T cell activation is an Immune Correlate of Risk in BCG vaccinated infants

Helen A. Fletcher[1,2], Margaret A. Snowden[3], Bernard Landry[3], Wasima Rida[4], Iman Satti[1],
Stephanie A. Harris[1], Magali Matsumiya[1], Rachel Tanner[1], Matthew K. O'Shea[1],
Veerabadran Dheenadhayalan[3], Leah Bogardus[3], Lisa Stockdale[1,2], Leanne Marsay[5],
Agnieszka Chomska[6], Rachel Harrington-Kandt[1], Zita-Rose Manjaly Thomas[1], Vivek
Naranbhai[7], Elena Stylianou[1], Fatoumatta Darboe[8], Adam Penn-Nicholson[8], Elisa Nemes[8],
Mark Hatheril[8], Gregory Hussey[8], Hassan Mahomed[8], Michele Tameris[8], J. Bruce McClain[3],
Thomas G. Evans[3], Willem A. Hanekom[8], Thomas J. Scriba[8], Helen McShane[1]

[1]The Jenner Institute, University of Oxford, Oxford, UK, OX3 7DQ; [2]London School of
Hygiene & Tropical Medicine, London, UK, W1CE7HT; [3]Aeras, Rockville, USA, MD 20850;
[4]Biostatistics Consultant, 1129 N. Illinois St, Arlington, USA, VA 22205; [5]Oxford Vaccine
Group, University of Oxford, Oxford, UK, OX3 7LE; [6]The Kennedy Institute, University of
Oxford, Oxford, UK, OX3 7LF; [7]Wellcome Trust Centre for Human Genetics, University of
Oxford, Oxford, UK, OX37BN; [8]South African Tuberculosis Vaccine Initiative, Institute of
Infectious Disease and Molecular Medicine, University of Cape Town, South Africa, 7935.

Corresponding author

Helen.fletcher@lshtm.ac.uk; helen.mcshane@ndm.ox.ac.uk

ABSTRACT

Vaccines to protect against tuberculosis (TB) are urgently needed. We performed a case–

control analysis to identify immune correlates of TB disease risk in Bacille Calmette-Guerin

(BCG) immunised infants from the MVA85A efficacy trial. Among 53 TB case infants and

205 matched controls the frequency of activated HLA-DR+ CD4+ T cells associates with

increased TB disease risk (OR 1.828, 95% CI 1.25-2.68, p = 0.002, FDR = 0.04, conditional

logistic regression). In an independent study of *M. tuberculosis*-infected adolescents,

activated HLA-DR+ CD4+ T cells also associate with increased TB disease risk (OR 1.387,

95% CI 1.068-1.801, p = 0.014, conditional logistic regression). In infants, BCG-specific T

cells secreting IFN-γ associate with reduced risk of TB (OR 0.502, 95% CI 0.29-0.86, p =

0.013, FDR = 0.14).. The causes and impact of T cell activation on disease risk should be

considered when designing and testing TB vaccine candidates for these populations.

INTRODUCTION

Tuberculosis (TB) causes higher mortality than any other infectious disease globally and

there is an urgent need for improved vaccines if we are to control the epidemic. The

incidence of TB disease in children under 2 years of age in the Western Cape of South

Africa is estimated to be 1.5% per annum [1]. Despite the widespread use of Bacille

Calmette-Guérin (BCG), we do not have an immune correlate of TB risk following

immunisation. Defects in cytokine signalling pathways for IFN-γ and IL-12 increase

susceptibility to mycobacterial infection in humans [2-6], suggesting a role for these cytokines

in protection. HIV infection is also a risk factor, with TB risk increasing as CD4 T cell counts

decline [7]. Animal studies have confirmed the importance of CD4 T cells and IFN-γ in

protection from TB disease [8,9] and have guided the development of new candidate TB

vaccines which increase immunity against *M. tuberculosis* through boosting of a Th1 type

cellular immune response [10]. However, results from an immune correlates study in BCG

vaccinated infants from the Western Cape of South Africa did not find BCG antigen specific Th1 cytokine secreting T-cells to be associated with reduced TB disease risk [11], and the Th1 boosting candidate TB vaccine MVA85A (Modified Vaccinia virus Ankara expressing Ag85A from *M. tuberculosis*) did not improve protection in BCG vaccinated infants [12]. To aid the development of more effective TB vaccines there is an urgent need to identify immune correlates of TB disease risk. Although MVA85A did not protect against TB disease beyond that conferred by BCG alone, all infants were vaccinated with BCG at birth and analysis of samples collected from infants subsequently diagnosed as TB cases in the trial provided an opportunity to identify correlates of TB risk [12]. We performed a case-control study on cryopreserved blood samples using immune assays, that measured both BCG-specific and non-specific immune responses with the aim of identifying immune variables which correlate with disease risk in BCG-vaccinated infants. In BCG-vaccinated infants activated HLA-DR+ CD4+ T cells associate with increased risk of TB and BCG-specific T cells secreting IFN-γ associate with reduced risk of TB disease over the next 3 years of life. The causes and impact of T cell activation should be considered when designing and testing candidate TB vaccines.

RESULTS

Primary analysis

Blood samples for immunologic assessment were obtained from all infants within 7 days prior to vaccination with either MVA85A or placebo (D0) and 28 days (D28) following vaccination (Figure 1). We performed ex vivo IFN-γ ELISPOT and mycobacterial growth inhibition assays to assess antigen specific T cell responses, performed ELISA to assess antibody responses and characterised cells using flow-cytometry with cell surface staining for Live/Dead cells followed by staining with CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD127 and HLA-DR.

Conditional logistic regression analysis was used to assess the association between D0 immune response and risk of TB disease. To adjust for multiple comparisons, the false discovery rate (FDR) was calculated for each variable using the method of Benjamini and Hochberg[13]. Of the 22 pre-specified immune response variables evaluated, the frequency of D0 activated HLA-DR+ CD4+ T-cells was associated with increased risk of TB disease (estimated odds ratio 1.12 per 1 unit increase, p = 0.002, FDR = 0.047) (Table 1 and Supplementary Fig. 1). The frequency of D0 CD4+ T-cells (estimated odds ratio 0.95 per 1 unit increase, p = 0.027, FDR = 0.198) and the magnitude of D0 BCG-specific T-cells secreting IFN-γ (estimated odds ratio 0.46 per 1 log10 unit increase, p = 0.03, FDR = 0.198) were associated with reduced risk of TB disease (Figure 2A and Figure 2C). For subsequent assessment of linearity of the immune prarameters, we classified immune responses into low, medium, and high categories based on tertiles of the data (Figure 2).

In a whole blood ICS assay, performed on a subset of infants, it was found that the response to BCG was predominantly a CD4+ T-cell mediated polyfunctional response characterised by the production of IFN-γ, TNF-α and IL-2 (Figure 3).

Exploratory analysis suggested that D28 post-boost vaccination Ag85A-specific IgG antibodies were also associated with reduced TB disease risk (estimated odds ratio 0.62 per 1 log10 unit increase, p = 0.019). None of the immune variables were able to predict TB disease risk with an area under the receiver operating characteristic (AUROC) above 0.8. The box-plot of each immune response variable, stratified by case/control status is shown in Figure 2 and Supplementary Fig. 2. Variables that did not associate with TB disease risk included D0 PPD IFN-γ ELISpot (estimated odds ratio 0.72, p = 0.453) and D28 Ag85A peptide IFN-γ ELISpot (estimated odds ratio 0.69, p = 0.183) and the D0 mycobacterial growth inhibition assay (MGIA) (estimated odds ratio, 0.31, p = 0.318) (Table 1). The magnitude of the IFN-γ ELISpot response to mycobacterial antigens was low. However, cells were functionally able to secrete IFN-γ as ~25% of infants responded to CMV stimulation with responses ranging to >1000 SFC/million PBMC (Supplementary Fig. 2).

Activated HLA-DR+ CD4+ and CD8+ T-cells were highly correlated with each other (R = 0.53 p<0.0001) and both populations were inversely correlated with total CD4+ T-cells (R = -0.427 p<0.0001, R = -0.391 p<0.0001 respectively) (Supplementary Table 1). There was a positive correlation between CMV IFN-γ ELISpot and the magnitude of HLA-DR+ CD8+ T-cells (R = 0.301 p<0.0001) (Supplementary Table 1).

Impact of MVA85A vaccination on immune correlates

There was no significant difference by MVA85A or placebo groups at either D0 or D28 when treatment group (MVA85A or placebo) was included as an interaction term in the analysis. Results for HLA-DR+ CD4+ T-cells including treatment group (MVA85A or placebo) as an interaction term in the analysis are shown in Supplementary Table 2.

The day 28 analysis is potentially confounded as Ag85A antibodies are induced by MVA85A immunisation and for 8 case infants the matched controls were all from the placebo arm and for 3 placebo infants the matching controls were all from the MVA85A arm. To explore the possibility of any confounding effect of mixed control infants from MVA85A and placebo arms the D28 conditional logistic regression analysis was performed separately on infants only from the MVA85A group and only the placebo group. Results are presented in Supplementary Table 3. There is no statistical significance for any immune variable when infants are analysed separately by study group (likely due to the reduced sample size only ~25 case infants in each analysis). However, the OR for each immune variable remains in the same direction as reported for the primary analysis.

Relative contribution to disease risk

To compare the relative effect size of the different immune response variables we used z-score transformation to scale each variable to a standard deviation of 1 and performed

conditional logistic regression on z-score transformed data (Supplementary Table 4). The

OR for Ag85A IgG adjusted from 0.62 to 0.74, for CD4+ T-cells from 0.95 to 0.65, for BCG

ELISpot from 0.46 to 0.502 and for HLA-DR+ CD4+ from 1.12 to 1.83.

, The relative effect of TB disease risk is therefore smallest for Ag85A IgG followed by CD4+

T cells, BCG ELISpot and greatest for HLA-DR+ CD4+ T-cells.

HLA-DR+ CD4+ T-cells in *M. tuberculosis*-infected adolescents

We retrieved cryopreserved PBMC from healthy, South African, *M. tuberculosis*-infected

adolescents previously enrolled into the Adolescent Cohort Study [14], to determine if

frequencies of HLA-DR expressing CD4+ T-cells were also associated with risk of

progression to TB disease in this independent cohort. HLA-DR expressing CD4+ T-cells

were measured in 61 samples from 30 adolescents who progressed to microbiologically

confirmed incident TB and in 132 samples from 59 matched controls who remained healthy.

Overall, frequencies of HLA-DR expressing CD4+ T-cells were higher in adolescents who

progressed to TB disease compared to matched controls who did not progress to disease

OR 1.387, 95% CI 1.068-1.801, p = 0.014 (Figure 4 and Table 2). Using conditional logistic

regression the difference between controls and progressors was significant at all time points

prior to diagnosis of TB disease (Table 2). However, the odds ratio increased at times closer

to TB diagnosis (Table 2).

Risk of TB disease by category of immune response

To assess the linearity assumption of the conditional logistic regression based on a

continuous immune response variable, we classified immune responses into low, medium,

and high categories based on tertiles of the data (Figure 2). Conditional logistic regression

was then used to analyse the association between immune response category and risk of

TB disease (Supplementary Table 3). For HLA-DR+ CD4+ T-cells the risk was greatest for higher responders (OR 1.97, 95% CI 0.86-4.46) and for BCG ELISpot (OR 0.2, 95% CI 0.009-0.91) and CD4+ T-cells (OR 0.41, 95% CI 0.17-1.01) the risk was lowest for higher responders, confirming the linearity of response. For Ag85A IgG, medium responders were at lowest risk at D0 (OR 0.28, 95% CI 0.0098-0.79) but at D28 the response was linear, with the lowest risk in the higher responders (OR 0.29, 95% CI 0.12-0.72).

To determine if there were any time dependent effects of immune correlates on risk of TB disease we examined TB disease risk by time for case infants. The risk of TB disease by time for each tertile of immune response category for D0 HLA-DR+ CD4+ T-cells, BCG-specific IFN-γ ELISpot response, CD4+ T-cells and Ag85A-specific IgG are shown in Figure 5. The main effect of BCG ELISpot appears to be early in the first 6-12 months of the follow-up period whereas the effect is more evenly distributed by time for the other immune variables (Figure 5).

Ag85A specific IgG antibody response over time

The Mann-Whitney test was used to assess the effect of time and vaccination status on Ag85A-specific IgG for the infant cases and corresponding controls with antibody data at either D0 or D28. The median OD (405 nm) at D0 was 1.477 (IQR 0.815-2.173) and did not differ by vaccination status (p = 0.51). MVA85A vaccinated infants experienced a median increase of 1.226 OD by D28 (p < 0.0001) while control infants experienced a mean increase of 0.425 (p = 0.02) (Figure 6). The difference between the vaccine and control group at D28 is highly significant (p < 0.0001). However, the exact quantity of antibody in the MVA85A group is unknown as the upper limit of detection of the ELISA is ~3 OD and many infants in the MVA85A group were near the upper limit of detection at D28.

DISCUSSION

In this primary correlates of risk analysis we have demonstrated that activated HLA-DR+ CD4+ T-cells were associated with subsequent risk of TB disease. This finding was replicated in an independent cohort of *M. tuberculosis*-infected adolescents who either progressed to active TB disease, or who remained healthy over 2 years of follow up. We have also shown that BCG-specific IFN-γ secreting T-cells measured by ELISpot assay were associated with a reduced risk of TB disease in infants. BCG-specific IFN-γ secreting T-cells were not measured in the adolescent cohort. The effect of BCG-specific IFN-γ secreting T-cells in infants was linear with higher numbers of IFN-γ secreting T-cells associated with a greater reduction in risk of TB disease. The main effect of IFN-γ secreting T-cells appeared to be in the first 6-12 months of follow-up, suggesting an early protective effect of BCG-specific IFN-γ secreting T-cells in infancy. Activated T-cells are known to be associated with HIV progression but this is the first report of an association between T-cell activation and TB disease risk [15-18]. Although BCG immunisation has been shown to drive early BCG-specific CD4+ T-cell activation in this infant population we do not know if BCG immunisation is driving T-cell activation or if other factors such as immunisation with EPI vaccines or infection with pathogens is contributing to T-cell activation [19]. Infection with CMV has been shown to drive T-cell activation in HIV-positive patients and we did observe a correlation between T-cell activation and CMV ELISpot response in this study [20,21]. In an immune phenotyping study, differences in T-cell activation were largely associated with environmental and not genetic factors, with CMV identified as the major microbial driver of immune variation [22].   The factors that drive variation in BCG efficacy include previous exposure to mycobacteria, age and proximity to the equator [23]; these factors have all been associated with increased T-cell activation [24-30]. The elevated CD4 T-cell activation observed in *M. tuberculosis*-infected progressors coincides with an elevated type I/II interferon signature, which is suggestive of incipient or sub-clinical TB (Zak et al., in press), suggesting a possible link between bacterial replication and T-cell activation. Primary CMV infection typically peaks at birth and again in adolescence [31,32] and may also be contributing to T-cell

activation in this adolescent cohort. However, it is likely that there are other, unknown factors driving immune activation in the adolescents, and that these may be different to those driving CD4 T-cell activation in infants. Ultimately, multiple mechanisms of CD4 T-cell activation may be associated with risk of TB.

The cellular immune response to BCG administered at birth in South African infants peaks at 6-10 weeks [19]. The samples collected here were 16-20 weeks post-BCG immunisation and we see an association between BCG-specific IFN-γ ELISpot response and reduced TB disease risk. The importance of an intact Th1 type cellular immune response in protective immunity has been demonstrated in human genetic studies and murine *M. tuberculosis* challenge experiments[2-6,8,9], however this is the first time a role has been demonstrated for magnitude of vaccine-induced IFN-γ secreting T-cells and reduced TB disease risk in a human immune correlates study [11]. A previous study in this infant population found no association of IFN-γ with TB disease risk [11]. Our study differs in several aspects which could explain the contrasting results including the time point (age) at which the response was measured (at peak or post-peak), IFN-γ assay used (ELISpot versus intracellular cytokine assay), sample type (PBMC versus whole blood), statistical design, more stringent TB case definition, and different control definition. In infants the magnitude of the MVA85A boosted Ag85A, PPD and BCG specific IFN-γ ELISpot response is lower than that of UK adults, children or adolescents [12,33-35]. It is possible that the lack of efficacy with MVA85A was due to insufficient boosting of a Th1 type immune response in infants.

An unexpected finding was the association of Ag85A-specific IgG measured at D28 (5-7 months of age) with reduced risk of TB. Ag85A-specific IgG increased from D0 to D28 in both treatment groups in this study. Antibody response are likely primed by BCG vaccination at birth. However, they could also be rising due to exposure to environmental mycobacteria. In a cohort of 66 BCG vaccinated infants in Turkey, PPD-specific IgG levels began to rise at 4 months of age and progressively increased through to the end of the study at 15 months of age [36]. Although there are no published studies describing BCG-specific antibody responses

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in South African infants, it is possible that Ag85A IgG would have a similar kinetic and would be rising in this infant cohort at the time of randomization.

Although the Ag85A IgG response was boosted after vaccination with MVA85A, there was no significant effect of vaccination status when included as a main effect in our D28 analysis. It is possible that the infant Ag85A antibody response induced by MVA85A was short-lived or that the protective effect of induced antibodies was masked as Ag85A IgG antibodies were also rising in the placebo group in this study. It is also possible that in BCG vaccinated infants Ag85A IgG is not directly linked with reduced risk but is correlated with an alternative protective immune mechanism induced by BCG. We did not see any significant differences using a mycobacterial growth inhibition assay (MGIA) between case and control infants. The lack of difference could be due to sample size or lack of sensitivity of the assay; however we did not use autologous serum in the MGIA assay and would not therefore have measured any potential effect of antibodies on the control of mycobacterial growth in infant PBMC.

The immune correlates identified in this study would ideally be confirmed in independent clinical studies in BCG-vaccinated infants. However, the infant population in the Western Cape in South Africa has the highest incidence of non-HIV associated TB recorded in the world at a rate of 1.39 per hundred person years[12]. With such a low incidence rate the MVA85A trial had to enrol 2797 infants who were followed up for a median of 24.6 months at a total cost of approximately 30 million US dollars to gain sufficient power to determine differences between the placebo and MVA85A arms. The finding that T-cell immunogenicity is reduced in infants compared with adults with MVA85A and other subunit vaccines in development, together with the realisation that an adolescent vaccine would have a greater impact on transmission, has led to a refocusing within the field on adolescents as the most important target population[37]. However, the TB incidence rate in adolescents and adults is even lower than in infants and a TB vaccine efficacy trial will therefore be more costly and lengthy in these populations than it was in infants [14].

Nonetheless, we have identified CD4+ T-cell activation as a potential correlate of TB disease risk in BCG-vaccinated infants and have confirmed this finding in an independent adolescent cohort. Moreover, we have identified BCG-specific IFN-γ T-cells as a correlate of reduced risk in infants, supporting the concept of Th1 boosting in TB vaccine design. We also identified Ag85A-specific IgG as a potential novel immune correlate of TB disease risk in BCG-vaccinated infants, suggesting a possible role for both Ag85A antibodies and BCG-specific IFN-γ T-cells in protection from TB disease. However, these responses may be impaired by T-cell activation and the impact of T-cell activation on disease risk should be considered when designing and testing TB vaccine candidates for these populations.

METHODS

Case control design

Infants who were BCG vaccinated within 7 days of birth and enrolled in an efficacy trial of the candidate TB vaccine MVA85A were included in this case-control study, ClinicalTrials.gov number NCT00953927 (Figure 1). HIV negative infants testing negative with QuantiFERON TB Gold In-tube test (Cellestis, Australia), and without known TB exposure were randomized at 4-6 months of age to receive a single intradermal dose of MVA85A or placebo (Candin™, a candida skin test antigen)[12]. The trial was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee, Oxford University Tropical Research Ethics Committee, and the Medicines Control Council of South Africa. Informed consent was obtained from the mothers of all infants.

Infants who met the primary case definition for TB disease (described in the Statistical Analysis Plan (SAP) in the Supplementary Information) were included as cases. For each case, three control infants were randomly selected from a pool of control infants. Infants from both the placebo and MVA85A immunised groups were combined into one control pool for

selection. Infants were included in the control pool if they did not convert to latent TB infection based on the QuantiFERON TB Gold In-tube test (Cellestis, Australia), had not received TB treatment, and had not received TB preventative therapy during study follow-up. Matching was based on gender, ethnic group, CDC weight-for-age percentile (± 10 points), and time on study (± 9 months). Infant PBMC samples stored in liquid nitrogen were available for 55 of 71 TB case infants and for 205 of 213 matched controls (Figure 1). Viability of thawed PBMC was assessed using flow cytometry with Live/Dead Violet stain (Invitrogen) and phytohemagglutinin (PHA) was included as a positive control for cell viability on ELISpot plates. For the majority of case infants controls were from both the placebo and MVA85A arms of the study. However, for 8 case infants the matched controls were all from the placebo arm and for 3 placebo infants the matching controls were all from the MVA85A arm. Controls for whom the case is missing a blood sample are excluded from analysis.

Immune response variables

A series of pilot experiments using cellular samples from the MVA85A efficacy trial were performed to select immune response variables for this study[38]. Infants selected for these pilot studies were non-case, non-control infants receiving either MVA85A or placebo[38]. Results are described by Harris *et al.*, and methods are summarised in the SAP[38]. As cell number was limited and the magnitude of the antigen specific immune response was low[12,38] we selected assays that measured different components of the immune response, and those assays which did not correlate with each other. Immune assays that were initially reviewed for inclusion in this correlates analysis and ultimately selected are listed in the SAP (Supplementary Information).

ELISpot responses were log10-transformed and where the response was zero, a value of 0.3 log10 was used. For analysis of IFN-γ samples were only included if PHA $\geq$ 1000 SFC/million PBMC. For all other analyses, samples were included if the percentage of live

T-cells was ≥ 50% or if flow cytometry was not performed on that sample then PHA ≥

1,000 SFC/million PBMC. Ag85A specific IgG in the plasma was measured as an

exploratory analysis by ELISA. The laboratory staff performing the assays were blinded to

case-control and vaccine group status of infant samples.

Ex vivo IFN-γ ELISpot assay

The *ex vivo* IFN-γ ELISpot assay was performed using a human IFN-γ ELISpot kit (capture

mAb -D1K) (Mabtech). Duplicate wells containing 3×10^5 PBMC were stimulated for 18

hours with antigen, Phytohemagglutinin (PHA) as a positive control or media alone as a

negative control. Antigens were: a single pool of Ag85A peptides, consisting of 66 15-mer

peptides, overlapping by 10 amino acids (2 µg/ml/peptide) (Peptide Protein Research); BCG

from pooled SSI vaccine vials (2×10^5 CFU/ml); purified protein derivative (PPD) from *M.

tuberculosis* (20 µg/ml) (SSI); pools of peptides with knows CD8 T-cell epitopes from flu,

Epstein-Barr virus (EBV), and cytomegalovirus (CMV) peptides (10 µg/ml/peptide) (Peptide

Protein Research); and combined TB10.3 and TB10.4 peptides (10 µg/ml/peptide) (Peptide

Protein Research). Results are reported as spot-forming cells (SFC) per million PBMC,

calculated by subtracting the mean of the unstimulated wells from the mean of antigen wells

and correcting for the numbers of PBMC in the wells. A response was considered positive if

the mean number of spots in the antigen well was at least twice the mean of the

unstimulated wells and at least 5 spots greater.

Cell surface flow cytometry

PBMC were washed and stained with 5µl Live/Dead Violet (Invitrogen) followed by surface

staining with the following titrated antibodies: 0.5µl CD3-AF700 (clone UCHT1, Ebioscience),

2µl CD4-APC (clone RPA-T4, Biolegend), 2µl CD8-Efluor605 (clone RPA-T8, Ebioscience),

2µl CD14-PE/Cy7 (clone HCD14, Biolegend), 2µl CD16-AF488 (clone 3G8, Biolegend), 1µl

CD19-PE/Cy5 (clone HIB19, Biolegend), 2μl CD25-APC/Cy7 (clone BC96, Biolegend), 2μl CD127-NC650 (clone eBioRDR5, Ebioscience), 15μl HLA-DR-PE (clone L243 Biolegend). Fluorescence minus one (FMO) controls were used to set gates for CD25, CD127 and HLA-DR. Samples were acquired on a BD LSR II flow cytometer. Results are presented as percentages of cells after gating out of dead cells and doublets. CD4+ and CD8+ T-cells were identified as CD3+ cells, while CD14+/− and CD16+/− cells were identified as CD3− and CD19− populations. CD25+ CD27− populations were gated on the CD4+ cells.

Mycobacterial growth inhibition assay

Duplicate 2-ml screw-cap tubes containing 1×10^6 PBMCs in 600 μl of medium were rotated at 37°C with   600 CFU of BCG Pasteur stock for 4 days. The PBMCs were then lysed with sterile water, and the lysate transferred to a Bactec MGIT supplemented with PANTA antibiotics and OADC enrichment broth. The tube was placed in a Bactec MGIT 960 and incubated until growth was detected (time to positivity).

Ag85A IgG ELISA

Nunc Immunoplates were coated overnight at 4°C with 50μL sodium carbonate buffer containing 1.5μg/mL recombinant antigen 85A protein. Plates and reagents were brought to room temperature and the plate washed 3 times with PBS, 5% Tween20 (v/v). Plates were blocked for 1 hour at room temperature with 100μL blocking buffer (PBS, 5% Milk Blocking (w/v). All serum samples were diluted 1/100 with PBS, 5% Milk Blocking (w/v) and 50μL of each diluted sample was plated in triplicate. Controls and plate blanks consisting of assay diluent alone with no serum were added in triplicate. Plates were sealed and incubated for 2 hours at room temperature. After washing 50μL of a 1:500 dilution of goat anti-human (KPL) detection antibody was added to each well for 1 hour at room temperature. For colour development 50μL TMB (BD) was added to each well. Plates were incubated for 15 minutes

in the dark before the reaction was stopped by adding 50uL of 2M Sulfuric Acid (Sigma) to each well. Absorbance was measured using a microplate reader at 450nm.

Statistical analysis

Prior to data analysis, a statistical analysis plan (SAP) was prepared in which primary and secondary analyses were defined and the methods of analysis were specified (Supplementary Information). Briefly, 22 primary immune variables were selected for inclusion in the case-control study (SAP Table 1B). For each variable, a conditional logistic regression analysis was used to assess the association between D0 immune response and risk of TB disease. To adjust for multiple comparisons, the false discovery rate (FDR) was calculated for each variable using the method of Benjamini and Hochberg[13]. FDR values less than or equal to 0.2 were considered significant. To assess the predictive value of an immune variable, the area under the receiver operating characteristic (AUROC) curve was computed. Immune variables with AUROC values above 0.80 were considered good predictors of TB disease risk.

Secondary analyses included conditional logistic regression analysis of D28 immune responses for each of the primary variables. Regression models with and without vaccination status (MVA85A or placebo) included as a main effect were examined. In addition to the primary variables, D0 and D28 Ag85A-specific IgG antibody levels were analyzed by conditional logistic regression.

Immune response variables that were significant at D0 or D28, were categorized as low, medium, or high based on tertiles and conditional logistic regression was performed to assess linearity. To explore any time dependent effect of immune correlates plots of the cumulative hazard function (Breslow estimator) for immune response variables (by tertile of immune response) were plotted for case infants only.

The change in antibody from D0 to D28 was assessed using generalized estimating equations as described in Yang and Tsiatis (2001)[39]. For our setting, the cluster variable was limited to participant ID and ignored the matching. However, only controls for whom the case had data were included. We also used an unstructured covariance matrix to describe the variance in antibody titers over time.

Outliers as defined by Tukey's rule were excluded from the primary analysis. Missing data were not imputed. For the Day 0 analysis, out of 3918 total measurements across 22 immune variables, 19 data points were identified as statistical outliers as described in the SAP (Supplementary Table 6). Following investigation of the raw data 12 out of 19 data points were retained and 7 data points excluded. Exclusions were due to high background in the ELISpot assay or PHA response <1000 SFC/million PBMC.

The Adolescent Cohort Study (ACS)

We used samples from the Adolescent Cohort Study (ACS) for validation of the finding that elevated HLA-DR+ CD4+ T-cells correlated with risk of TB. The ACS was a prospective epidemiological cohort study of *M. tuberculosis* infection and TB disease in adolescents, aged 12-18 years, from the Western Cape of South Africa [14]. A total of 6,363 adolescents were recruited at high schools and followed for 2 years. Latent *M. tuberculosis* infection was diagnosed by tuberculin skin test or QuantiFERON Gold in Tube assay and PBMC were collected at enrolment and end of study (24 months) in all, and in an active follow-up group also at 6, 12 and 18 months (Zak et al., in press). Participants suspected of having TB were investigated through sputum smears, culture and chest X-ray. A protocol-defined incident case of TB was any case confirmed by two or more sputum smears positive for acid-fast bacilli and/or one positive sputum culture for *M. tuberculosis*. After excluding individuals with a previous history of TB disease, we examined the frequency of HLA-DR expressing CD4+ T-cells in 61 progressor samples (from 30 progressor adolescents) and 132 control samples

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(from 59 matched control adolescents). All available PBMC samples from progressors and matched controls were included in the analysis. Controls were matched on age, gender, ethnicity and high school. HLA-DR expressing CD4+ T-cells were measured in PBMC by first gating on singlet cells, then selecting cells with consistent fluorescence during acquisition (time gate), followed by a side scatter/CD45 gate to select lymphocytes. Thereafter CD14- CD3+ T-cells and then CD4+ CD8− cells were selected. Frequencies of HLA-DR expressing CD4+ T-cells are reported as a proportion of total live CD4+ T-cells. Data were analysed using conditional logistic regression as described above comparing progressors to matched controls.

TABLE LEGEND

Table 1. Conditional Logistic Regression

FIGURE LEGENDS

Figure 1. Cohort of infants from the MVA85A efficacy trial included in this immune correlates case-control study.

At 4-6 months of age blood was collected from HIV-negative, HIV unexposed BCG vaccinated infants with no active or chronic illnesses (including suspected TB), and with no household exposure to an adult who had active TB disease. Infants were then randomized to receive either MVA85A or placebo and followed for 2-3 years. TB cases (n = 71) were defined by a positive culture for *M. tuberculosis* or presence of *M. tuberculosis* DNA using a certified molecular diagnostic assay; or a stringent composite endpoint which included symptoms, radiological signs and an exposure history (see Endpoint 1 definition in SAP). Control infants (n = 213) had no evidence of TB exposure, no evidence of TB infection on

IGRA and were matched (3 controls to 1 case) on age, race, time on study and CDC weight percentile.

Figure 2. Distribution of significant immune variables in TB case and controls from the infant MVA85A vaccine trial.

Immune variables $p<0.05$ at D0 or D28 are shown. Infants are stratified according to TB case (red) or control (grey) status. The 50th percentile of the immune variable is indicated by a horizontal line. The shape of the point indicates whether an infant was in the placebo (round) or MVA85A (diamond) arm of the study. There were no significant differences in immune variables when infants were stratified by study arm. Combined low, medium, and high immune responses at D0 (panels A, C, E) or D28 (panels B, D, F) were used to divide the all samples (both case and control groups) into thirds; medium third is indicated by the grey horizontal bar. The log frequency of live CD3+ cells expressing CD4, CD4 and HLA-DR or CD8 and HLA-DR respectively. SFC/million PBMC is the number of IFN-γ spot forming cells per million PBMC (only results from infants with a PHA response greater than 1000 SFC/million are shown). Optical density was measured by ELISA at wavelength 405 nm.

Figure 3. BCG-specific CD4 T-cell response in whole blood

Frequencies of cytokine-expressing BCG-specific CD4-positive T-cells expressing IFN-γ, TNF-α, IL-2 or IL-17 measured by whole blood intracellular cytokine staining 28 days after administration of placebo (n = 19) or MVA85A (n = 17). Only p-values < 0.05 shown. Mann-Whitney test, not adjusted for multiple comparisons.

Figure 4. Frequencies of HLA-DR expressing CD4+ T-cells in latently *M. tuberculosis* infected adolescents.

Frequencies of HLA-DR expressing CD4+ T-cells in *M.tb* infected adolescents who progressed to TB disease (red) (progressors, n = 61 samples from 30 progressors) compared with matched controls who did not progress to disease (grey) (controls, n = 132 samples from 59 controls).

Figure 5. Risk of TB disease over time by magnitude of immune response (TB cases only)

Hazard of disease over time for HLA-DR+ CD4+ T-cell, BCG ELISpot, CD4+ T-cell and Ag85A IgG OD immune response magnitude is shown for TB case infants. All immune responses were measured at D0 prior to immunisation with MVA85A. For each immune parameter TB cases were stratified into subgroups divided into thirds according to immune response level (low blue, mid green, and high yellow). The plots show the cumulative hazard of TB over time for the 3 immune level groups.

Figure 6. Ag85A IgG is boosted by MVA85A but is also increased in the placebo group from D0 to D28

Ag85A-specific IgG stratified by vaccine group (MVA85A D0 n=79, MVA85A D28 n=107, placebo D0 n=88 and placebo D28 n=95). Lines represent median responses; round placebo, diamond MVA85A, red case and grey control.. Although Ag85A IgG is boosted by MVA85A, Ag85A-specific IgG is significantly higher at D28 when compared to D0 in both the placebo and MVA85A groups.

Table 1. Conditional Logistic Regression

Estimated Odds-Ratio (OR) of TB disease from a Conditional Logistic Regression of Day 0 immunological variable							
Quantitative variable	N	Cases	Est OR*	95% CI	P value	FDR value	AUROC
CD3+ T cell	186	50	0.98	(0.95, 1.02)	0.394	0.656	0.542
CD4+ T cell	186	50	0.95	(0.92, 0.99)	0.027	0.198	0.592
CD4+HLADR+ T cell	186	50	1.12	(1.04, 1.2)	0.002	0.047	0.619
CD4+CD25+CD127-	186	50	0.82	(0.54, 1.25)	0.356	0.647	0.550
CD8+ T cell	186	50	1.04	(0.99, 1.09)	0.159	0.399	0.556
HLA-DR+ CD8+ T cell	186	50	1.02	(1.00, 1.04)	0.056	0.279	0.579
CD14+CD16+ e	186	50	1.00	(0.80, 1.25)	0.994	0.994	0.448
CD14+CD16-	186	50	1.01	(0.93, 1.09)	0.857	0.994	0.505
CD19+ B cell	186	50	1.04	(0.99, 1.09)	0.144	0.399	0.580
BCG MGIA	80	30	0.31	(0.03, 3.08)	0.318	0.585	0.529
85A ELISpot	125	40	NA	NA	NA	NA	NA
BCG ELISpot	138	43	0.46	(0.23, 0.93)	0.03	0.198	0.591
PPD ELISpot	90	32	0.72	(0.3,1.7)	0.453	0.639	0.586
TB10.3/10.4 ELISpot	90	32	0.40	(0.13,1.31)	0.131	0.314	0.567

EBV ELISpot	100	34	0.98	(0.34, 2.82)	0.966	0.994	0.5
CMV ELISpot	126	40	0.87	(0.54, 1.41)	0.582	0.776	0.52
FLU ELISpot	100	34	NA	NA	NA	NA	NA
GAM.DEL (putative)	186	50	1.41	(0.93, 2.12)	0.104	0.399	0.561
NK.16NEG (putative)	186	50	0.92	(0.79, 1.08)	0.331	0.647	0.541
NK.16POS (putative)	186	50	0.99	(0.89, 1.1)	0.851	0.994	0.507
CD14+CD16+ /CD3+	186	50	1.37	(.05,38.72)	0.854	0.994	0.515
CD14+CD16- /CD3+	186	50	0.96	(0, 13609)	0.993	0.994	0.442
Exploratory Variable							
Ag85A IgG	145	46	.70	(0.46, 1.08)	0.106		.589
Estimated Odds-Ratio (OR) of TB disease from a Conditional Logistic Regression of Day 28 immunological variable							
CD3+ T cell	200	52	0.99	(0.96,1.03)	0.746	0.871	0.511
CD4+ T cell	200	52	0.98	(0.94, 1.02)	0.344	0.871	0.526
HLA-DR+ CD4+ T cell	200	52	1.12	(1.05, 1.19)	0.001	0.013	0.653
CD4+CD25+CD127-	200	52	0.68	(0.37, 1.25)	0.281	0.871	0.528
CD8+ T cell	200	52	1.02	(0.96, 1.08)	0.423	0.871	0.555
HLA-DR+ CD8+ T cell	200	52	1.02	(1.00, 1.04)	0.031	0.346	0.604
CD14+CD16+	200	52	0.92	(0.74, 1.15)	0.516	0.871	0.534

CD14+CD16-	200	52	0.99	(0.92, 1.07)	0.752	0.871	0.502
CD19+ B cell	200	52	1.01	(0.96, 1.07)	0.610	0.871	0.538
BCG MGIA	74	29	0.49	(0.05, 4.60)	0.458	0.871	0.548
85A ELISpot	165	49	0.69	(0.4,1.19)	0.183	0.871	0.552
BCG ELISpot	165	49	0.70	(0.4, 1.21)	0.202	0.871	0.557
PPD ELISpot	146	46	0.56	(0.31, 1.01)	0.055	0.404	0.575
TB10.3/10.4 ELISpot	146	46	0.99	(0.4, 2.47)	0.990	1	0.499
EBV ELISpot	155	48	1.20	(0.49, 2.96)	0.688	0.871	0.507
CMV ELISpot	163	49	1.08	(0.72, 1.61)	0.723	0.871	0.523
FLU ELISpot	154	48	1.00	(0.11. 8.87)	1	1	0.494
GAM.DEL (putative)	200	52	0.98	(0.74, 1.31)	0.866	0.953	0.508
NK.16NEG (putative)	200	52	1.08	(0.94, 1.23)	0.245	0.871	0.527
NK.16POS (putative)	200	52	0.96	(0.85, 1.08)	0.464	0.871	0.532
CD14+CD16+ /CD3+	200	52	0.03	(0,923.33)	0.608	0.871	0.494
CD14+CD16- /CD3+	200	52	0.45	(0.02, 9.45)	0.497	0.871	0.529
Exploratory Variable							
Ag85A IgG	188	56	0.62	(0.41, 0.93)	0.019		0.615

NA = model did not converge; results not available

*represents the odds ratio for a 1 unit change in the immune response

Table 2. Conditional logistic regression of HLA-DR+ CD4+ T-cells in the Adolescent Cohort Study

Time to TB diagnosis	N	progressors	OR	95% CI	P value
Any	193	61	1.387	1.068, 1.801	0.014
All controls and progressors < 18 months	173	50	1.402	1.067, 1.843	0.015
All controls and progressors < 12 months	140	34	1.537	1.106, 2.135	0.01
All controls and progressors < 6 months	118	16	1.501	1.047, 2.152	0.027

SUPPLEMENTARY INFORMATION

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Author contribution

HF contributed to conception, design, analysis, acquisition and drafting. HM, MS, BL, WR, MH, GH, HM, MT, JBM, TE, WH, TS contributed to conception, design, analysis and drafting. IS, SH, MM, RT, MO, DV, LB, LS, LM, AC, RK, ZMT, VN, ES, FD, APN, EN contributed to design, analysis, acquisition and drafting.

Conflict of financial interests

HM was previously a shareholder in the Oxford-Emergent Tuberculosis Consortium (OETC), a joint venture established for the development of MVA85A (OETC no longer exists).
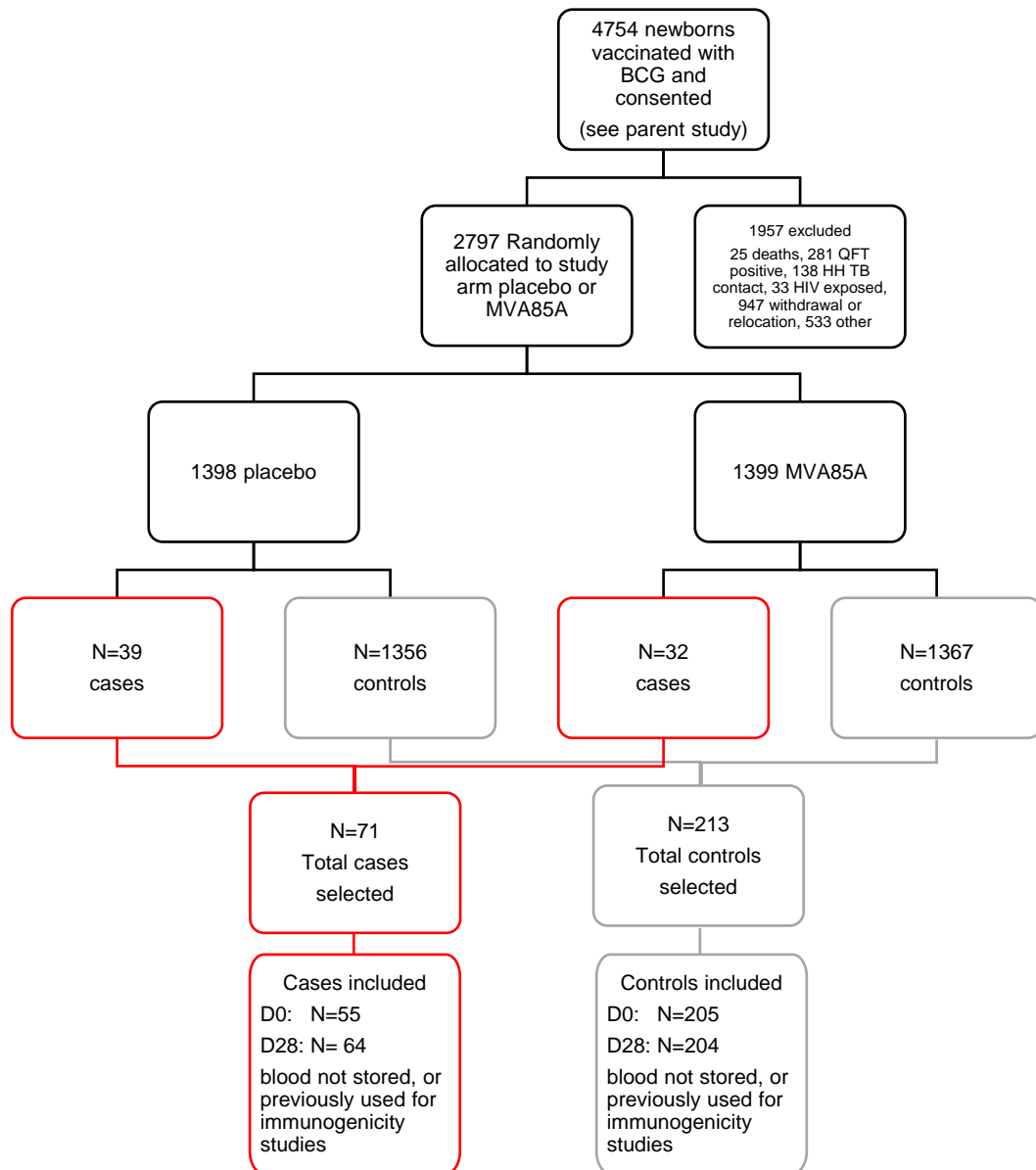
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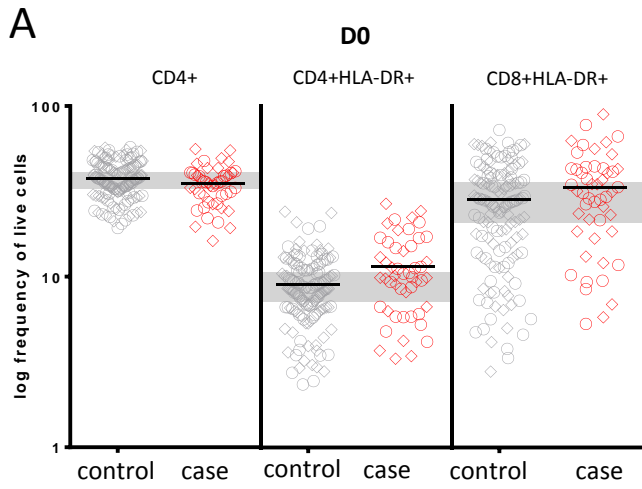
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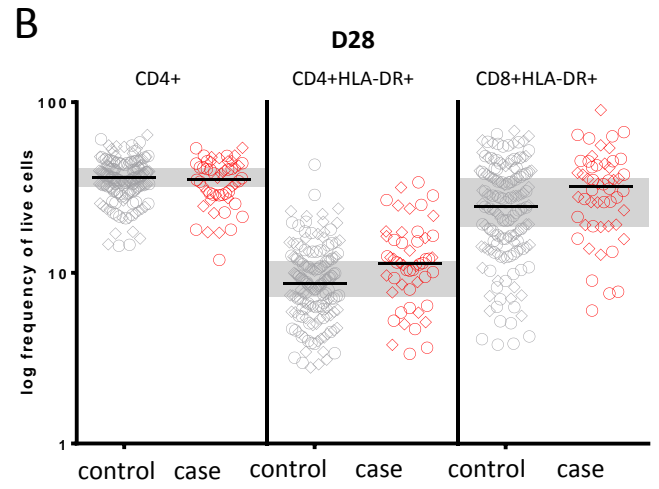
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4754 newborns
vaccinated with
BCG and
consented
(see parent study)

2797 Randomly
allocated to study
arm placebo or
MVA85A

1957 excluded
25 deaths, 281 QFT
positive, 138 HH TB
contact, 33 HIV exposed,
947 withdrawal or
relocation, 533 other

1398 placebo

1399 MVA85A

N=39
cases

N=1356
controls

N=32
cases

N=1367
controls

N=71
Total cases
selected

N=213
Total controls
selected

Cases included
D0: N=55
D28: N= 64
blood not stored, or
previously used for
immunogenicity
studies

Controls included
D0: N=205
D28: N=204
blood not stored, or
previously used for
immunogenicity
studies

A

D0

CD4+ CD4+HLA-DR+ CD8+HLA-DR+

log frequency of live cells

100

10

1

control case control case control case

Immune response %

	low	mid	high		low	mid	high		low	mid	high
control	31	32	37		38	38	24		37	32	31
case	42	34	24		28	36	36		26	34	40

B

D28

CD4+ CD4+HLA-DR+ CD8+HLA-DR+

log frequency of live cells

100

10

1

control case control case control case

Immune response %

	low	mid	high		low	mid	high		low	mid	high
control	31	37	32		36	29	35		37	32	31
case	42	21	37		20	20	60		23	37	40

C

BCG IFNγ ELISPOT D0

SFC/million PBMC

400

300

200

150

100

50

0
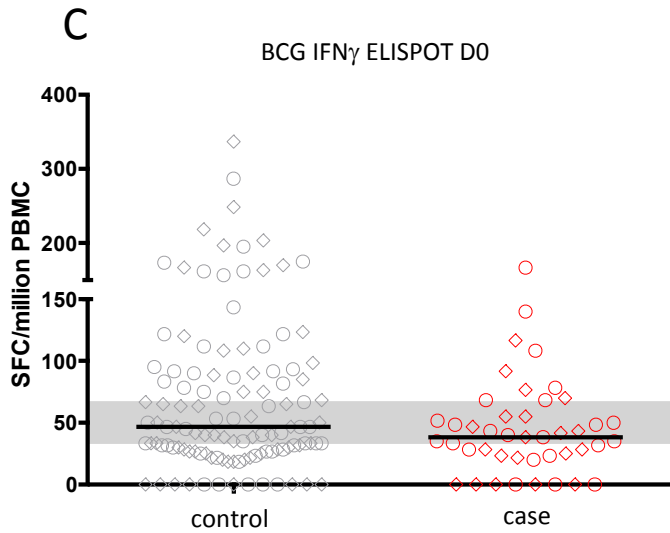
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Immune response %

	low	mid	high
control	30	31	39
case	38	40	22

D

BCG IFNγ ELISPOT D28

SFC/million PBMC

400

300

200

150

100

50

0
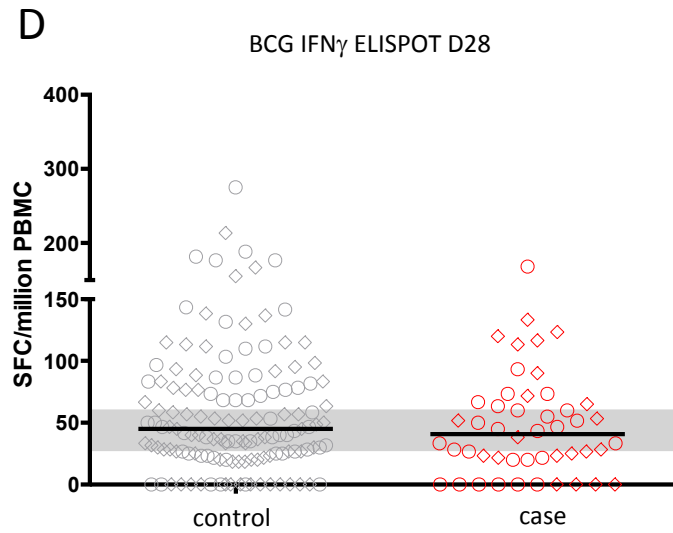
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Immune response %

	low	mid	high
control	32	33	35
case	45	29	26

E

Ag85A IgG D0

OD 405 nm

4

3

2

1

0
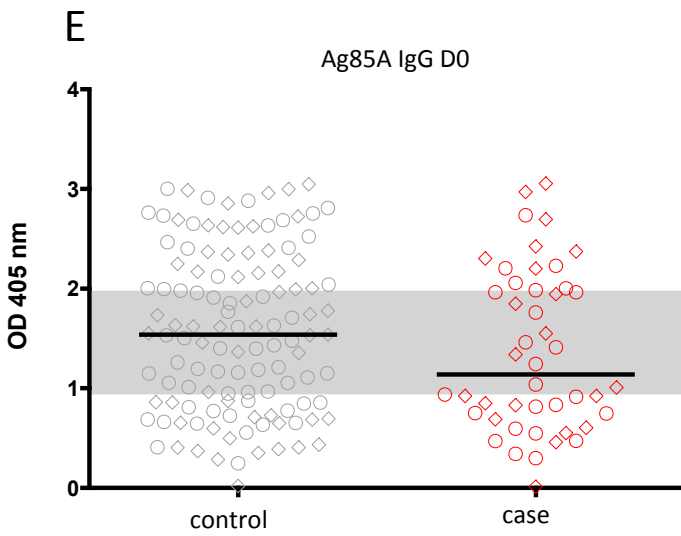
control case

Immune response %

	low	mid	high
control	29	36	47
case	44	26	28

F

Ag85A IgG D28

OD 405 nm

4

3

2

1

0
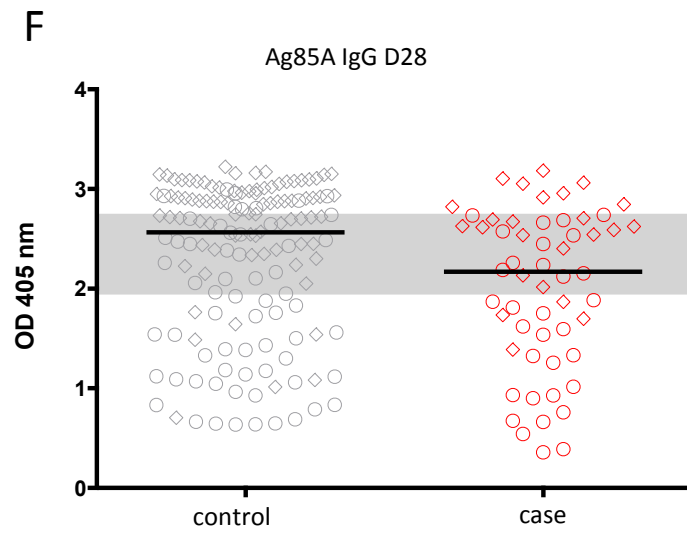
control case

Immune response %

	low	mid	high
control	30	30	40
case	43	41	16