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Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific CD4⁺ memory T lymphocyte populations

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Abstract

In the search for effective vaccines against intracellular pathogens such as HIV, tuberculosis and malaria, recombinant viral vectors are increasingly being used to boost previously primed T cell responses. Published data have shown prime-boost vaccination with BCG-MVA85A (modified vaccinia virus Ankara expressing antigen 85A) to be highly immunogenic in humans as measured by *ex vivo* IFN- γ ELISPOT. Here, we used polychromatic flow cytometry to investigate the phenotypic and functional profile of these vaccine-induced *Mycobacterium tuberculosis* (*M.tb*) antigen 85A-specific responses in greater detail. Promisingly, antigen 85A-specific CD4⁺ T cells were found to be highly polyfunctional, producing IFN- γ , TNF- α , IL-2 and MIP-1 β . Surface staining showed the responding CD4⁺ T cells to be relatively immature (CD45RO⁺ CD27^{int}CD57⁻); this observation was supported by the robust proliferative responses observed following antigenic stimulation. Furthermore, these phenotypic and functional properties were independent of clonotypic composition and epitope specificity, which was maintained through the different phases of the vaccine-induced immune response. Overall, these data strongly support the

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use of MVA85A in humans as a boosting agent to expand polyfunctional *M.tb*-specific CD4⁺ T cells capable of significant secondary responses.

Keywords

CD4⁺ T cells; Human; Recombinant viral vectors; Tuberculosis

Introduction

It is estimated that three billion people are latently infected with *Mycobacterium tuberculosis* (*M.tb*), with around two million deaths attributable to tuberculosis (TB) per annum [1]. HIV co-infection dramatically increases the risk of developing active TB disease [2]. Available antibiotic chemotherapy regimens are becoming less effective in the face of emerging multidrug-resistant *M.tb* strains [3]. The most efficient way to control any infectious disease is through prevention, and there is an urgent need for a successful TB vaccine.

BCG vaccination was first introduced in 1921. Immunisation with BCG in infancy provides protection against childhood forms of disseminated TB and leprosy and is cost-effective [4]; however, it is ineffective in protecting against adult pulmonary disease, particularly in TB endemic regions [5]. A prime-boost vaccination strategy that encompasses the benefits of BCG combined with a potent boosting agent could provide an economic and sustainable strategy to increase anti-mycobacterial immunity and reduce the burden of TB disease [6].

Recombinant poxviral vectors are increasingly being used in the development of novel vaccination strategies against intracellular pathogens such as HIV, malaria and TB. Poxviral vectors are particularly attractive due to their ability to induce strong cellular immune responses despite high levels of attenuation [7] and to efficiently express foreign genes [8]. Modified vaccinia Ankara (MVA), a non-replicating vector, has been shown to be safe and immunogenic in humans, especially when used as a boosting agent [9, 10]. Promising results have also been obtained using MVA-based constructs as therapeutic vaccines in HIV-1 [11] and human papilloma virus (HPV) infection [12].

We have developed an immunisation strategy using BCG to prime and a recombinant MVA expressing antigen 85A (MVA85A) to boost. Antigen 85A, or mycolyl transferase, is involved in mycobacterial cell wall biosynthesis [13] and is a component of the immunodominant antigen 85 complex. Antigen 85A is present in all strains of BCG and is highly conserved amongst all mycobacterial species, thereby allowing natural boosting of specific immune responses *via* environmental exposure to non-pathogenic mycobacteria [14].

In small animals, antigen 85A is a key target of the immune responses induced by BCG and is protective when administered via DNA vaccination, inducing both Th1 and CTL responses in BALB/c mice [15]. In humans, 85A is a major target antigen recognised by T cells from infected individuals [16] and induces both CD4⁺ and CD8⁺ T cell responses in

healthy TB contacts [17]. HLA-A2-restricted CD8⁺ T cells have also been found in a high proportion of BCG-immunised individuals [18].

Following promising pre-clinical efficacy data [19, 20], McShane *et al.* [21] showed a BCG-MVA85A prime-boost regimen in healthy adults to be highly immunogenic. Vaccination with BCG-MVA85A induced significantly higher levels of circulating IFN- γ -secreting cells than either BCG or MVA85A alone. *In vitro* depletion studies showed that this response was attributable to the CD4⁺ T cell fraction. Screening for fine specificity against the 66 15mer peptides that span the antigen 85A protein suggested that a range of HLA class II molecules can present 85A-derived epitopes for immune surveillance [21]. In this light, the ability of BCG-MVA85A immunization in humans to induce high levels of IFN- γ -producing CD4⁺ T cells is promising. However, emerging evidence in a number of systems suggests that more comprehensive qualitative assessments of the cellular immune response might allow more accurate identification of protective T cell populations [22, 23].

The protective components of anti-tuberculous immunity are incompletely defined. The importance of adaptive cell-mediated immunity, in particular CD4⁺ T cells, in resistance to TB disease is highlighted by increased TB infection rates in HIV-seropositive cohorts [24] and data from murine models [25]. Within the CD4⁺ T helper cell compartment, Th1-polarised immune responses are central to protection against TB disease [26, 27]. The immunomodulatory cytokines IFN- γ and TNF- α contribute to the recruitment of monocytes and granulocytes [28] and activate the antimicrobial activity of macrophages [26]. IFN- γ -targeted gene knockout mice exhibit increased susceptibility to virulent mycobacterial challenge [29], whilst in humans genetic mutations of the IL-12-dependent IFN- γ production pathway confer susceptibility to poorly pathogenic mycobacterial species [30]. TNF- α has been shown to be critical for granuloma formation in mice [31], and in humans, targeted anti-TNF- α therapy for chronic inflammatory conditions can lead to reactivation of latent tuberculosis [32].

Other T cell subsets contribute to the adaptive immune response to *M.tb*, although their roles are less well defined. CD8⁺ T cells appear to mediate immune surveillance of latent TB infection [33] and to be involved in macrophage activation [34]. Disruption of the MHC class I antigen processing and presentation pathway compromises survival following *M.tb* challenge [35]. Gamma/delta T cells [36] have been found in large influxes at sites of infection and dominate production of IL-17 in mice [37] and appear to be important in the control of bovine TB [38]. The CD1-restricted presentation of glycolipids derived from mycobacterial cell walls may also be important [39].

The aim of this study was to perform an evaluation of the functional characteristics (proliferative capacity and production of IFN- γ , IL-2, TNF- α and MIP-1 β), maturation phenotype and clonal composition of *M.tb*-specific CD4⁺ T cell populations induced by BCG-MVA85A vaccination in healthy adults, as a precursor to planned efficacy studies in target populations.

Results

Antigen 85A-specific CD4⁺ T cell populations exhibit polyfunctional capabilities

Recent advances in flow cytometry have allowed more detailed characterisation of antigen-specific T cell populations at the single cell level [40]. We used polychromatic flow cytometry to expand previous work to investigate which lymphocyte subsets are responsible for the production of IFN- γ and if other effector cytokines are produced following BCG-MVA85A vaccination in humans. The surface mobilization of CD107a as a marker of degranulation [41] was examined, together with the intracellular production of the effector cytokines IFN- γ , IL-2 and TNF- α and the chemokine MIP-1 β by *M.tb* antigen-specific CD4⁺ T cell populations (Fig. 1).

High levels of cells producing IL-2, TNF- α and MIP-1 β in addition to IFN- γ were observed in 6/6 subjects (100%) analysed at wk 1 post-MVA85A boost. The frequency of IFN- γ ⁺ CD4⁺ T cells measured by flow cytometry showed a highly significant correlation coefficient of 0.736 ($p < 0.0005$; Spearman's nonpara-metric test) with the frequency of cells secreting IFN- γ observed in the direct *ex vivo* ELISPOT assays (data not shown). The response profile was dominated by the production of IFN- γ and TNF- α . At wk 1 100% of responding (antigen 85A-specific) CD4⁺ T cells produced both TNF- α and IFN- γ . Production of TNF- α within responding CD4⁺ cells was maintained at 100% up to wk 24. IFN- γ production within responding CD4⁺ cells was maintained at 100% up to wk 8 and by wk 24 post-MVA85A had dropped to 50%. Virtually all IFN- γ ⁺ CD4⁺ T cells were multifunctional: only one subject at a single time point contained cells single-positive for IFN- γ , and these were at a frequency of less than 0.025% of the total CD4⁺ T cell response. Over 50% of the responding CD4⁺ T cells produced IL-2 up to wk 24. The production of MIP-1 β was maintained over a shorter time period but still showed a substantial increase following MVA85A vaccination. Responding cells were classified as 4+, 3+, 2+ or 1+ populations according to the expression profile of the functional markers IFN- γ , IL-2, TNF- α and MIP-1 β . At wk 1 post-MVA85A, the response was dominated by a 4+ population, which produced IFN- γ , IL-2, TNF- α and MIP-1 β and accounted for >40% of responding CD4⁺ T cells. At wk 2, 8 and 24, a 3+ population producing IFN- γ , IL-2 and TNF- α formed the majority of the response.

Following reports of cytolytic CD4⁺ effector T cells [42], we looked for the surface mobilization of CD107a following antigen stimulation. We observed low-level increases in the mobilization of CD107a within the CD4⁺ T cell subset for subject 514 (0.11%) and subject 501 (0.03%) at wk 2 post-MVA85A vaccination compared to pre-MVA85A levels. No change in CD107a mobilisation was observed in 4/6 subjects. CD107a was not included in the functional profile analyses.

Polyfunctional 85A-specific CD4⁺ T cell populations are relatively immature

Using mAb against the surface markers CD45RO, CD27 and CD57, we assessed the maturation status of responding CD4⁺ T cell populations (Fig. 2). As expected, all responding CD4⁺ T cells expressed high levels of the CD45RO isoform, a tyrosine phosphatase splice variant expressed on antigen-experienced cells [43]. Intermediate

expression levels of CD27 (CD27^{int}), a molecule used to define the differentiation status of antigen-specific T cell populations [44] were found in 3+ and 4+ IL-2-producing populations. A reduction in expression of CD27 was observed in the 3+ and 2+ populations that did not produce IL-2 (Fig. 2B, C). CD57, a marker associated with terminally differentiated populations with limited proliferative capacity and an increased sensitivity to apoptosis [45], was expressed at low levels on all of the dominant responding populations.

Robust proliferative capacity of *M.tb*-specific CD4⁺ T cell populations

Following the observation that cells were predominantly polyfunctional and functionally heterogeneous, we wanted to assess the proliferative capacity of MVA85A-induced CD4⁺ T cell populations, primarily because the ability to proliferate following antigen recall is a fundamental requirement for memory T cells [46]. CFSE dilution assays were performed on this cohort with data reported as the cell division index (CDI), the ratio of stimulated to unstimulated proliferation for purified protein derivative tuberculin (PPD-T) and antigen 85A complete peptide pool. PPD-T and antigen 85A peptide-specific proliferative responses were seen in CD4⁺ T cells in 5/5 (100%) of subjects analysed (Fig. 3). The median *M.tb*-specific response increased initially following MVA85A boosting at wk 1 and subsequently decreased to baseline levels by wk 8. However, by wk 24, the proliferative response again increased to reach levels equal to or greater than those seen at wk 1. The CDI for antigen 85A peptide pool stimulation at wk 2 was significantly greater than pre-MVA85A vaccination levels ($p < 0.04$; Wilcoxon signed rank test for matched pairs).

Pilot studies in our laboratory have shown that a decrease in the CDI of up to a 50% can be observed when analysing cryopreserved PBMC compared to freshly isolated PBMC. Due to logistical constraints, the analyses in this study were all performed on cryopreserved PBMC and, as such, the results may underestimate the true proliferative capacity of CD4⁺ T cells from BCG-MVA85A-vaccinated individuals.

The polyfunctional response profile is independent of clonotypic composition and epitope targeting

The functional profile of responding T cell populations has been shown to be dependent to some extent on the avidity properties of individual clonotypes for their corresponding cognate antigens [47, 48]. To determine whether 85A-specific CD4⁺ T cell responses were restricted to a particular subset of clonotypes, we sorted antigen-activated populations from two subjects by flow cytometry and conducted a molecular analysis of *TCRB* gene expression using an unbiased template-switch anchored RT-PCR [49]. Two distinct patterns of clonotypic usage were observed (Fig. 4). In subject 501, a relatively polyclonal responding population was observed, with a diverse architectural profile at the primary sequence level. In contrast, the 85A-specific CD4⁺ T cell population from subject 514 was more oligoclonal and exhibited a highly skewed TCR repertoire; specifically, almost half of the constituent clonotypes expressed TCRBV12–3 combined with a SLXE motif in CDR3 positions 3–6. This profound TCR bias seemed likely to reflect a restricted response to a particular epitope within the targeted protein [50], and this consideration prompted us to examine the fine epitope specificity of the 85A-specific CD4⁺ T cell populations in subjects 501 and 514.

Mapping of the fine specificity of the response to antigen 85A by IFN- γ ELISPOT revealed that these two subjects exhibit differential epitope targeting patterns (Fig. 5). Remarkably, although the magnitude of responses decreases from wk 1 to wk 24 post-boost (as expected), the epitope specificity displayed at wk 1 is maintained at wk 24 in both individuals.

Discussion

The aim of vaccination is to induce long-lasting populations of immune cells capable of mounting a protective secondary response upon pathogen encounter. Given the evidence to date, it seems likely that one criterion for a successful TB vaccine will be the ability to elicit a potent antigen-specific CD4⁺ T cell population. Used as a post-BCG boosting vaccine, MVA85A has been shown to be highly immunogenic in humans, eliciting substantial populations of CD4⁺ T cells capable of secreting IFN- γ in direct *ex vivo* assays [21]. Although this is a promising finding, it is becoming increasingly clear in several systems that simple measurements of response magnitude in isolation are insufficient predictors of immune protection. Data from long-term nonprogressor cohorts infected with HIV-1 have shown that the ability of responding CD8⁺ T cells to produce multiple effector cytokines may hold the key to immune control [51]. It is now clear that CD4⁺ T cells can elicit multiple direct effector functions including cytotoxic activity [42, 52]. Furthermore, studies in mice have shown that vaccine-induced polyfunctional (IFN- γ ⁺IL-2⁺TNF- α ⁺) CD4⁺ T cell populations provide protection against *Leishmania major* challenge. Additionally, these polyfunctional CD4⁺ T cell populations were found to produce more cytokine on a per-cell basis than the monofunctional cells [53].

Here, we report that MVA-boosted antigen 85A-specific CD4⁺ T cell populations were polyfunctional, with production of TNF- α , IFN- γ and IL-2 being maintained by over 50% of responding CD4⁺ T cells up to 24 wk following MVA85A administration. There can be little doubt of the importance of TNF- α and IFN- γ in TB immunity [29–32, 54, 55], whilst the cytokine IL-2 can induce proliferation and acquisition of effector function in activated CD4⁺ and CD8⁺ T cells [56, 57]. The ability of a cell to produce IL-2 has also been used to distinguish memory T cell subsets [58] and may reflect antigen load *in vivo* [59, 60]. Therefore, the sustained production of TNF- α , IFN- γ and IL-2 and the polyfunctional nature of the 85A-specific CD4⁺ T cells following BCG-MVA85A vaccination in humans is particularly encouraging.

Antigen 85A-specific CD4⁺ T cell populations exhibited a relatively immature memory T cell pheno-type (CD45RO⁺CD27^{int}CD57⁻). A decrease in CD27 expression appears to be associated with the loss of IL-2 production. This may be evidence of an association between a functional characteristic and a phenotypic marker within this cell population. Further analysis of the maturation phenotype of responding populations is required to confirm this observation. This phenotype may explain the low levels of CD107a surface mobilisation observed. In the two subjects that did exhibit some changes in CD107a expression, it would be interesting to examine the ability of these cells to produce granzyme and to elicit direct cytotoxic activity.

CD27, CD57 and CD45RO were selected for use since the expression of these markers is stable under both freeze/thaw and antigen stimulation conditions. The primary goal of incorporating differentiation markers was to distinguish between naïve and memory T cells.

As opposed to functional markers, there is still controversy about correlations between differentiation state of antigen-specific cells and clinical markers; indeed, inconsistent data using surface molecules to classify memory T cell populations have been reported [61, 62].

CFSE dilution assays revealed that 85A-specific CD4⁺ T cells were capable of extensive proliferation, consistent with a relatively immature memory T cell phenotype. The large range of diversity in the observed proliferative responses is likely to reflect, at least in part, the varying levels of previous exposure to environmental mycobacterial species that individuals may experience.

Interestingly, in the two individuals studied, TCRB CDR3 analysis suggested that functional attributes were independent of antigen targeting or clonotypic composition, thereby indicating a generic effect of MVA85A boosting on antigen-specific memory CD4⁺ T cells. Using the IFN- γ ELISPOT assay to map the fine specificity of the 85A response may incur a bias, since at wk 24 not all antigen 85A-specific CD4⁺ T cells produced IFN- γ ; however, it did reveal that the epitope specificity against the vaccine insert displayed during the acute response was maintained over time.

This study describes the functional, phenotypic and clonotypic profile of the induced T cell response from clinical studies with a promising, highly immunogenic candidate TB vaccine. When administered to healthy adults, this vaccination strategy induces CD4⁺ T cell populations with the capacity to produce a range of effector cytokines and to proliferate following antigen encounter. The age range of the volunteers and the time interval between BCG and boosting with MVA85A in this study was quite broad. However, we have no evidence from other studies that the time interval between BCG and MVA85A administration influences the ability of MVA85A to boost the *ex vivo* IFN- γ ELISPOT responses.

Until efficacy data with this or other candidate vaccines in humans is available, we are unable to assess mechanistically the contribution of different T cell populations to protective immunity. However, our data indicate that BCG-MVA85A-induced *M.tb*-specific CD4⁺ T cell populations are well equipped to play a role in protective immunity and provide compelling evidence to support the advancement of this vaccination strategy into efficacy studies. Further analysis using these techniques in future vaccine trials will also show whether the promising findings reported here are reproducible in the target population.

Materials and methods

Vaccine study participants

BCG-vaccinated adults were recruited under protocols approved by the Oxfordshire Research Council Ethics Committee, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00427830) ID NCT00427830. The age range for inclusion was 18–55. All subjects tested seronegative for HIV, hepatitis B virus and

hepatitis C virus and had a Heaf test reaction not greater than grade II at screening. Subjects received a single intradermal inoculation with MVA85A at a dose of 5×10^7 PFU. The median time between BCG vaccination and immunization with MVA85A was 18 years (range: 0.5–38 years). From the original study group, a subset of six individuals was selected for more detailed evaluation of vaccine-induced T cell responses according to the magnitude of responses and cell availability.

PBMC preparation

Blood samples were taken for up to 12 months following MVA85A vaccination. PBMC were separated by standard Ficoll-Hypaque (Sigma-Aldrich, UK) density gradient centrifugation and cryopreserved in 90% hea-inactivated endotoxin-tested FCS (BioSera Ltd, UK) with 10% DMSO (Sigma-Aldrich) according to standard protocols.

Antibodies

Fluorochrome-conjugated antibodies or antibody-conjugated quantum dots were used in pre-determined optimal concentrations. The staining panel used to assess intracellular cytokine production and expression of maturation markers contained the following mAb: CD3-allophycocyanin (APC) Cy7, IFN- γ -FITC, MIP-1 β -PE and TNF- α -PECy7 (all from BD Pharmingen, USA); CD4-PECy5.5 (Caltag, USA); CD45RO-Texas red-PE (TRPE; Beckman Coulter, USA); CD107a-Alexa680, CD14-pacific blue, CD19-pacific blue, CD27-Cy5PE, CD57-quantum dot (QD)565 and CD8-QD705 conjugated according to standard protocols (<http://drmr.com/abcon/index.html>). Unconjugated mAb were obtained from BD Biosciences, and quantum dots were obtained from Molecular Probes, Invitrogen. The CFSE proliferation assay used the directly conjugated mAb CD4-pacific blue (E-bioscience, San Diego, CA) and CD3-cascade yellow (DakoCytomation, UK). The staining panel used for sorting of antigen-activated CD4⁺ T cells for clonotype composition analysis used mAb against CD3-FITC (BD Pharmingen), CD4-PECy5.5, CD8-QD705, CD14-pacific blue, CD19-pacific blue, CD25-PE and CD69-APC (both from BD Pharmingen). Following staining, fixed cells were stored at 4°C in the dark and analysed within 24 h for all experiments shown.

Intracellular cytokine staining assay

PBMC were thawed and rested overnight in R10 medium [RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin sulphate and 1.7 mM sodium glutamate (all from Gibco, Invitrogen, UK)] with DNase I (10 U/mL; Roche Diagnostics, USA). The following morning, PBMC were adjusted to 1×10^6 cells/mL in R10 supplemented with 1 μ g/mL α CD28, 1 μ g/mL α CD49d (both from BD Biosciences), 0.7 μ l/mL monensin (GolgiStop, BD Pharmingen), pre-titred α CD107-Alexa680 and 10 μ g/mL brefeldin A (Sigma-Aldrich). Stimulated PBMC were incubated at 37°C/5% CO₂ for 6 h with either 10 μ g/mL PPD-T, 2 μ g/mL antigen 85A complete peptide pool (66 15mer peptides overlapping by 10 amino acids; 2 μ g/mL final concentration for each individual peptide) or 5 μ g/mL staphylococcal enterotoxin fragment B (Sigma-Aldrich). Unstimulated PBMC were used to assess non-specific cytokine production. Following stimulation, PBMC were washed in PBS containing 1% FCS and 0.1% sodium azide and then stained with the amine-reactive LIVE/DEAD fixable violet dead cell stain kit (Molecular Probes, Invitrogen)

[63] and mAb against CD4, CD14, CD19, CD27, CD45RO and CD57. Subsequently, PBMC were washed, permeabilized (Cytofix/Cytoperm Kit, BD Pharmingen) according to the manufacturer's instructions, stained for CD3, CD8 (to account for down-regulation) and cytokines/chemokines (IFN- γ , TNF- α , IL-2 and MIP-1 β), washed and fixed in 1% paraformaldehyde. Cells were analysed using a modified FACS Aria (Becton Dickinson). Responses were analysed using FlowJo version 8.2 (Tree Star Inc, USA). Initial gating used a forward scatter height (FSC-H) versus side scatter area (SSC-A) gate to capture small lymphocytes. Events were then gated through forward scatter area (FSC-A) *versus* FSC-H to remove doublet events. Non-viable, CD14⁺ and CD19⁺ cells were excluded using a dump channel *versus* CD3. Following this, events were sequentially gated through CD3⁺, CD8⁻ and CD4⁺ *versus* IFN- γ . The Boolean gate platform was used with individual function gates to create all possible response pattern combinations. Pestle version 1.2 (Mario Roederer, Vaccine Research Center, NIAID, NIH) was used to prepare data for the subsequent analysis of functional response profiles.

CFSE proliferation assay

Proliferation assays were conducted using CFSE, a fluorescent cytoplasmic tracking dye that can be used to monitor progressive cell divisions [64]. Purified PBMC were thawed and resuspended in R10-AB [complete medium prepared using 10% human AB endotoxin-tested sera (BioSera Ltd)] with 10 U/mL DNase I (Ambion, Applied Biosystems, UK) and rested for a minimum of 2 h at 37°C/5% CO₂. Cells were then washed in sterile PBS (Sigma-Aldrich) prior to labelling with 0.75 μ M CFSE (Molecular Probes, Invitrogen) for 8 min. Staining was quenched by washing twice in R10-AB. CFSE-labelled cells were re-suspended at a concentration of 2×10^6 cells/mL and cultured for 6 days at 37°C/5% CO₂ with the following antigens: 20 μ g/mL PPD-T (purified protein derivative tuberculin; Statens Serum Institut, Denmark), 2 μ g/mL antigen 85A complete peptide pool or 5 μ g/mL staphylococcal enterotoxin fragment B (Sigma-Aldrich). Unstimulated PBMC were used to assess background proliferation levels. Following harvest on day 6, PBMC were washed in PBS containing 1% FCS and stained with surface antibodies against CD3 and CD4. Cells were analyzed with a CyAn ADP flow cytometer (DakoCytomation, Denmark). Between 200 000 and 500 000 total events were collected for each sample. Data analysis was performed using FlowJo version 8.2. Longitudinal results are expressed as the CDI, which is defined as the median percent of CFSE^{dim} cells in stimulated wells divided by the median percent of CFSE^{dim} cells in unstimulated wells.

Flow cytometric cell sorting of viable antigen-specific CD4⁺ T cells

PBMC were thawed and rested for 2 h as before. Cell numbers were adjusted to 2×10^6 PBMC/mL in R10 supplemented with 1 μ g/mL α CD28 and 1 μ g/mL α CD49d, then incubated overnight at 37°C/5% CO₂ with 2 μ g/mL antigen 85A complete peptide pool or left unstimulated. After incubation, PBMC were washed and then stained with LIVE/DEAD fixable violet dead cell stain kit and mAb against CD3, CD4, CD8, CD14, CD19, CD25 and CD69. Monocytes, B cells and non-viable cells were excluded from the analysis using a gating tree as described above. CD25⁺CD69⁺CD4⁺ T lymphocytes were sorted at 75 PSI using a modified FACS Aria directly into RNeasy lysis buffer (Qiagen, Crawley, UK) and stored at -80°C prior to clonotype analysis; sort purity was >99% in all cases.

Analysis of clonotypic composition

Cells sorted on the basis of activation marker up-regulation were subjected to molecular analysis of *TCRB* gene expression as described previously [49]. Briefly, mRNA was extracted (Oligotex kit; Qiagen, USA), and a template-switch anchored RT-PCR was performed using a 3' *TCRB* constant region primer (5'-GCTTCTGATGGCTCAAACACAGCGACCTC-3'). Amplicons were ligated into pGEM-T easy vector (Promega Corporation, USA) and cloned by transformation of competent DH5 α *E.coli*. Selected colonies were amplified by PCR with standard M13 primers and sequenced. A minimum of 50 clones per sample were generated and analysed. Individual chromatograms were assessed using Sequencher 4.7 (Gene Codes Corporation, USA), and nucleotide comparisons were used to establish clonal identity. The IMGT nomenclature system is used throughout [65].

Ex vivo IFN- γ ELISPOT assay

Ex vivo IFN- γ ELISPOT assays were performed as previously described [21]. Briefly, freshly isolated PBMC were suspended in complete medium [R10; RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin sulphate and 1.7 mM sodium glutamate (all from Gibco, Invitrogen)] at a concentration of 3×10^6 PBMC/mL. An aliquot of 100 μ L of this cell suspension was plated directly into each well of the ELISPOT plate (MAIP S4510; Millipore UK Limited). Individual antigen 85A 15mer peptides at 10 μ g/mL were used to stimulate cryopreserved PBMC for 18 h at 37°C/5% CO $_2$, with phytohaemagglutinin (10 μ g/mL; Sigma-Aldrich) as a positive control. Wells without stimuli were used as background controls. Individual peptides were ranked according to previously obtained data (data not shown), and the 22 most immunogenic were selected; all 66 peptides could not be assayed individually due to limitations in cell availability. A count was required to be at least double the background and five counts greater than background to be considered positive.

Data analysis and statistical comparisons

The data analysis program Simplified Presentation of Incredibly Complex Evaluations (SPICE, version 3.1; Mario Roederer, Vaccine Research Center, NIAID, NIH) was used to analyse data and generate graphical representations of functional T cell responses using background-deducted polychromatic flow cytometric data. Non-specific background became extremely low when combinations of functions were examined. A threshold of 0.01% was used to avoid systematic bias incurred by zeroing negative values. Values <0.01% were set to zero. The distribution of negative values in the background-subtracted data set was used to select this value. To assess the statistical significance between pre- and post-MVA85A time points, the Wilcoxon signed rank test for matched pairs was performed using STATA Statistical Software, Release 9.0. 2005 (Stata Corporation, College Station, TX). Correlation coefficients were obtained with Spearman's rank correlation test using SPSS v12 for Windows.

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Abbreviations

CDI	cell division index
M.tb	Mycobacterium tuberculosis
MVA	Modified vaccinia Ankara
MVA85A	modified vaccinia virus Ankara expressing antigen 85A
PPD-T	purified protein derivative tuberculin
TB	tuberculosis

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Figure 1.

85A-specific CD4 $^{+}$ T cells are polyfunctional. Production of CD107a, IFN- γ , IL-2, MIP-1 β and TNF- α was assessed following antigenic stimulation of cryopreserved PBMC using polychromatic flow cytometry. (A) Representative bivariate plots from subject 501 showing *M.tb* antigen 85A-specific functional responses (upper panels) compared to background responses (lower panels). (B) Frequency of responding CD4 $^{+}$ T cells positive for IFN- γ , IL-2, MIP-1 β and TNF- α pre-MVA85A vaccination and at wk 1, 8 and 24 post-MVA85A vaccination. All subjects are combined into one group for analysis. Boxes represent

interquartile ranges; the line in the middle of the box represents the median, and minimum/maximum lines are shown. (C) Functional composition of the CD4⁺ T cell response. Responses are grouped and colour-coded according to the number of functions. The pie charts summarize the fractions of the total response that are positive for a given number of functions. Every possible combination of functions is shown on the *x*-axis. Individual data points and median percentage of the total CD4⁺ response (open bars) with 3rd quartile are shown for each of the functional species. As in (B), all subjects are combined into one group. No responses were seen in the CD8⁺ T cell subset (data not shown).

Figure 2.

Polyfunctional 85A-specific CD4⁺ T cell populations are relatively immature. Surface markers were included in the polychromatic flow cytometry panel to investigate the phenotype of responding 85A-specific CD4⁺ T cells. (A) Representative individual responses for subject 514 are shown. Every possible functional combination is shown on the *x*-axis; the absolute frequencies (total CD4⁺ T cell response) of the four most frequently occurring dominant functional species are shown as colour-coded bars. (B) For subject 514, the CD27 *versus* CD45RO phenotype of the dominant response profiles are shown as colour-coded dots overlaid on density plots showing the phenotype of the total CD4⁺ population. Comparable results were obtained in all six subjects. (C) The median fluorescence intensity of CD27 expression within the four colour-coded dominant responding populations for each of the six subjects (with median line) is shown. (D) Again for subject 514, the CD27 *versus* CD57 phenotype of the dominant response profiles are shown as colour-coded dots overlaid on density plots showing the phenotype of the total

CD4⁺ population. Comparable results were obtained in all six subjects. (E) CD27 *versus* CD57 phenotype of a CD8⁺ T cell subset to allow comparison of (B) with a CD57⁺ phenotype (not seen in CD4⁺ T cell subset).

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Figure 3.

M.tb antigen-specific CD4⁺ T cells are capable of robust proliferative responses. CFSE dilution assays were performed on cryopreserved PBMC. (A) Representative histogram plots from subject 503 showing PPD-T- (upper panels) and 85A- (lower panels) stimulated proliferative responses (black line) overlaid on the unstimulated response (solid grey). (B) Median responses to PPD-T (solid line) and 85A (broken line); data are presented as CDI values. (C) Individual CDI values for all subjects at all time points analysed, with median values and interquartile ranges. (D) Median differences between time points with 95% confidence intervals and *p* values are provided. Missing data values indicate that stored PBMC were not available.

ID	TCRBV	CDR3	TCRBJ	%Freq
501 Seq: 58	4-1	CASSQEQTGGRYEQY	2-7	21
	27	CASRGDRRISTDTQY	2-3	14
	5-1	CASSRGQGAMYGYT	1-2	14
	7-9	CASSLAGANVLT	2-6	5
	7-2	CASSWDRGNTIY	1-3	5
	2	CASSEATGAVEQF	2-1	3
	29-1	CSVGEDSRNTEAF	1-1	5
	27	CASRLEGEETQY	2-5	3
	4-2	CASSQVSKEQY	2-7	7
	6-2	CASSWDRGRGNQPQH	1-5	2
	20-1	CSARDIYVRAAETQY	2.5	2
	2	CASSGVGGGETQY	2.5	2
	28	CASSFNSVNEKLF	1.2	2
	18	CASSQGASNYGYT	1-2	2
	7-9	CASSFRMGVGKLF	1-4	2
	29-1	CSVEGSGANVLT	2-6	2
	9	CASSVRVNYGYT	1-2	2
	20-1	CSAPRGANSPLH	1-6	2
	4-2	CASSQEGQGANYGYT	1-2	2
	20-1	CSANGASGKSTDTQY	2-3	5
514 Seq: 88	12-3	CASSLIEGQPQH	1-5	69
	12-3	CASSSLENTEAF	1-1	6
	4-3	CASRPVQGASRYSTDTQY	2-3	5
	12-3	CASSLLEGIQQF	2-1	6
	3-1	CASSPLTSGGANTQY	2-3	2
	7-2	CASSSPSGANVLT	2-6	2
	10-1	CASNRPNQPQH	1-5	2
	12-3	CASSLIEKQAQH	1-5	2
	5-1	CASSLGAQLNTEAF	1-1	1
	7-3	CASSSMGARGTNEQF	2-1	1
	12-3	CASTRTGGGYEQY	2-7	1
	12-3	CASSSLEGLEQY	2-7	1
	30	CAWSDFYEQY	2-7	1

Figure 4.

Clonotype composition analyses for subjects 501 and 514. Molecular analysis of *TCRB* gene expression was conducted on antigen 85A-stimulated CD4⁺CD25⁺CD69⁺ T cells. The percentage frequency of each clonotype and the total number of sequenced clones are shown, together with CDR3 amino acid sequence, TCRBV and TCRBJ usage. For subject 501 in the antigen 85A-stimulated sample, 582 events (0.42% of CD4⁺ T cells) were collected; in the unstimulated control, 18 events (0.036% of CD4⁺ T cells) were collected. For subject 514 in the antigen 85A-stimulated sample, 1197 events (0.44% of CD4⁺ T cells) were collected; in the unstimulated control, 37 events (0.044% of CD4⁺ T cells) were collected.

Figure 5.

Differential clonotype usage is reflected in epitope targeting patterns. IFN- γ ELISPOT was performed on cryopreserved PBMC using individual 85A 15mer peptides. Fine specificity epitope mapping of 85A responses are shown for subject 501 at (A) wk 1 and (C) wk 24 and for subject 514 at (B) wk 1 and (D) wk 24 post-boost.