of whom 36 were selected to be successively included into cohorts 1 to 3 (Fig. 1). All received a single intradermal administration of either MTBVAC or BCG in a randomized, double-blind manner. All volunteers (n=35) except one from MTBVAC 5×10^4 completed the D210 safety follow-up visit and 34 subjects responded to the safety phone call at one year. Immunogenicity data were completed at D210 for 35 subjects. Demographics were comparable across the four vaccine groups (the three MTBVAC dose groups and BCG group) (Supplementary Table 1 in the Supplementary Appendix).

Safety and reactogenicity

Vaccination with MTBVAC or BCG was generally well tolerated. There was no clinically relevant difference with regards to local and systemic AEs between the highest MTBVAC dose and BCG group (both at 5×10^5 CFU/0.1mL) (Fig. 2). A trend towards a higher incidence of local AEs in the MTBVAC groups in parallel to the dose of vaccine administered suggested a dose-dependency from D0 to D6 (Fig. 2A). Specifically, pain and induration were less frequently reported at the injection site after MTBVAC (all groups) than after BCG. Local or systemic AEs after D6 were rare, mostly vaccine non related, without any correlation with type or dose of vaccine administered (Fig. 2B, D). From D0 to D210, there were only two local grade 3 AEs: pain at injection site at D0 and at D2 in volunteers from MTBVAC 5×10^3 . A total of 16 grade 3 systemic AEs occurred, all vaccine unrelated. There was no SAE in any of the groups. Biological safety tests were generally within the normal range with no clinically relevant difference between groups.

Local discharges occurred after D6 in ten volunteers, six from BCG group (median duration of discharge 17.5 days, range 1 to 174 days) and four from MTBVAC 5×10^5 (median duration of discharge 21.5 days, range 1 to 89 days) (Fig. 2A, B). Positive cultures of mycobacteria from the injection site were observed in six of ten volunteers, four from BCG and two from MTBVAC 5×10^5 . One volunteer from MTBVAC 5×10^5 had a positive culture, but without discharge (local, benign ulceration only). Samples from urine and stool were all negative.

Immunogenicity

EAST-6, CFP-10 and PPD ELISPOT assays

All volunteers were negative by ELISPOT for ESAT-6, CFP-10 and PPD at D0 as well as at D210 (end of active follow-up study) (Fig. 3). A transient increase in CFP-10 specific IFN γ response over the limit of positivity (≥ 55 SFU/million PBMC) for the ELISPOT assay was observed in two subjects from MTBVAC 5×10^4 and in one subject of MTBVAC 5×10^5 at D28. ELISPOT to PPD peaked at D28, without reaching significance in any of the groups (Fig. 3).

Whole Blood Assay and Intracellular Cytokine Staining

We assessed the kinetics of CD4 $^+$ and CD8 $^+$ T-cell responses to vaccines by tracking the expression of IFN γ , TNF α or IL-2 upon stimulation with live MTBVAC or BCG. Cytokine positive CD4 $^+$ T-cells were induced after vaccination with MTBVAC in a dose-dependent manner (Fig. 4A, B, C) as well as with BCG (Fig. S2A, B, C in the Supplementary Appendix). At the dose of 5×10^5 , IFN γ and IL-2 positive CD4 $^+$ T-cells proportion and the number of responders were higher (although not statistically significant) after MTBVAC vaccination than after BCG, with a peak at D28. No difference between groups was observed for TNF α expressing CD4 $^+$ T-cells.

Poly- and mono-functional CD4⁺ and CD8⁺ responses were both induced. Pie charts showed that globally the highest proportion of polyfunctional CD4⁺ T-cells was observed in response to live MTBVAC in MTBVAC 5x10⁵ as compared to BCG (Fig 5A). Particularly, three-cytokine positive CD4⁺ memory T-cell response was significantly enhanced at D28 both after live MTBVAC and BCG stimulation (Fig. 4D, Fig. S2D). This observation was confirmed when we separately analyzed memory mono-, bi- and trifunctional CD4⁺ T-cells (Fig. 5B, C). Interestingly, a CD8⁺ T-cell response was present after stimulation with live BCG in both MTBVAC and BCG vaccinated groups (Fig. 5D), but was not detectable in response to *in vitro* stimulation with live MTBVAC (data not shown). CD8⁺ T-cell response was in this case mainly monofunctional (IFN γ or TNF α positive, data not shown).

Furthermore, we examined the response of both central and effector memory separately in MTBVAC 5x10⁵ and BCG (Fig. S3 in the Supplementary Appendix). A robust central memory CD4⁺ T-cell (T_{CM}) response was induced in both equivalent vaccine dose groups after stimulation with live MTBVAC and BCG. The effector memory CD4⁺ T-cell (T_{EM}) response was weaker in the two vaccine groups (equivalent doses), the strongest after stimulation with live MTBVAC suggesting that both MTBVAC and BCG vaccination might favour a central memory response induction.

Taken together, these data show that the response to live MTBVAC or BCG in both vaccine groups at equivalent dose was similar, although polyfunctional CD4⁺ T-cell as well as T_{CM} CD4⁺ T-cell responses appeared to be stronger after MTBVAC vaccination.

DISCUSSION

For the first time, a single intradermal dose of an attenuated *M. tuberculosis* has been administered as a vaccine to human volunteers, with a highly safe profile, comparable to BCG in healthy adults. No evidence of dissemination to spleen or lymphatic system was shown, based on clinical criteria (careful and repeated palpation of lymph node areas and spleen). These sites were checked carefully and systematically since lymph nodes were the only sites where the persistence of MTBVAC and BCG was shown in preclinical animal studies ⁹.

In any development of new TB vaccines, the potential interference of the vaccine with IGRA diagnostic tests has to be examined. This is particularly relevant to MTBVAC, the first live vaccine in clinical experimentation that expresses ESAT-6 and CFP-10, two antigens integrated in IGRA tests ^{9,13}. No positive responses to these antigens were found at the end of the follow-up period in any of the MTBVAC-vaccinated groups. However, CFP-10 induced a transient response in three MTBVAC-vaccinated volunteers at D28, which coincides with the peak of the immune response observed in the whole blood assay. These data show that even though ESX-1 secretion system of *M. tuberculosis* is impaired in MTBVAC¹³, intracellular expression of CFP-10 at least could still elicit some specific immune responses in some individuals upon vaccination. Future efficacy studies may help to determine whether this could represent an advantage in protection against TB. Of note, some subunit vaccines in development are based in part on ESAT-6 to improve protection in BCG-vaccinated or latently infected individuals⁸. Taken together, the negativity of IGRA tests seven months after MTBVAC immunization is a key element for progressing MTBVAC to efficacy and prevention of infection trials in high burden countries, such as South Africa¹⁹.

As secondary endpoint, MTBVAC demonstrated promising immunogenic properties. WBA was used to evaluate the response to live mycobacteria as it represents a more

relevant *in vitro* model than PBMC based antigen stimulation assays, mirroring an *in vivo* condition where all blood components of the immune system are present for an optimal antigen presentation and T-cell stimulation. MTBVAC vaccinated volunteers developed a dose-response dependent induction of polyfunctional (IFN γ ⁺, TNF α ⁺, IL-2⁺) MTBVAC- and BCG-specific CD4⁺ T-cells. In addition, MTBVAC 5x10⁵ induced a higher numbers of responders than BCG, although statistical significance could not be reached. Furthermore, although non significant, a memory CD8⁺ T cell response was induced after stimulation with live BCG, a phenomenon that will need more experimental work to be better understood. The marked enhancement of polyfunctional CD4⁺ T-cell response to live MTBVAC in MTBVAC vaccinees suggests at least a qualitative if not only quantitative difference between the two vaccines. Although we have currently no argument to translate this difference into protective activity, it will be interesting in future trials to examine whether this finding may have some value as biomarker of protection.

Experimental animal studies assessing the efficacy of novel TB vaccines have reported an association between mycobacteria specific polyfunctional T-cells co-expressing IFN γ , TNF α and IL-2 and protection against TB²⁰. However, clinical data in newborns in a high-burden area in South Africa did not reveal any correlation between frequency of cytokine-expressing specific T-cells and BCG-induced protection²¹. Moreover, Kagina et al did not distinguish effector and memory T-cells²¹. In this regard, our data suggest that MTBVAC may favour a T-cell central memory (T_{CM}) response rather than an effector memory in contrast to BCG²². Because of its capacity to proliferate and differentiate into effector cells, the T_{CM} cell subset is essential for the maintenance of the long term immunological memory. Interestingly, adoptive transfer studies in mice have shown that efficient protection in the lungs against an aerosol challenge with *M. tuberculosis* was mainly dependent on T-cells expressing a T_{CM} phenotype ²³.

Moreover, subunit vaccine H56 in the relatively low proinflammatory adjuvant IC31 induced a strong T_{CM} response in non human primates, which resulted in efficient limitation of *M. tuberculosis* infection and reduced rates of clinical disease, in particular pulmonary pathology and extrapulmonary dissemination²⁴. These observations may seem paradoxical, but may suggest that in TB a better protection may potentially be associated with a long lasting immunity provided by T_{CM} rather than terminally differentiated T_{EM} cells ^{25,26}.

The absence of PhoP-dependent immunomodulatory lipids, as well as lack of virulence cell-wall lipids PDIM may explain the higher immunogenicity of MTBVAC. In this regard, PDIM mutants of *M. tuberculosis* have been shown to co-localize with acidic compartments suggesting better antigen processing and presentation²⁷. In addition, MTBVAC expresses genes lost in *M. bovis* and BCG, some of them described as major antigens in human⁷, possibly contributing to the improved immunogenicity of this vaccine. Ongoing analysis is now aiming to evaluate the specific T cell responses to defined antigens differentially present in MTBVAC and/or BCG.

So far, no correlate of protection against TB has been identified. Therefore, efficacy studies in high-burden countries are crucial to determine the real value of new TB vaccines. Despite the failure of subunit vaccine MVA85A to improve BCG efficacy in infants in a Phase 2b trial in South Africa, this first efficacy trial since BCG has paved the way for testing new vaccines in this population²⁸. Other subunit vaccine candidates are progressing towards efficacy trials including M72F²⁹. On the other hand, live recombinant *M. bovis* BCG VPM1002³⁰ is the most advanced of the live vaccine strategies in the current vaccine pipeline now in Phase 2 clinical trials. MTBVAC can now be added to this list. Taken together, the excellent safety profile and encouraging immunogenicity results of this current phase 1 with the live attenuated MTBVAC vaccine support further clinical development in different target populations in high

burden countries. A phase 1b trial for safety and immunogenicity in healthy newborns in South Africa was recently approved. Further plans to apply a large array of immunogenicity endpoints in higher number of volunteers (adults and adolescents) are underway, with the aim to more deeply detail and potentially distinguish immunogenicity between MTBVAC and BCG in TB-endemic countries.

Contributors

Authors' contribution

FS (Principal Investigator), RA, DM, NA, JD, EP and CM contributed to study design. RA, RC, OK, VSM, ACT, CEM, NR, KJ, LV conducted the study. RA performed immunological data statistical analysis. KJ did the microbiological work. FS, RA, DM, NA, CM wrote the paper.

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536

FIGURE LEGENDS

Fig. 1. Screening, Enrollment, Vaccinations, and Follow-Up. Subjects fulfilling the inclusion criteria were enrolled and randomly allocated (on a 3:1 basis) in a dose escalation manner to three cohorts, with a total of 12 participants per cohort comprising 9 subjects injected with MTBVAC 5×10^3 , or 5×10^4 , or 5×10^5 CFU and 3 subjects with BCG 5×10^5 CFU. After unblinding, 3 MTBVAC groups and one BCG group, each of 9 subjects, were identified. All available study data and samples were used for study analyses.

Fig. 2. Local and systemic AEs. Safety was assessed based on a collection of solicited local (injection site pain, erythema, induration, pruritus, discharges, scar) and systemic AEs (headache, musculoskeletal pains, fatigue, digestive symptoms, fever) as well as unsolicited AEs. A) Local AEs within 7 days of vaccination (diary card). B) Systemic AEs within 7 days of vaccination, C) Local AEs from D7 to D210 after vaccination, D) Systemic AEs from D7 to D210 after vaccination. "Other" indicates unsolicited AEs not includable in the local or systemic categories mentioned. AEs severity was described as indicated in M&M. Bars indicate the number of patients with AE, whatever the grade of severity. ■ MTBVAC G1 (group 1) 5×10^3 , ■ MTBVAC G2 5×10^4 , ■ MTBVAC G3 5×10^5 , ■ BCG 5×10^5 CFU/mL.

Fig. 3. TB ELISPOT assay. ELISPOT was performed on the day of screening and at days 0, 28 and 210. Panels show peripheral blood mononuclear cells (PBMCs) responses to ESAT-6, CFP-10 and *M. tuberculosis* purified protein derivative (PPD) in spot forming units (SFU) per million cells. All volunteers were negative for ESAT-6, CFP10 and PPD at D210, the end of active follow-up.

Fig. 4. Intracellular cytokine staining of MTBVAC specific CD4⁺ responses in whole blood assay. CD4 T-cells expressing IFN γ (Panel A), IL-2 (Panel B), TNF α

(Panel C) or all 3 cytokines simultaneously (Panel D). Data are expressed as % total CD4⁺ T-cells at D0, D28, D90 and D210. Numbers above the x axis indicate the number of responders per group at indicated time points. Friedman ANOVA test with Dunn's post-test was used to compare responses within groups at different time-points with D0. Numbers on top of the panels indicate *p* values of Friedman ANOVA; stars indicate *p* values of post-tests, * *p*<0.05, ** *p*<0.01, *** *p*<0.001. Between groups comparisons (Kruskall-Wallis test) did not show any significant difference between BCG and MTBVAC groups.

Fig. 5. Polyfunctional responses in MTBVAC 5x10⁵ and BCG groups. Panel A shows the proportions of CD4⁺ T-cells producing any combination of IFN γ , IL-2 and TNF α in response to MTBVAC or BCG as pies. Panels B and C show the frequencies of memory CD4⁺ and Panel D the frequencies of memory CD8⁺ cells producing one, two or three cytokines in response to MTBVAC (B) or to BCG (C and D). Friedman ANOVA test with Dunn's post-tests was used to compare responses within groups at different time-points with D0. Numbers on top of the panels indicate *p* values of Friedman ANOVA, stars indicate *p* values of post-test * *p*<0.05, ** *p*<0.01, *** *p*<0.001, **** *p*<0.0001. Between groups comparisons (Kruskall-Wallis test) did not show any significant difference between BCG and MTBVAC groups.

Figure 1

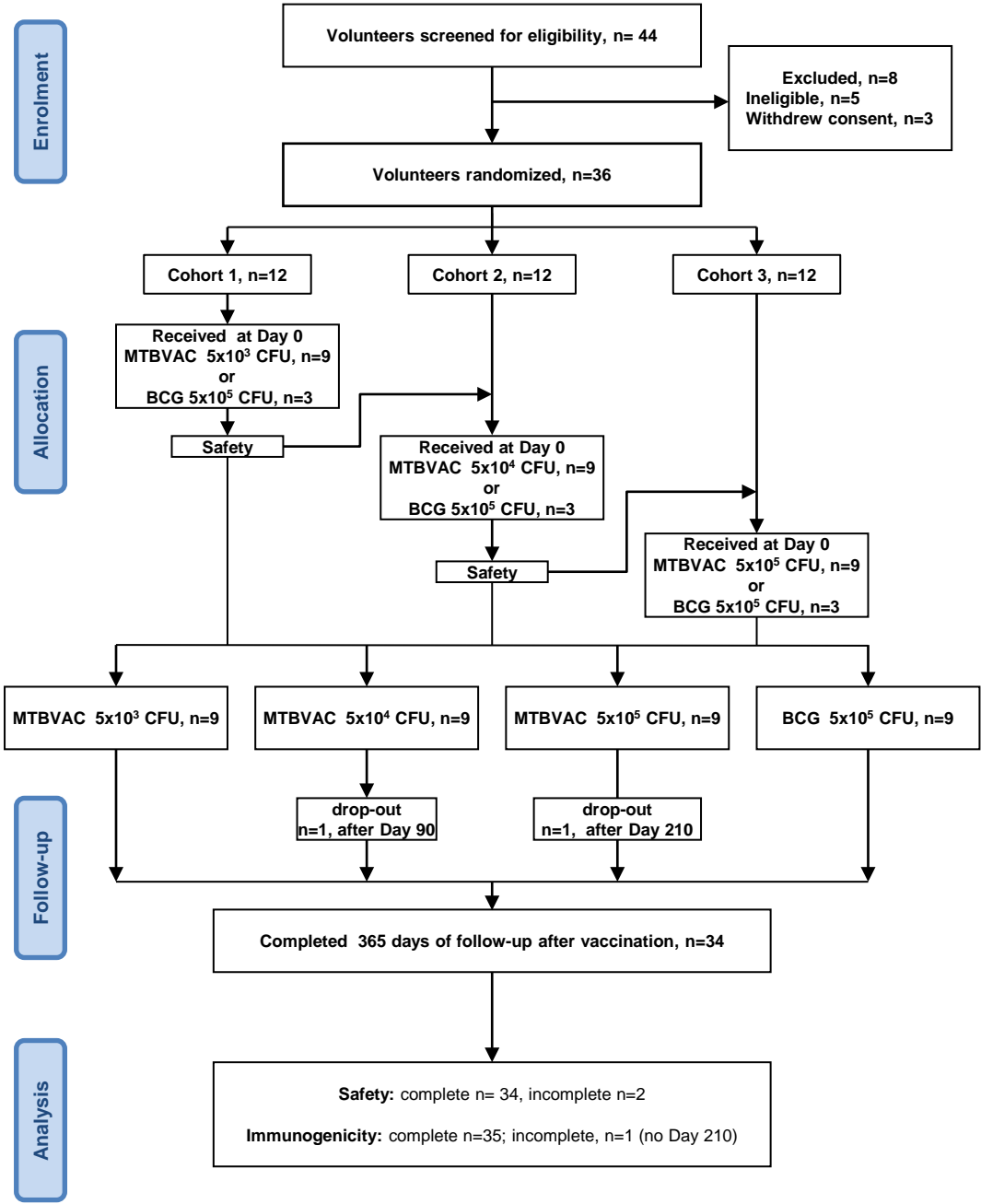


Figure 2

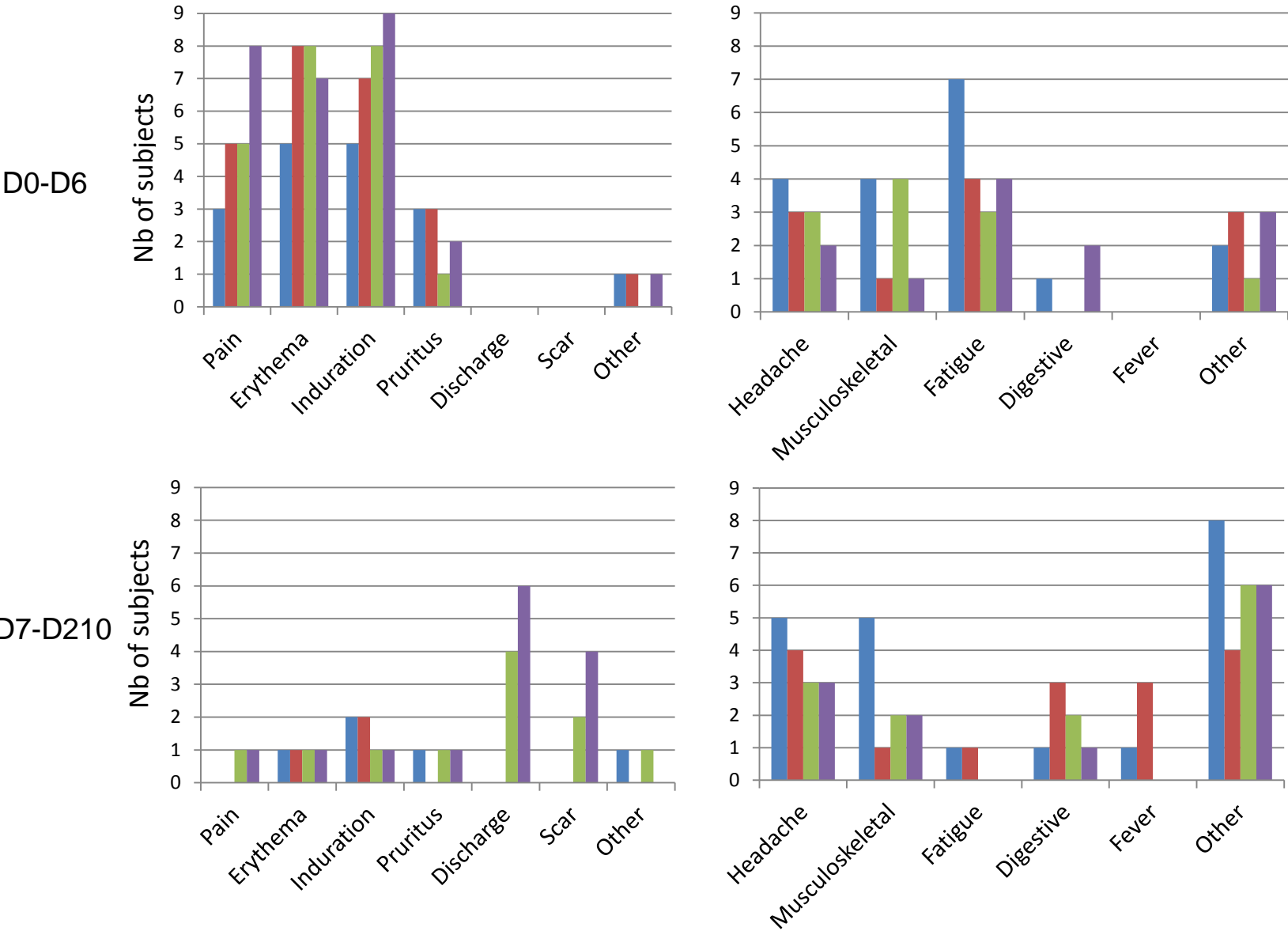
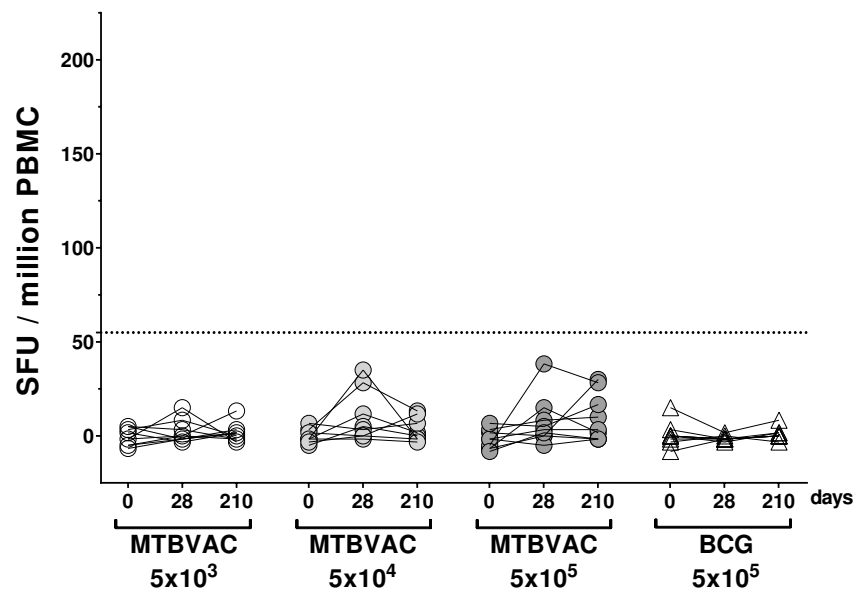
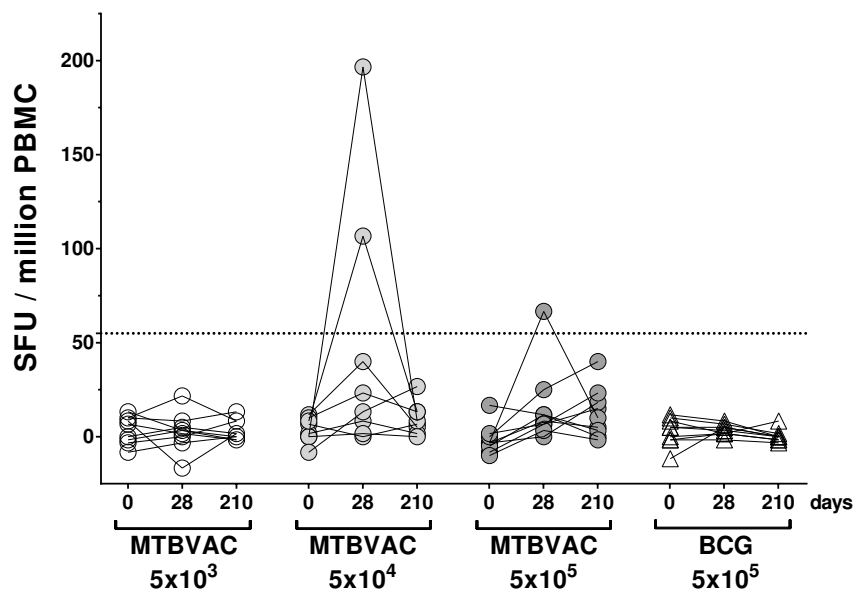


Fig 2

Figure 3
ESAT6



CFP10



PPD

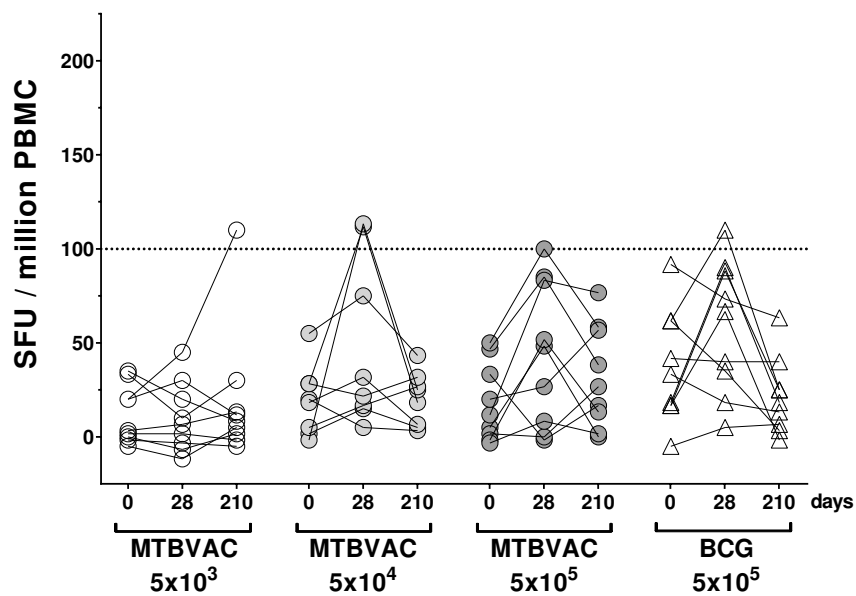


Figure 4

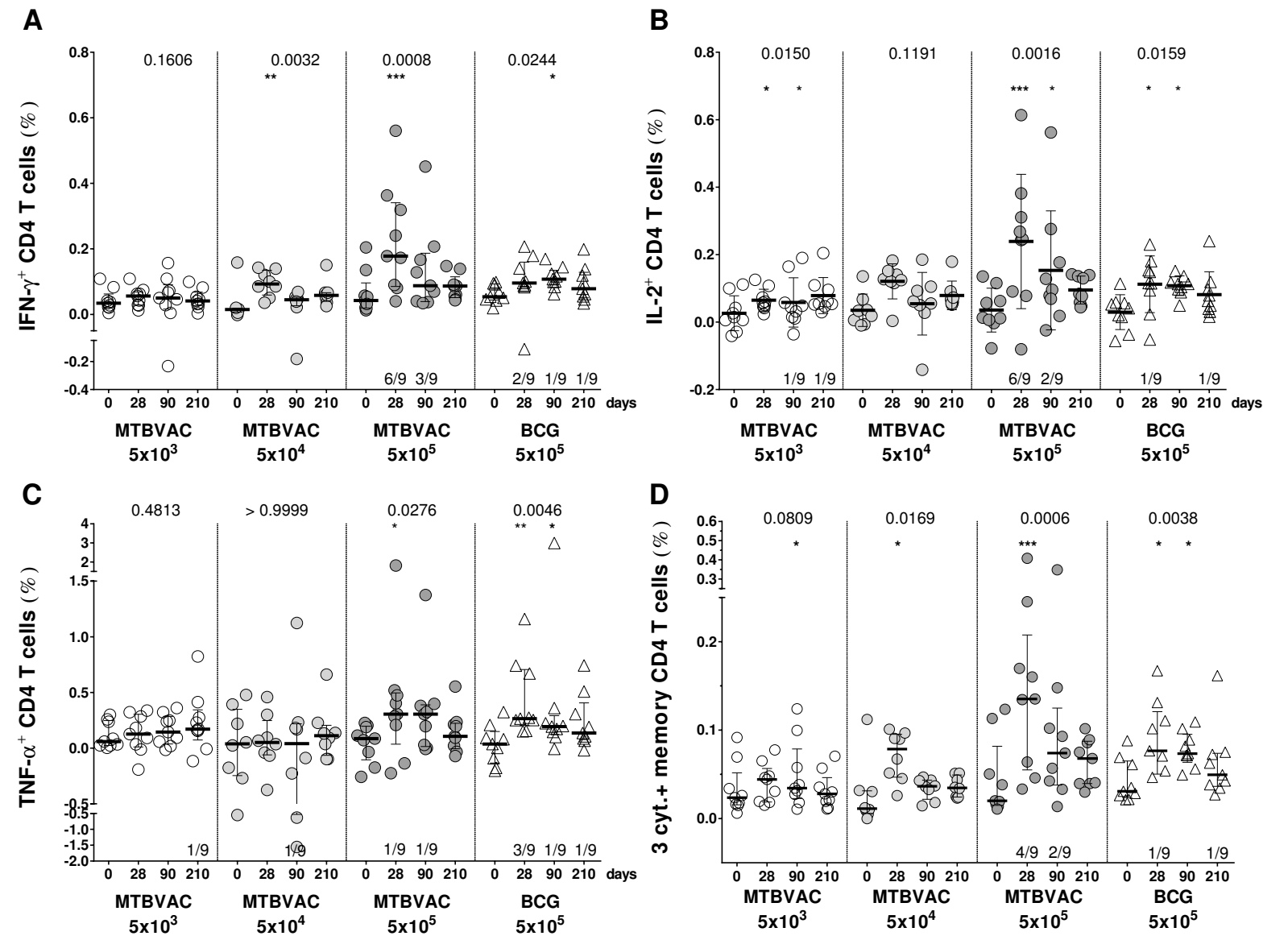


Figure 5 A

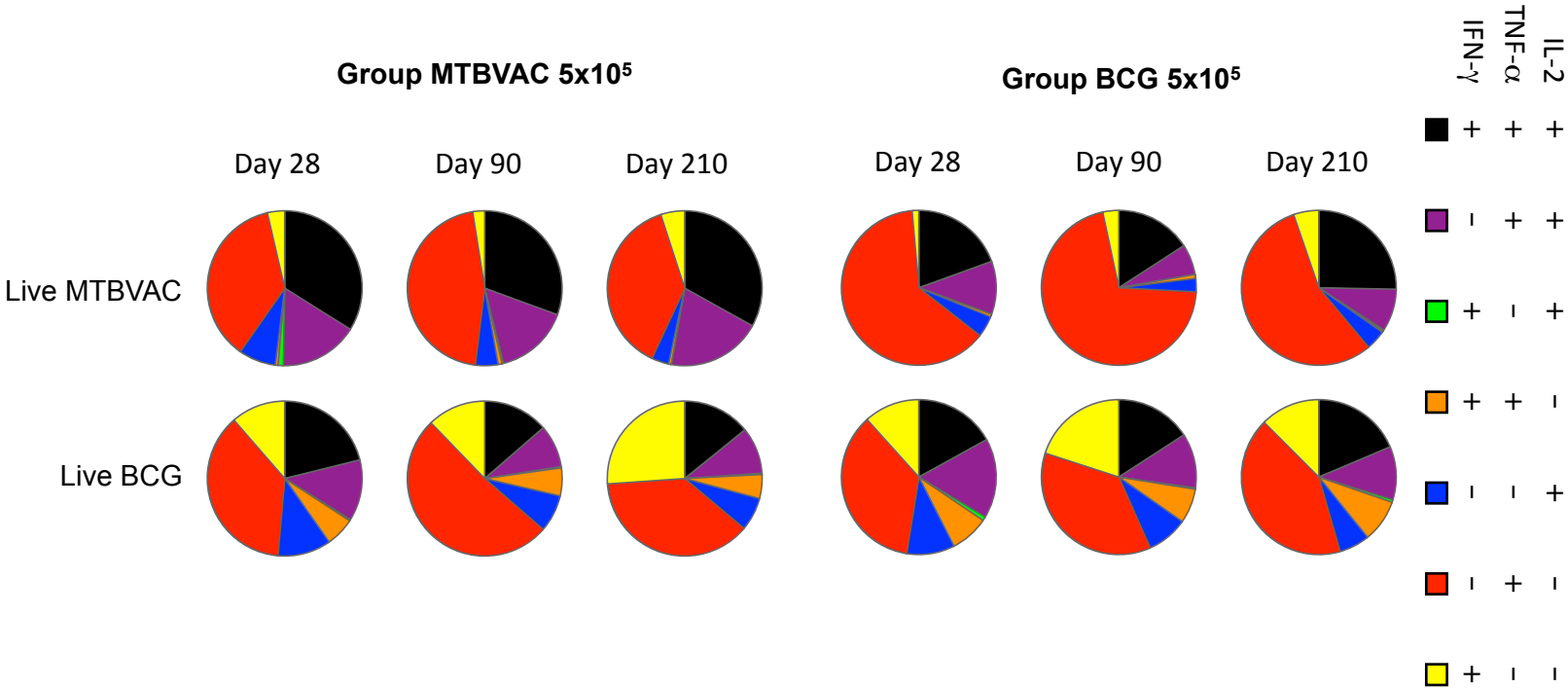


Figure 5 BCD

