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Inhibition of mycobacterial growth *in vitro* following primary but not secondary vaccination with BCG

Running title: Growth inhibition following BCG vaccination

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Abstract

Despite the widespread use of the BCG vaccine there are more than 9 million new cases of tuberculosis (TB) every year and there is an urgent need for better TB vaccines. TB vaccine candidates are selected for evaluation based in part on the detection of an antigen-specific IFN-γ response. The measurement of mycobacterial growth in blood specimens obtained from subjects immunized with investigational TB vaccines may be a better *in vitro* correlate of *in vivo* vaccine efficacy. We performed a clinical study with 30 UK adults who were followed for 6 months to evaluate the ability of both a whole blood and a novel PBMC based mycobacterial growth inhibition assay to measure a response to primary vaccination and revaccination with BCG. Using cryopreserved PBMC we observed a significant improvement in mycobacterial growth inhibition following primary vaccination, but no improvement in growth inhibition following revaccination with BCG ($p<0.05$). Mycobacterial growth inhibition following primary BCG vaccination was not correlated with PPD antigen specific IFN- γ ELISPOT responses. We demonstrate that a mycobacterial growth inhibition assay can detect improved capacity to control growth following primary immunization but not revaccination with BCG. This is the first study to demonstrate that an *in vitro* growth inhibition assay can identify a difference in a vaccine response comparing both primary and secondary BCG vaccination, suggesting that *in vitro* growth inhibition assays may serve as better surrogates of clinical efficacy than those assays currently used for the assessment of candidate TB vaccines.

Keywords: Tuberculosis, TB, Vaccine, immune response,

Introduction

There are more than 9 million new cases of TB every year, primarily affecting young adults in the most productive years of their life. Despite the existence of effective tools for the diagnosis and treatment of TB, the global incidence remains high, driven primarily by the increase in HIV-associated TB in Africa (1). The only available vaccine for TB, Bacille Calmette-Guérin (BCG), was first used as a human vaccine in 1921 and is one of the most widely administered vaccines in the world. Although widely practiced the administration of BCG to newborns as recommended by WHO, while affording protection against severe infant TB, leads to protection against later pulmonary disease which is variable and mostly poor (2). There is an urgent need to develop an improved vaccine. Over the last decade, 16 candidate vaccines have entered clinical trials (3). However, the progression of candidate vaccines from pre-clinical studies and early phase I trials to larger and more expensive efficacy trials is frustratingly slow. In part this is due to the nature of the disease, as only 1 in 10 people infected with TB develop disease, which may occur decades after initial infection. It is therefore necessary to prioritise which candidate vaccines are to be tested in efficacy trials at the available field sites. The selection of candidate vaccines for efficacy testing will be based in part on safety and immunogenicity in early clinical trials. The identification of a biomarker or biosignature of protective efficacy which could be used to select vaccine candidates for further study would greatly facilitate vaccine development and have a major impact on the field. Several groups have demonstrated the potential use of *ex vivo* mycobacterial growth inhibition assays in both *Mycobacterium tuberculosis* (MTB) exposed and BCG-vaccinated cohorts (4-9). However, despite more than a decade of use, these assays

have not been adopted by TB vaccine developers, in part because of concerns regarding reproducibility of these assays. To date, TB vaccine developers have focused on the discovery of T cell signatures of vaccine effectiveness. However, due to the recent failure of T cell signatures to differentiate protected from non-protected individuals (10), there has been a resurgence of interest in the utility of mycobacterial growth inhibition assays (MGIA) as an alternative route of assessing vaccine induced protection.

Here, we describe the findings of a clinical study to evaluate the ability of both a whole blood and a peripheral blood mononuclear cell (PBMC) based MGIA to measure a response to primary and repeat BCG vaccination in UK adults. The use of functional assays for the early identification of promising vaccine candidates is essential if we are to expedite the development of a new TB vaccine and use limited resources most effectively.

Materials and Methods

Ethics statement

Participants were recruited under a protocol approved by the Oxfordshire Research Ethics Committee (OxREC A). Written informed consent was obtained from all individuals prior to enrolment in the trial.

Study design and participants

This was a non-randomized, clinical study in healthy adults with a) no history of BCG vaccination or b) a history of prior BCG vaccination more than six months before study

enrolment (Figure 1). Volunteers were screened and enrolled only if routine haematology and biochemistry measures were within the normal range and if they were negative on serology for HIV, HBV and HCV. Volunteers were aged 18-50 with no evidence of latent MTB infection, as determined by an in-house IFN-γ ELISPOT assay which measures responses to ESAT-6 and CFP-10 peptides at screening. Enrolled participants attended clinic on three occasions prior to vaccination with BCG. These three pre-vaccination visits were to determine the baseline response and assay variability over-time in healthy adult volunteers. Volunteers were then vaccinated with a single dose of ~ 2-8 x 10^5 CFU BCG-SSI (Staten Serum Institute), administered intradermally over the deltoid region of the arm. Following vaccination with BCG, participants were followed-up and blood was taken at weeks 4, 8, and 24 to determine the longitudinal response following immunisation.

Immunological assays

PBMC were isolated from heparinised whole blood and the *ex vivo* IFN-γ ELISPOT was performed as previously described (11). PBMC were isolated by centrifugation of whole blood over 15 ml LymphoPrep™ (Axis-Shield) in a LeucoSep™ tube (Greiner Bio-One) according to manufacturer's instructions. The *ex vivo* IFN-γ ELISPOT assay was used to assess antigen specific responses by incubating PBMC (0.3 x 10^6) overnight for 18 hours with 20 μg/ml purified protein derivative (PPD) from MTB (SSI). Positive Staphylococcal Enterotoxin B (SEB) and negative (media only) control wells were included for each participant.

Bacterial strains and culture

We used BCG Pasteur (donation from Ann Rawkins, Health Protection Agency, Porton Down, UK) as the immune target in our growth inhibition assays. BCG Pasteur was cultured in 6 x Bactec Mycobacteria growth indicator tube (MGIT) tubes for 8 days. Mycobacterial cultures were then pooled, aliquoted and frozen at -80°C, as previously described, to provide stocks for use in this study (4).

Whole blood growth inhibition assay

The whole blood growth inhibition assay was performed using blood from 18 of the 30 volunteers as previously described (4). Duplicate tubes containing 300 ul of whole blood were incubated on a rotator at 37°C with 300 ul RPMI seeded with ~150 CFU of mycobacterial stock culture (BCG Pasteur) for 4 days. Blood cells were then lysed with sterile water and the lysate containing mycobacteria transferred to a BACTEC MGIT tube supplemented with PANTA™ antibiotics and OADC™ enrichment broth (all from Becton Dickinson). The tube was placed in a BACTEC MGIT 960 and incubated until growth detected (time to positivity). Using a standard curve enables conversion of time to positivity (TTP) of a sample tube into an initial mycobacterial inoculum volume (Supplementary Figure 1). This inoculum volume is then converted to CFU (50µl of inoculum = 3.50E+04 CFU/ml). Duplicate MGIT tubes are seeded with ~150 CFU of mycobacterial stock culture and placed directly into the Bactec MGIT 960 on day 0 of the blood culture to be used as growth control tubes. The CFU count of each sample tube is divided by the CFU count of the growth control tube, divided by 4 days and log converted. This gives the delta log growth per day for each sample tube. Software to

146 calculate change in bacillary viability based on MGIT TTP written by one of the authors
147 (RSW) is available on request. In one case, to show data expressed as TPP and delta
148 log growth per day, see Supplementary Figure 2 compared with Figure 4. The number
149 of viable mycobacteria recovered following incubation of PBMC was lower than
150 expected due to the effect of residual penicillin and streptomycin in culture media. The
151 predicted growth was 2.5 x 10^5 CFU and the actual growth was 250 CFU. A difference
152 of approximately 3 logs (0.9013 delta log growth per day) (Supplementary Figure 3). To
153 calculate growth inhibition in PBMC samples 0.9013 delta log growth per day was
154 subtracted from the control tube to better reflect the number of viable mycobacteria
155 used in the assay.
156
157 **PBMC based growth inhibition assay**
158 We adapted the whole blood assay described above for use with cryopreserved PBMC.
159 PBMC from 19 of the 30 volunteers were thawed and rested overnight at 37°C in RPMI
160 containing benzonase (10 U/ml, Novagen), 10% pooled human AB serum, L-glutamine,
161 penicillin and streptomycin. After the overnight rest cells were counted, washed and
162 resuspended in the above media without benzonase or antibiotics, but with HEPES. The
163 percentage viability of recovered cells was 70-90% per vial. Duplicate 2ml screw cap
164 tubes containing 1 x 10^6 PBMC in 600ul of media were incubated on a rotator at 37°C
165 with ~600 CFU of BCG Pasteur stock for 4 days. PBMC were then lysed with sterile
166 water and the lysate containing mycobacteria transferred to a BACTEC MGIT tube
167 supplemented with PANTA™ antibiotics and OADC™ enrichment broth. The tube was

placed in a BACTEC MGIT 960 and incubated until growth detected (time to positivity). Data were analysed as described for the whole blood assay above.

Data analysis

Statistical analysis was performed using SPSS and Graphpad Prism. One-way Anova and Students T- tests were used to determine significant differences in growth inhibition. Mann-Whitney and Wilcoxon signed rank were used to determine differences in ELISPOT responses. Spearmans Rho was used to determine correlations between growth inhibition and immune response. Assay variability was assessed by calculating the co-efficient of variation.

Results

Study participants

There were 30 participants enrolled into the study, of whom 15 had been previously vaccinated with BCG (mean of 17.8 years prior to enrollment). The remaining 15 had no previous history of BCG vaccination (Figure 1). All volunteers received a single intradermal immunization of BCG-SSI. Demographic characteristics of study participants are summarised in Table 1 and are similar to those reported in previously published studies (11, 12).

IFN-γ ELISPOT response to PPD in BCG vaccinated volunteers

In order to compare immunological responses in the BCG vaccinated subjects with growth inhibition, IFN-γ responses were first measured by ELISPOT. There was a

significant increase in the magnitude of the IFN-γ ELISPOT response to PPD following both primary vaccination and revaccination with BCG ($p<0.0001$, Figure 2A and B). The peak IFN-γ ELISPOT response was at 4 weeks following immunization and had returned to baseline by 24 weeks for both groups (Figure 2A and B). When comparing the two groups we found that the IFN-γ ELISPOT responses were significantly higher in those volunteers revaccinated with BCG at baseline, week 4 and week 24 ($p<0.05$, Table 2). This is consistent with PPD IFN-γ ELISPOT responses observed following BCG vaccination in previously published studies (11).

Measurement of Growth Inhibition following BCG vaccination

A mycobacterial growth inhibition assay using a Bactec MGIT to detect growth (22) was modified (see Methods) and used to measure growth inhibition using both whole blood and cryopreserved PBMC from volunteers previously vaccinated with BCG and volunteers who had no history of BCG vaccination. Growth inhibition was measured in 9 volunteers using the whole blood assay and 10 volunteers using the PBMC assay with 5 volunteers tested using both assays. Using whole blood there was a trend for enhanced growth inhibition comparing BCG vaccinated and naïve subjects but the difference was not significant (Figure 3A). Using cryopreserved PBMC there was significantly more mycobacterial growth inhibition comparing PBMC from BCG vaccinated subjects with BCG naïve subjects ($p<0.05$, Figure 3B).

Following primary BCG vaccination we observed enhanced mycobacterial growth inhibition compared with pre-vaccination responses in whole blood collected 8 weeks following vaccination in 7 of 9 volunteers tested ($p<0.05$, T-Test), although the response

was not significant when tested using one-way ANOVA (Figure 4A; for comparison with time to positivity data see Supplementary Figure 2A). In subjects with a history of BCG vaccination we did not detect increased mycobacterial growth inhibition in whole blood after BCG revaccination (Figure 4B; for comparison with time to positivity data see Supplementary Figure 2B).

Using PBMC cryopreserved from the same volunteers we observed a significantly enhanced mycobacterial growth inhibition at both 4 and 8 weeks, following primary vaccination with BCG ($p<0.05$ Wilcoxon, Figure 4C). All volunteers displayed enhanced mycobacterial growth inhibition following BCG immunization, for 6 volunteers this was at both weeks 4 and 8. In subjects with a history of BCG vaccination we did not detect increased mycobacterial growth inhibition in cryopreserved PBMC after BCG revaccination (Figure 4D).

The magnitude of observed growth inhibition was significantly greater when using cryopreserved PBMC compared to whole blood at both week 4 and 8 following vaccination with BCG. To confirm the ability of the PBMC assay to detect a difference pre and post vaccination with BCG we repeated the experiment using PBMC from the same 8 volunteers we used in the whole blood assay. We also ensured that PBMC were not exposed to antibiotics at any point in the procedure as was the case with the PBMC data shown in Figure 3B. Differences pre and post vaccination with BCG were only observed when we lowered the inoculum volume to 250 CFU in 1 x 10^6 PBMC (Figure 5). This suggests that BCG vaccine mediated control of mycobacterial growth may be overwhelmed at higher doses of viable mycobacteria and that growth inhibition

in the whole blood assay may be further improved if a lower dose of mycobacteria is used.

The reproducibility of the growth inhibition assay, in these studies was assessed as described in Methods for both whole blood and cryopreserved PBMC using samples collected on 3 consecutive weeks at baseline in our trial (Figure 1). The mean Co-efficient of Variation (CV) and 95% confidence intervals for cryopreserved PBMC and whole blood were 7.7 (6-9.4) and 24.5 (15.3-33.7) respectively. Using Intraclass Correlation (ICC) with a two-way mixed model where 1 indicates perfect agreement and 0 indicates no agreement there was fair agreement among the 3 measures in whole blood (.466) and good agreement between the 3 measures in PBMC (.625). The data demonstrates that there is reasonable reproducibility in the growth inhibition assays.

Mycobacterial growth inhibition does not correlate with IFN-γ ELISPOT responses

Next, we searched for a relationship between the induction of an antigen specific T-cell response in BCG vaccinated volunteers and the ability to control mycobacterial growth *in vitro*. There was no correlation between mycobacterial growth inhibition in whole blood and the IFN-γ ELISPOT response at recruitment into the study or following primary vaccination or revaccination with BCG. Also, there was no correlation between mycobacterial growth in PBMC and the IFN-γ ELISPOT response following primary vaccination with BCG.

However, in the group with a history of BCG vaccination, there was a correlation with growth inhibition at 4 weeks and the peak IFN-γ ELISPOT response at 4 weeks

(p<0.005, Figure 6A). This indicates that a T-cell response may be contributing to control of mycobacterial growth following revaccination with BCG, although the effect is likely to be modest or partial as revaccination with BCG did not result in a significant improvement in growth inhibition when compared to baseline.

We also observed that, a strong IFN-γ ELISPOT response at baseline, prior to revaccination with BCG, was correlated with reduced mycobacterial growth inhibition at 24 weeks following revaccination (Figure 6B). Boosting T-cell responses by revaccination with BCG may reduce the long-term ability to control BCG growth, perhaps as a consequence of T-cell exhaustion or T-cell regulation although these were not measured in the current study.

Discussion

Using a mycobacterial growth inhibition assay we observe that subjects with a history of BCG vaccination control the growth of mycobacteria more effectively than those who have never been immunized. In addition, primary BCG vaccination leads to inhibition of mycobacterial growth up to 8 weeks following immunization whereas revaccination with BCG does not improve, and may reduce, the ability of cells to control mycobacterial growth. In the UK, primary vaccination with BCG protects with an efficacy of 80% (13). There have been no clinical trials of intradermal BCG revaccination in the UK but the lack of change in TB incidence following withdrawal of BCG revaccination (Finland) (14) and lack of efficacy in clinical trials of BCG revaccination in other countries (Malawi, Brazil, Chile, Hong Kong) (15-19) has shown that revaccination with BCG does not improve protection from TB disease. Our *in vitro* mycobacterial growth inhibition data

282 are consistent with the epidemiological data which indicate that primary vaccination, but
283 not revaccination with BCG, can improve protection against TB disease.
284
285 There is an urgent need for a biomarker which can be used to identify potentially
286 protective vaccines or vaccine combinations early in clinical development which could
287 accelerate regulatory approval of clinical studies and ultimately the approval of new TB
288 vaccines. The capacity of these assays to detect inhibition of mycobacterial growth in
289 both historically and recently BCG vaccinated subjects warrants further evaluation in
290 different study populations and in phase I and II clinical trials of candidate TB vaccines.
291 An assay used for regulatory approval would have to be validated according to the
292 principles of the International Conference on Harmonization (ICH Harmonized Tripartite
293 Guidelines) which encompass accuracy, precision (repeatability, intermediate precision,
294 reproducibility), specificity and linearity. To begin to determine if it would be possible to
295 validate an MGIA we determined the intra assay variability of both the whole blood and
296 PBMC based MGIA. Tuomela *et. al*. suggest that for assay validation a CV of less than
297 50% is acceptable variation for the measurement of a bacterial target of a cell based
298 assay (20). In this report, both the whole blood and the PBMC MGIA's had a CV less
299 than 50% over repeated sampling prior to immunization. Volunteers were recruited over
300 a 12 month period and multiple aliquots of frozen mycobacterial stock were used in the
301 whole blood assay. Variations in mycobacterial stock viability, in volunteer blood and
302 variability in the week-to-week performance of the assay could have contributed to the
303 overall higher variability of the whole blood assay when compared to the PBMC assay
304 which was run in just two batches in this study. We saw the greatest improvement in

growth inhibition at 8 weeks following primary vaccination with BCG indicating that this is the optimum time point for measurement of growth inhibition following vaccination with a live, replicating mycobacteria. We could not detect growth inhibition at 24 weeks. It is probable that the optimum time for measurement of growth inhibition will have to be determined during early phase clinical trials for each candidate TB vaccine.

Earlier studies assessing four mycobacterial growth inhibition assays, two whole blood and two PBMC based assays, observed optimum growth inhibition at 8 weeks and 6 months following revaccination with BCG (6, 21). Differences between our study and those of Hoft *et. al.* and Cheon *et. al.* include the use of a different growth inhibition assay protocol, a different BCG vaccine strain, enrollment of a UK study population where primary BCG vaccination is known to have an efficacy of 80%, and a longer interval between primary and revaccination with BCG. Cheon *et. al*. found that blood from only 4 of 10 subjects from the USA inhibited mycobacterial growth following primary vaccination with BCG whereas PBMC from 7 of the 8 UK subjects with no history of BCG vaccination tested in our study inhibited mycobacterial growth 8 weeks following primary BCG vaccination.

To determine if a BCG vaccine-induced immune response was correlated with growth inhibition we compared the PPD antigen specific IFN-γ T cell response, measured using an *ex vivo* ELISPOT assay, with mycobacterial growth inhibition. Revaccination with BCG induces a greater number of PPD antigen specific T cells, when compared to primary vaccination with BCG, yet this greater number of T cells does not result in an improved capacity to control mycobacterial growth *in vitro.* This is consistent with other

reports which have found that IFN-γ is not correlated with mycobacterial growth inhibition following BCG vaccination of either infants or adults (6, 7). The peak immune response was at 4 weeks following revaccination with BCG and this was the only time point at which the IFN- γ T cell response was associated with control of mycobacterial growth in PBMC. It is thought that prior exposure to environmental mycobacteria or MTB can interfere with the establishment of protective immunity following immunization with BCG (22, 23). An immune response to PPD can be induced by previous BCG vaccination, exposure to environmental mycobacteria or exposure to MTB itself. Barreto *et. al*. reported that the efficacy of BCG revaccination was 33% in a population where there was low exposure to environmental mycobacteria and MTB (the Brazilian city of Salvador) but that efficacy was lost in older children, who were more likely to have a response to PPD at the time of vaccination, and also lost in cities where there was higher exposure to environmental mycobacteria and MTB (23). These findings are consistent with our growth inhibition assay findings, in which we see that a higher baseline immune response to PPD is associated with lack of control of mycobacterial growth at 24 weeks following revaccination. In this study, we have not identified the immune mechanism of *in vitro* growth inhibition but, consistent with other reports, have shown that measurement of vaccine induced IFN-γ secreting T cells alone does not reflect the overall capacity of cells from vaccines to control mycobacterial growth.

Due to the lack of an immune correlate for protection against TB, IFN-γ remains the primary measure of TB vaccine immunogenicity in humans. Vaccine candidates are mostly selected for further development based on efficacy in preclinical animal models, safety and the induction of an IFN-γ response in early clinical trials (24). In this report,

we describe an alternative assay that could potentially allow direct comparison of vaccines across clinical trials and animal species. As this assay requires no specific immune reagents or antigen stimulation, it could be used for the early assessment of a wide range of candidate TB vaccines. Since the BACTEC MGIT is found in many clinical laboratories, an automated version of the growth inhibition assay can be implemented by investigators performing TB vaccine studies in human subjects as described in this report as well as in animal models of TB (25). Further, as this assay appeared to mimic the findings of clinical efficacy trials, detecting improved capacity to control mycobacterial growth following primary immunization but not revaccination with BCG, it may be a better surrogate of clinical efficacy than existing assays used for the assessment of candidate TB vaccines.

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Figure Legends

Figure 1. Outline of clinical study showing schedule of blood collection and BCG vaccination.

Figure 2. T cell responses in adults receiving primary vaccination with BCG or BCG revaccination. PPD antigen specific T cells were detected in PBMC from healthy, BCG naïve (panel A) or previously BCG vaccinated (panel B) adults receiving ~2-8 x 10^5 CFU of BCG-SSI. T cells were stimulated overnight with PPD and responses were detected using an ex vivo IFN-γ ELISPOT assay. Values were determined by Wilcoxon matched-pairs signed rank when compared to baseline (before BCG vaccination).

Figure 3. *In vitro* growth inhibition in BCG vaccinated and BCG naïve subjects using both whole blood and cryopreserved PBMC. A) The ability of whole blood from 8 BCG naïve and 10 previously BCG vaccinated volunteers to inhibit mycobacterial growth at baseline is compared. There was a trend for enhanced mycobacterial growth inhibition in the BCG vaccinated group but this was not significant. B) The ability of cryopreserved PBMC from 9 BCG naïve and 10 BCG vaccinated volunteers to inhibit mycobacterial growth were assessed. Significantly more growth inhibition was observed in the PBMC cultures from previously BCG vaccinated subjects ($p>0.05$, T-Test). Boxplots show the lowest datum within 1.5 interquartile range of the 25th quartile, and the highest datum still within 1.5 interquartile range of the 75th quartile (Tukey).

492 Figure 4. Mycobacterial growth in the blood and PBMC of adults receiving primary
493 vaccination with BCG or following revaccination with BCG. Mycobacterial growth was
494 measured in whole blood (panels A and B) and cryopreserved PBMC (panels C and D)
495 of healthy, BCG naïve (panel A and C) or previously BCG vaccinated (panel B and D)
496 adults receiving ~2-8 x 10^5 CFU of BCG-SSI. Mycobacterial growth in samples collected
497 at baseline (before BCG vaccination) was compared to growth in samples collected at
498 4, 8 and 24 weeks post-vaccination. One-way Anova was used to test for significance
499 followed by a paired T-Test. Boxplots show the lowest datum within 1.5 interquartile
500 range of the 25th quartile, and the highest datum still within 1.5 interquartile range of the
501 75^{th} quartile (Tukey).
502
503 Figure 5. Growth inhibition in PBMC pre and post primary vaccination with BCG. To
504 confirm that growth inhibition can be measured in frozen PBMC without antibiotics from
505 subjects vaccinated with BCG we repeated the assay using PBMC from the same 8
506 subjects used in the whole blood growth inhibition assay. Growth inhibition was
507 measured pre vaccination and 8 weeks post vaccination.
508
509 Figure 6. Correlation of mycobacterial growth with the IFN-γ ELISPOT response to PPD.
510 In cryopreserved PBMC collected 4 weeks following immunisation with BCG (panel A)
511 there was a significant correlation with the inhibition of mycobacterial growth and the
512 number of PPD antigen specific IFN-γ secreting T-cells. Subjects from both primary
513 and revaccination groups are included in panel A. In the revaccination group (panel B)
514 a higher PPD antigen specific IFN-γ ELISPOT response at baseline (prior to

vaccination) was associated with reduced capacity to control mycobacterial growth.

Spearmans correlation.

518 Table 1. Demographics of volunteers enrolled into the study

	Group	
	BCG **n = 15**	**BCG-BCG** **n = 15**
Male, no (%)	5 (33)	8 (53)
Median age, years (range)	28 (18-55)	27.5 (19-53)
Average time since BCG vaccination, years (range)	-	17.8 (3-38)

519

520

521 Table 2. Comparison of immune response between groups of volunteers receiving
522 primary BCG vaccination or revaccination with BCG

	Group BCG	**Group BCG-BCG**	**P value Mann Whitney**
	SFC/10^6 PBMC Median (IQR) n=15	SFC/10^6 PBMC Median (IQR) n=15	BCG vs. BCG-BCG
Week 0	37(14-60)	173 (74-281)	<0.005
Week 4	210 (79-516)	520 (330-1038)	<0.05
Week 8	185 (83-858)	245 (159-883)	ns
Week 24	53 (32-117)	261(85-521)	<0.05

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