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IMMUNOLOGICAL ASPECTS

Individual-level factors associated with variation in mycobacterial-specific immune response: Gender and previous BCG vaccination status

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SUMMARY

Introduction: A more effective tuberculosis (TB) vaccine is needed to eliminate TB disease. Many new vaccine candidates enhance the immunogenicity of the existing vaccine, Bacillus Calmette–Guérin (BCG). Understanding BCG induced immune variation is key to developing a new vaccine.
Aims: We aimed to establish if individual-level covariates were associated with cell-mediated immune response (interferon gamma (IFN-γ)) at vaccine trial enrolment (baseline) in a long-term retrospective analysis (LTR) and after BCG vaccination in a short-term prospective analysis (STP).
Methods: Four covariates were analysed: gender, country, BCG vaccination history and monocyte/lymphocyte cell count ratio. Univariable and multivariable linear regression were conducted on IFN-γ response at baseline for LTR, and area under the curve (AUC), 24 week and peak IFN-γ response for STP.
Results: Previous BCG vaccination was strongly associated with higher IFN-γ response at baseline (LTR analysis) (p-values < 0.05). Being male showed a weak association with higher baseline response (p-value $= 0.1$). BCG revaccination was strongly associated with a larger response increase than primary-vaccination (AUC & peak p-values < 0.01), but did not differ at 24 weeks (STP analysis). All other covariates were non-significant (p-values > 0.1).
Conclusion: This analysis suggests that previous BCG vaccination and gender are associated with durable IFN-γ responses. Vaccine trials may need to stratify by BCG vaccination history and gender.

1. Introduction

Tuberculosis disease (TB) caused by the organism *Mycobacterium tuberculosis* (*M.tb*), remains a substantial global health problem with approximately 9 million people developing active disease and 1.5 million TB-related deaths in 2013 [1]. This is despite nearly 70 years of widespread use of the only licensed TB vaccine, Bacillus Calmette–Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, which has exhibited variable efficacy [2]. Novel TB vaccines are considered an essential tool to meet the WHO goal of TB elimination by 2050 [3,4], and many candidates utilise a BCG prime-boost strategy.

It has been proposed that the observed variation in BCG efficacy could be attributed to individual-level factors that influence host mycobacterial-specific immune responses [5,6]. Factors that have been shown to be consistent in their influence of such responses include: latitude, which is known to be associated with varying

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exposure to non-tuberculous mycobacteria (NTM) [7], and *M.tb*-specific sensitization of the immune system through previous BCG vaccination [8]. Additional factors that have shown consistent influence include age at vaccination and BCG strain [5].

Another factor that may influence the mycobacterial-specific immune response is gender. TB prevalence surveys report a higher occurrence of disease in males than females [9], which is thought in part to be due to differences in the immune response between the sexes [10], in addition to social aspects [11]. However, so far, investigations into the effects of gender on the mycobacterial immune response have shown equivocal results [12,13] and few TB vaccine immunogenicity or efficacy trials have reported results stratified by gender.

In addition, recent evidence has shown that the ratio of host monocyte to lymphocytes cells (ML ratio) was associated with risk of TB disease [14–16]. Naranbhai et al. observed that in HIV positive, South African adults on combination antiretroviral therapy, this relationship was nonlinear, i.e. low and high, compared to moderate, ML ratios were associated with a higher risk of TB [14]. Little investigation has been made into how ML ratio may affect mycobacterial-specific immune responses and further insight into this relationship could potentially inform targeted TB vaccine strategies.

New detailed longitudinal immune response data to BCG vaccination has recently become available due to an increase in research into new TB vaccines in which BCG vaccination was used as a control [17]. These detailed data have the potential to give new insights into how individual-level factors alter the immune response to BCG.

Our aim was to consider how individual-level factors affect BCG immunogenicity as measured by tuberculin purified protein derivative (PPD) stimulated interferon gamma (IFN-γ) response following vaccination. Utilizing new immunological data allowed us to provide a more detailed analyses of the immune response than previous studies, which have focused on long-term responses with less detail of short-term dynamics.

2. Methods

In this study, two analyses were performed on data from participants included in new TB vaccine (BCG-booster) trials in which participants were given a new TB vaccine or BCG as a control measure. The data from the BCG control arms were used in this analysis.

Our first analysis aimed to determine which individual level covariates were associated with increased PPD antigen-specific IFN-γ immune response at enrolment to the trials. In this analysis, IFN-γ responses measured at enrolment to the trial (and before BCG vaccination was administered) is referred to as the 'baseline response'. This was a cross-sectional analysis of previously BCG vaccinated or BCG-naïve trial participants, and is referred to as the 'long-term retrospective' or 'LTR' analysis.

The second analysis aimed to determine which covariates were associated with IFN-γ immune response over a short period, following BCG vaccination. This analysis was conducted using data from the prospective follow-up of study participants, who had either been revaccinated or primary-vaccinated with BCG immediately following baseline screening and were followed up for 24 weeks post vaccination. This is referred to as the 'short-term prospective' or 'STP' analysis.

2.1. Data and materials

In this study we used data from seven vaccine trials involving BCG (Table 1). The available data were on HIV negative and *M.tb* naïve participants (see references in Table 1 for HIV and *M.tb* latency testing procedures). Data on haematological parameters were based on routine laboratory haematology testing at baseline and only those participants with values within normal limits were included in clinical trials.

IFN-γ response was measured using a standardized ex vivo IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) assay which quantifies IFN-γ secreting CD4+ T cells as spot forming units (SFU) per million peripheral blood mononuclear cells (PBMCs) using PPD as a stimulant. The same ELISPOT method including plates, antibody kits, antigens, developing reagents, washing method, ELISPOT reader and ELISPOT counting method were used across all UK trials and all South African trials. South African researchers visited the UK laboratory for ELISPOT training and reagents for the ELISPOT assay were shipped from UK to South Africa for these studies. As these BCG studies were conducted as part of a series of Phase I clinical trials with MVA85A all lab protocols and lab reagents were harmonized as far as possible between UK trials and between UK and South African trial. For the exact laboratory methodology see [17–20].

2.2. Covariates

The four individual-level factors (covariates) included in this analysis were country (UK or South Africa), gender, BCG vaccination history at baseline and baseline ML ratio. ML ratio data were not available for three of the studies (two UK trials and the South African trial, Table 1). For details on how BCG-vaccination history was determined see original trial methods [17–20]. BCG vaccination history was categorised into "never" and 10 year time-periods since vaccination with the reference group as 1–9 years since BCG vaccination. Age was not included as a covariate as it was colinear with BCG vaccination history.

2.3. Statistical analysis

The analyses were performed using linear regression. Firstly, a univariable model analyses was conducted referred to as the 'unadjusted' analysis, followed by multivariable model; the 'fully adjusted' analysis. Analysis was conducted using R [21]. A p-value of ≤ 0.05 was considered as strong evidence for an association with the outcome.

All outcomes were log transformed (natural log) as data were right-skewed and the residuals verified to justify this transformation. The effect measures are the anti-logged regression slope parameters, the associated 95% confidence interval (CI) and p-value. For the categorical covariates (country, gender and BCG vaccination history), these represent the ratio of the geometric means (GM) of the IFN-γ response outcome variable compared to the reference group. For the continuous covariate, ML ratio, the effect measure represents the increase in GM of the IFN-γ response outcome variable for an increase in 0.1 ML ratio (as ML ratio is bound by zero and one), assuming a linear trend in ML ratio.

Additionally, due to previous research that found a nonlinear relationship to exist between ML ratio and risk of TB disease [14], both linear and quadratic regression models were fitted to establish if a similar relationship existed between IFN-γ response and ML ratio (see Supplementary Material for example, Table S2). Analysis of variance (ANOVA) was used to assess if a non-linear relationship more adequately described this association.

2.3.1. Long-term retrospective (LTR) outcome variables

Baseline IFN-γ responses were used as the outcome variable in the long-term analysis. All four individual-level covariates were considered in the analysis. In the BCG vaccination history covariate,

Table 1
Demographic and trial information for participants included in long-term retrospective (LTR) and short-term prospective (STP) analyses. Trial information was split by BCG vaccine history where possible.

Vaccine trials no.	No. of participants	Country	Male (%)	Median age (IQR) years	Previous BCG vaccination (median years since (IQR))	Blood haematological: median (IQR) % of cells in whole blood		Median ML ratio (IQR)	Included in LTR/STP analysis	Reference
						Monocytes	Lymphocytes			
NCT00480688	11	UK	3 (27%)	25 (8.5)	None (NA)	0.30 (0.15)	1.76 (0.43)	0.20 (0.05)	LTR/STP	[17]
NCT00480714	6	UK	2 (33%)	25 (0)	None (NA)	0.36 (0.21)	1.90 (0.32)	0.19 (0.16)	LTR/STP	[17]
NA*	14	UK	4 (29%)	23.5 (9)	None (NA)	0.56 (0.18)	1.89 (0.41)	0.31 (0.18)	LTR/STP	[36]
	14		7 (50%)	23 (8.5)	Yes (15 (0.6))	0.57 (0.18)	1.66 (0.34)	0.29 (0.07)		
NCT00654316	13	UK	4 (29%)	25 (11)	Yes (12 (11))	0.34 (0.17)	1.53 (0.42)	0.24 (0.10)	LTR/STP	[18]
NCT00427453	10	UK	3 (30%)	24 (7.3)	None (NA)	NA	NA	NA	LTR	[19]
NCT00427830	15	UK	7 (47%)	27 (10.5)	Yes (21 (7))	NA	NA	NA	LTR	[17]
NCT00460590	4	South	2 (50%)	40.5 (4.8)	None (NA)	NA	NA	NA	LTR	[20]
	14	Africa	3 (21%)	33.5 (12)	Yes (33.5 (12))	NA	NA	NA		
Aggregated	101	–	35 (35%)	26 (11)	21 (17.3)	0.41 (0.24)	1.70 (0.51)	0.22 (0.11)	–	–

* Trial number was not available for this trial at time of analysis. Three participants from the first four trials (those included in both the LTR and STP analyses) did not have full STP data, so were only included in the LTR. NA = not available. IQR = Interquartile range; ML = Monocyte/lymphocyte.

the groups represent time since previous BCG vaccination with the group "never" representing those who were BCG naïve at baseline.

2.3.2. Short-term prospective (STP) outcome variables

To investigate the short-term response, IFN-γ responses at baseline and 4, 8 and 24 weeks post BCG vaccination were used and summarized using the following three statistics as outcome variable: area under the curve (AUC), peak and the 24 week (referred to as 'sustained') IFN-γ responses. The AUC summarises the total change in IFN-γ response over 24 weeks post BCG vaccination and was calculated using the R package "Kulife" [22].

For the STP analysis, unadjusted and fully adjusted regression was conducted separately for the three outcomes. For the STP analysis, an additional analysis was also carried out for peak and 24 week response, whereby adjustment for baseline IFN-γ responses was conducted, known as the 'partially adjusted' analysis. This was not adopted for the AUC outcome variable, as the AUC calculation is standardized by the baseline value, so adjustment for the effect is not necessary.

In the STP analysis, the categories defined for the BCG vaccination history covariate correspond to years since previous BCG vaccination before receiving BCG at enrolment into the trial. The group 'never', corresponds to being 'primary vaccinated' at enrolment. As all trials used in the STP analysis were UK based, all individual-level covariates except country were included.

3. Results

101 participants were included in this analysis (Table 1). Seven vaccine clinical trials were used in the LTR analysis; four of those also had data available for the STP analysis (Table 1 and Table S1). Participants were either vaccinated with BCG (56 participants), at a median of 21 years (interquartile range (IQR) = 17.3) before baseline or BCG naïve at baseline (45 participants). The median of the ML ratio was 0.22 (IQR = 0.11). The distribution of ML ratio amongst the population can be found in Figure S1.

3.1. Long-term retrospective (LTR) analysis

All 101 participants were included in the LTR analysis. All covariates were included in the fully adjusted analysis, except for ML ratio as data on this measure were not available for some of the trials (data were only available for 58 participants (Table 1)).

For male participants, the unadjusted GM ratio of the IFN-γ response at baseline was nearly twice that of females (GM ratio 1.97, 95% CI (1.03, 3.77)) (Table 2), and remained weakly associated

Table 2
Long-term retrospective (LTR) analysis: results of the linear regression analysis on baseline IFN-γ responses (SFU/mill cells) against individual-level covariates.

Covariates (n)	Geometric mean of IFN-γ response	Unadjusted GM ratio (95% CI), *p*-value		Fully adjusted* GM ratio (95% CI), *p*-value	
Country					
South Africa (18)	65.62	1	–	1	–
UK (83)	47.56	0.73	(0.32, 1.65), 0.63	1.02	(0.41, 2.55), 0.97
Gender					
Female (66)	39.82	1	–	1	–
Male (35)	78.45	1.97	(1.03, 3.77), 0.04	1.76	(0.96, 3.25), 0.07
BCG vacc history					
1–9 yrs (8)	133.54	1	–	1	
10–19 yrs (13)	121.28	0.91	(0.25, 3.28)	0.74	(0.20, 2.70)
20–29 yrs (19)	80.58	0.60	(0.18, 2.01)	0.57	(0.17, 1.93)
30 + yrs (12)	94.93	0.71	(0.19, 2.62)	0.72	(0.17, 3.13)
Never (49)	24.27	0.18	(0.06, 0.54), <0.001†	0.18	(0.06, 0.52), <0.001†
ML ratio (58)		0.89‡	(0.58, 1.38), 0.61	–	–

As an example of the GM of the IFN-γ response by ML ratio two values were chosen from the range of ML ratio (Table 1) to represent high and low values and the GM calculated using the unadjusted GM ratio value in the above table. As such, the GM for the IFN-γ response for a ML ratio of 0.1 and 0.3 were 52.03 and 50.88, respectively. Abbreviations: IFN-γ = Interferon gamma; vacc = vaccination; GM = geometric mean; yrs = years; ML = Monocyte/lymphocyte.

* Adjusted for all variables in the model except ML ratio.

† *p*-value for all categories of BCG vaccination history covariate using an ANOVA summary.

‡ Represents the value of the change in GM of the IFN-γ response for an increase in 0.1 of ML ratio.

after adjustment for country and years since BCG vaccination (GM ratio 1.76, 95% CI (0.96, 3.25)).

For BCG-naïve participants ('never'), a GM ratio of their IFN-γ response at baseline of 0.18 (95% CI (0.06, 0.54)) was found, compared to that of the reference group of 1–9 years since BCG vaccination (Table 2) and remained strongly associated after full adjustment (GM ratio 0.18, 95% CI (0.06, 0.52)). GM IFN-γ response was similar for 10–19, 20–29 and 30+ years since BCG vaccination, compared to 1–9 years since BCG vaccination (Table 2).

There was no evidence of an association between IFN-γ response at baseline and ML ratio in the linear or quadratic analyses (Table 2, Table S2). Neither was there an association between IFN-γ response and country (Table 2).

3.2. Short-term prospective (STP) analysis

Data from 55 participants, all UK adults, were available for the STP analysis. The IFN-γ responses over the 24 week follow-up period, by primary or revaccination status, are shown in Figure 1.

All analyses (unadjusted, partially and fully adjusted) suggested there was no association between gender or ML ratio and AUC (Table 3), 24 week response (Table 4) or peak response (Table 5).

Being primary-vaccinated ('never' in Table 3) was strongly associated with a lower AUC in the unadjusted analysis with a GM ratio of 0.16 (95% CI (0.06, 0.44)) (Table 3 and Figure 1). This association remained strong after adjustment for baseline IFN-γ response, gender and ML ratio (GM ratio 0.22, 95% CI (0.07, 0.68)). No other groups in the BCG vaccination history covariate were associated with AUC.

BCG vaccination history was strongly associated with 24 week response in the unadjusted analysis, specifically: primary-vaccinated participants had lower 24 week responses (GM ratio 0.14, 95% CI (0.06, 0.36)) ('never' in Table 4) compared to the reference group. After full adjustment, this association remained but was weaker (GM ratio 0.29, 95% CI (0.07, 1.12)). The partially adjusted analysis showed changes in the GM ratio for all covariates (Table 4). Most notably, the GM ratio for those who were primary vaccinated increased from 0.14 (95% CI (0.06, 0.36)) to 0.25 (95% CI (0.09, 0.69)) compared to the reference group in the unadjusted and partially adjusted analyses, respectively.

Primary-vaccinated participants had lower peak IFN-γ response compared to the reference group in the unadjusted analysis (GM ratio 0.24, 95% CI (0.14, 0.39)) (Table 5). This remained after full adjustment for all covariates (GM ratio 0.32, 95% CI (0.15, 0.68)) (Table 5). The partially adjusted analysis did not significantly change this value (GM ratio 0.29, 95% CI (0.16, 0.51)), indicating a minimal affect of baseline response on the association between BCG vaccination history and peak response (Table 5).

4. Discussion

We investigated the impact of multiple individual-level covariates on the mycobacterial-specific immune response pre- and post- BCG vaccination. Being male or previously BCG vaccinated was associated with higher IFN-γ response at baseline. BCG revaccination resulted in a larger initial increase in immune response than primary-vaccinated participants, but response was not significantly different at 24 weeks. All other covariates (country and ML ratio) were non-significant.

Differences in TB disease notification rates between the genders have been well documented and are thought to be a result of both social and biological factors [10]. In our analysis we found a weak association between male gender and higher IFN-γ levels at baseline in the long term retrospective (LTR) analysis. This could be linked to sex hormones causing differences in gender-associated immune responsiveness, specifically those of IFN-γ [10,23]. Our results are consistent with previous studies that show women have significantly lower IFN-γ response after PPD stimulation than men (after adjustment for age, BMI and *M.tb* infection) [13] as well as less strong tuberculin skin testing results [24]. However, these results remain somewhat surprising as reported disease incidence tends to be higher in men [9]. This could imply that disease burden differences may be due to behavioural, rather than biological, reasons or that a balanced immune response is

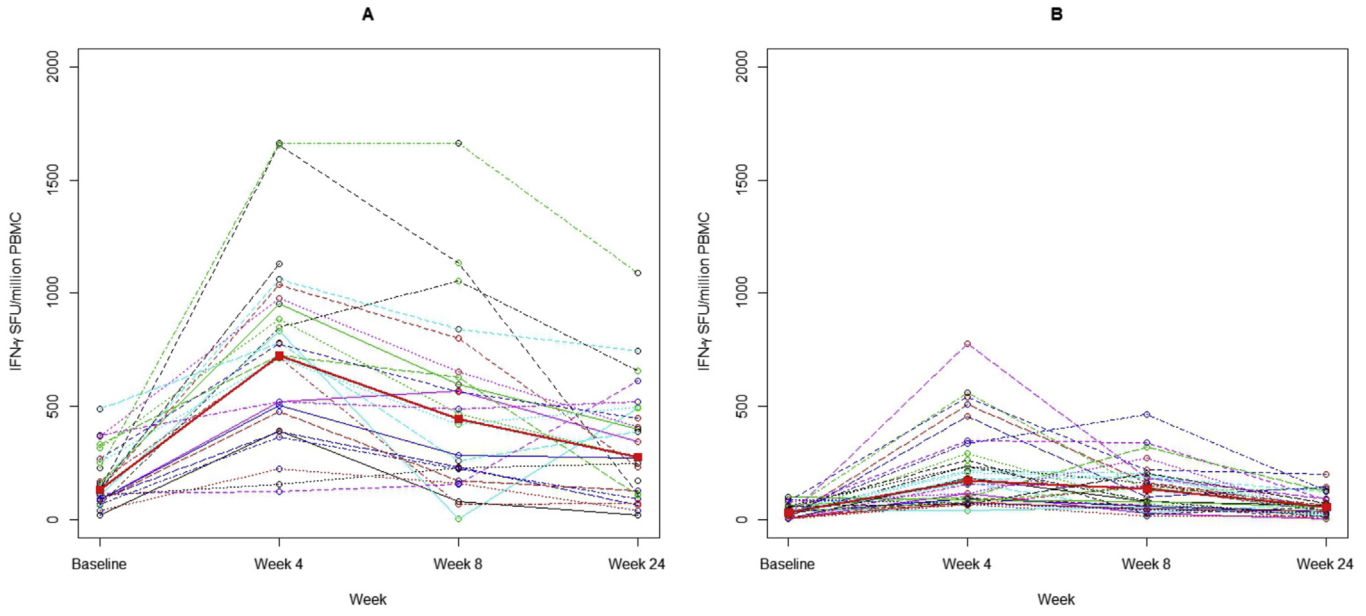

Figure 1. Longitudinal IFN-γ responses for the Short-term prospective (STP) analysis for 55 participants. BCG revaccinated (A) and primary-vaccinated (B). The bold red line represents the median values of each group at each time point. X-axis is not to scale. Abbreviations: IFN-γ = Interferon gamma; SFU = spot forming unit; PBMC = peripheral blood mononuclear cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Short-term prospective (STP) analysis: results of the linear regression analysis on AUC.

Area under the curve (AUC)					
Covariates (n)	Geometric mean of AUC	Unadjusted GM ratio (95% CI), *p*-value		Fully adjusted* GM ratio (95% CI), *p*-value (n = 43†)	
Baseline IFN-γ response (55)	329.03	1.00	(0.99, 1.01), 0.49	1.00	(0.99, 1.01), 0.76
Gender					
Female (36)	280.13	1	–	1	–
Male (19)	446.30	1.59	(0.73, 3.49), 0.24	1.04	(0.55, 1.96), 0.91
BCG vacc history‡					
1–9 yrs (8)	1156.66	1	–	1	–
10–19 yrs (10)	579.88	0.50	(0.15, 1.63)	0.45	(0.16, 1.27)
20–29 yrs (7)	384.62	0.33	(0.09, 1.21)	0.70	(0.19, 2.50)
Never (30)	187.84	0.16	(0.06, 0.44), 0.002§	0.22	(0.07, 0.68), 0.01
ML ratio (43)		1.16¶	(0.85, 1.58), 0.33	1.09¶	(0.82, 1.45), 0.53

Using a similar analysis of GM of IFN-γ response by ML ratio as in Table 2; GM for the AUC for a ML ratio of 0.1 and 0.3 were 111.57 and 115.01, respectively. Abbreviations: IFN-γ = Interferon gamma; vacc = vaccination; GM = geometric mean; yrs = years; ML = Monocyte/lymphocyte.
* Adjusted for all variables in the model.
† Due to missing ML ratio data.
‡ Prior to BCG vaccination in trial.
§ *p*-value for all categories of BCG vaccination history covariate using an ANOVA summary.
¶ The value of the change in GM of the AUC for an increase in 0.1 of ML ratio.

Table 4
Short-term prospective (STP) analysis: results of the linear regression analysis on 24 week IFN-γ response.

24 week IFN-γ response							
Covariates (n)	Geometric mean of 24 week IFN-γ response	Unadjusted GM ratio (95% CI), *p*-value		Partially adjusted (for baseline IFN-γ response) GM ratio (95% CI), *p*-value		Fully adjusted* GM ratio (95% CI), *p*-value (n = 43†)	
Baseline IFN-γ response (55)	87.44	1.01	(1.01, 1.01), <0.001	–	–	1.00	(0.99, 1.01), 0.17
Gender							
Female (36)	83.11	1	–	1	–	1	–
Male (19)	96.29	1.16	(0.51, 2.64), 0.72	1.12	(0.57, 2.21), 0.74	0.69	(0.32, 1.51), 0.35
BCG vacc history‡							
1–9 yrs (8)	274.13	1	–	1	–	1	
10–19 yrs (10)	212.37	0.78	(0.26, 2.32)	0.84	(0.29, 2.42)	1.05	(0.30, 3.73)
20–29 yrs (7)	214.43	0.78	(0.24, 2.59)	0.68	(0.21, 2.16)	1.55	(0.32, 7.35)
Never (30)	38.91	0.14	(0.06, 0.36), <0.001§	0.25	(0.09, 0.69), <0.001§	0.29	(0.07, 1.12), >0.05§
ML ratio (43)		1.08¶	(0.71, 1.65), 0.72	1.14¶	(0.81, 1.59), 0.45	1.04¶	(0.73, 1.47), 0.83

Using a similar analysis of GM of the IFN-γ response by ML ratio as in Table 2; GM for the 24 week response for a ML ratio of 0.1 and 0.3 were 125.08 and 126.01, respectively. The GM ratio for the baseline IFN-γ response covariate in the partially adjusted analysis is not included here, but all were similar to the unadjusted analysis value (approximately 1 and *p*-value<0.05). Abbreviations: IFN-γ = Interferon gamma; vacc = vaccination; GM = geometric mean; yrs = years; ML = Monocyte/lymphocyte.
* Adjusted for all variables in the model.
† Due to missing ML ratio data.
‡ Prior to BCG vaccination.
§ *p*-value for all categories of BCG vaccination history covariate using an ANOVA summary.
¶ The value of the change in GM of the 24 week response for an increase in 0.1 of ML ratio.

Table 5
Short-term prospective (STP) analysis: results of the linear regression analysis on peak IFN-γ response.

Peak IFN-γ response measured over 24 week follow-up							
Covariates (n)	Geometric mean of peak IFN-γ response	Unadjusted GM ratio (95% CI), *p*-value		Partially adjusted (for baseline IFN-γ response) GM ratio (95% CI), *p*-value		Fully adjusted* GM ratio (95% CI), *p*-value (n = 43†)	
Baseline IFN-γ response (55)	343.08	1.00	(1.00, 1.01), <0.001	–	–	1.00	(0.99, 1.01), 0.14
Gender							
Female (36)	336.38	1	–	1	–	1	–
Male (19)	356.90	1.06	(0.64, 1.76), 0.81	1.05	(0.68, 1.59), 0.83	0.89	(0.58, 1.36), 0.57
BCG vacc history‡							
1–9 yrs (8)	853.93	1	–	1	–	1	
10–19 yrs (10)	595.42	0.70	(0.38, 1.28)	0.71	(0.39, 1.30)	0.72	(0.35, 1.45)
20–29 yrs (7)	588.23	0.70	(0.36, 1.32)	0.65	(0.34, 1.24)	0.75	(0.32, 1.76)
Never (30)	201.06	0.24	(0.14, 0.39), <0.001§	0.29	(0.16, 0.51), <0.001§	0.32	(0.15, 0.68), <0.001§
ML ratio (43)		1.05¶	(0.82, 1.35), 0.70	1.09¶	(0.89, 1.32), 0.38	1.01	(0.84, 1.22), 0.89

Using a similar analysis of GM of the IFN-γ response by ML ratio as in Table 2; GM for the peak response for a ML ratio of 0.1 and 0.3 were 536.34 and 564.72, respectively. The GM ratio for the baseline IFN-γ response covariate in the partially adjusted analysis is not included here, but all were similar to the unadjusted analysis value (approximately 1 and *p*-value < 0.05). Abbreviations: IFN-γ = Interferon gamma; vacc = vaccination; GM = geometric mean; yrs = years; ML = Monocyte/lymphocyte.
* Adjusted for all variables in the model.
† Due to missing ML ratio data.
‡ Prior to BCG vaccination.
§ *p*-value for all categories of BCG vaccination history covariate using an ANOVA summary.
¶ The value of the change in GM of the peak response for an increase in 0.1 of ML ratio.

required to protect against TB disease and in males, a higher immune response may lead to detrimental exaggerated inflammatory responses [25].

There is uncertainty in the duration of protection of efficacy following BCG vaccination. In our Long-term retrospective (LTR) analysis, we found previous BCG vaccination was associated with a higher IFN-γ response at baseline, which supports results from several previous studies [8,26]. We also found, in both LTR analysis and short-term prospective (STP) analysis, no difference between any IFN-γ response if vaccinated any time between 10 and 30 years ago versus less than 10 years ago, suggesting that there may be no difference in the immune response generated at one year and up to 30 years after primary vaccination. These results suggest that BCG vaccination induces a durable memory response. However, previous studies have shown that IFN-γ responses following BCG vaccination can wane [27]. In order to more precisely assess the possibility of a waning response in our data, the BCG vaccination history covariate could be stratified into smaller groupings. However, with the current dataset size, this would impact on the statistical power of the analysis. The duration of a BCG immune response is complex and currently, not fully understood. As such, more trials to measure this specific immune response may be necessary.

In our STP analysis, we found that revaccination with BCG was associated with an increase in total (AUC) over 24 weeks and peak (taking into account baseline levels) IFN-γ response. However, it was not associated with higher IFN-γ response (when baseline responses were taken into account) at 24 weeks. Our in-depth characterization of this short term effect is supported by previous work at single time points that showed initial increases following revaccination with BCG [25–27] that were not sustained at 24 [28] or 52 weeks [29,30]. This suggests that revaccination with BCG has an impact on overall IFN-γ levels in the short term (<24 weeks) but may have little effect on IFN-γ levels long term (>24 weeks).

All other covariates were non-significant. This may be due to small numbers of participants or no association.

Recent evidence has revealed a complex relationship between ML ratio and risk of TB disease [14]. We explored if this could be explained by a link between ML ratio and IFN-γ responses. However, our results showed that ML ratio had no association on IFN-γ response in the LTR or STP analyses. This difference could be explained by HIV status amongst our participant population compared to previous work [14].

There are a number of limitations of our work. Most importantly, we chose to use IFN-γ-expressing cells as our marker of immune response. Whilst the presence of IFN-γ has been shown to be important in protection against *M.tb* infection [31], it has not proven to be a correlate of protection for TB disease [32,33]. It is, however, one of the most commonly used measures of TB vaccine immunogenicity we have [34]. The use of IFN-γ-expressing cells as our sole indicator of immunogenicity has benefits in its simplicity, and was the only outcome for which data was available to us. Other studies are being carried out that may give a more in depth view of the immune response to BCG in which a more complex "biosignature" is being investigated [34]. Secondly, our work was limited to the data available from the seven TB vaccine trials, which restricted the covariates available and the size of the participant cohorts. For example, HIV positive and latently infected individuals were excluded. Thirdly, in the outlined laboratory procedure [17–20] a 16-h ELISPOT assay was chosen, which may have potentially missed central memory CD4+ T-cells as they require a longer period of antigen re-stimulation to generate IFN-γ [35]. As such, our responses may underestimate the true "memory" cell presence, specifically at the later time point of 24 weeks.

The implications of our results are as follows. Our results show that previous BCG vaccination generates a higher immune response and this may complicate the interpretation of immunological results of new TB vaccine clinical trials, and support stratification of vaccine trial results by previous TB vaccination status, as is carried out previously [17,26]. In addition, if replicated in future analysis, our results also suggest that future TB vaccine trials may need to also stratify their analysis by gender. Moreover, to potentially capitalize on the impact of higher immune response due to previous vaccination and to improve upon the variable efficacy of BCG, it has recently been suggested that revaccination with BCG may increase efficacy [36]. Our findings showed that revaccination with BCG, whilst providing a higher IFN-γ peak response, did not increase IFN-γ at 24 weeks over the levels measure in primary-vaccinated participants. This provides more evidence to support the WHO policy not to revaccinate with BCG [37].

As an extension to our analysis, the time between the long-term retrospective (LTR) and short-term prospective (STP) analysis could be considered (for example, a number of years via a phase II/III clinical trial), which is not addressed here. Knowledge of this may indicate why we see a gender effect in the long term and not in the short-term and give further insight into the duration of BCG immunogenicity. Moreover, the link between ML ratio and TB disease is an exciting development in the search for informative TB risk factors and further work with additional detailed datasets should be conducted on the immunology driving this relationship. To improve upon the methods used in our STP analysis, mathematical models could be adopted to explore the underlying mechanisms behind the dynamics. The impact of the covariates on key immune system parameters would then be analysed.

5. Conclusion

The research conducted in this analysis aimed to establish, using new detailed mycobacterial-specific immune response data, which, if any, individual level covariates alter the immune response over the long term or shortly after BCG vaccination. This analysis suggests that previous BCG vaccination and gender are associated with durable IFN-γ responses. The results of this analysis imply that future vaccine trials should consider stratifying the trial population for analysis by gender and BCG vaccination history.

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Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.10.002.

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