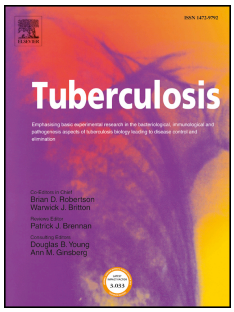

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Impact of Individual-level Factors on *Ex vivo* Mycobacterial Growth Inhibition: Associations of Immune Cell Phenotype, Cytomegalovirus-specific Response and Sex with Immunity Following BCG Vaccination in Humans

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Abstract

Understanding factors associated with varying efficacy of Bacillus Calmette-Guérin (BCG) would aid the development of improved vaccines against tuberculosis (TB). In addition, investigation of individual-level factors affecting mycobacterial-specific immune responses could provide insight into confounders of vaccine efficacy in clinical trials. Mycobacterial growth inhibition assays (MGIA) have been developed to assess vaccine immunogenicity *ex vivo* and provide a measure of immune function against live mycobacteria. In this study, we assessed the impact of immune cell phenotype, cytomegalovirus (CMV)-specific response and sex on *ex vivo* growth inhibition following historical BCG vaccination in a cohort of healthy individuals (n=100). A higher frequency of cytokine-producing NK cells in peripheral blood was associated with enhanced *ex vivo* mycobacterial growth inhibition following historical BCG vaccination. A CMV-specific response was associated with T-cell activation, a risk factor for TB disease and we also observed an association between T-cell activation and *ex vivo* mycobacterial growth. Interestingly, BCG-vaccinated females in our cohort controlled mycobacterial growth better than males. In summary, our present study has shown that individual-level factors influence capacity to control mycobacterial growth following BCG vaccination and the MGIA could be used as a tool to assess how vaccine candidates may perform in different populations.

Keywords: tuberculosis vaccine, BCG, growth inhibition assay, cytomegalovirus, sex, NK cell

Introduction

Tuberculosis (TB) is the number one cause of death from an infectious disease worldwide and it is currently estimated that a quarter of the world population is infected with *Mycobacterium tuberculosis* (*Mtb*) [1, 2]. The introduction of Bacillus Calmette-Guérin (BCG) vaccination and chemotherapy in the past century provided optimism to fight the disease. Despite this, drug-resistant TB is now a major risk to global health security, and BCG as the only licensed vaccine for TB is known to have a variable efficacy against contagious adult pulmonary TB [3, 4]. BCG remains as the most widely used vaccine worldwide, primarily because it provides good protection against TB in children [3]. Understanding factors associated with varying BCG protection could aid the development of improved vaccination practice as well as novel vaccines against TB.

It has been proposed that the observed variation in BCG efficacy is attributed to individual-level factors which influence host mycobacteria-specific immune responses [5-7]. In a recent systematic review, protection following BCG vaccination was shown to vary according to the geographical latitudes in which the vaccine was given. In the UK, a country where exposure to environmental mycobacteria and/or *Mtb* is regarded to be lower (latitude > 40^{o}), BCG is known to provide efficacy of up to 80% against pulmonary TB [8] and vaccination of school-aged children could provide protection for more than 20 years [9]. Another factor that may influence the mycobacteria-specific immune response is sex. Globally, TB case rates are much higher in men than in women, as reflected by a global male to female ratio (M:F) of 1.7 for case notifications in 2016 [10]. Males contribute to 65% of TB cases worldwide and although it is thought that socioeconomic and cultural factors are contributing to the observed sex bias, differences in the immune responses between the sexes also play a role [11, 12]. It is generally acknowledged that females exhibit more robust immune responses towards infection and vaccination compared to males [13]. In the context of susceptibility to TB, differences in immune cells frequencies and functions have been thought to contribute to higher TB rates in males [14]. With regard to BCG vaccination, there is currently limited evidence concerning the impact of sex on its protective effect against pulmonary TB in adults. Interestingly, BCG is thought to provide a non-specific protective effect against unrelated pathogens, thus contributing in reduction of overall cause of mortality, and this effect is more pronounced in females rather than males [15-17].

Recently, Fletcher *et al*. found that T-cell activation is an immune correlate of risk of TB disease in BCG-vaccinated infants in a study enrolling a large cohort of infants [18]. Chronic exposure to antigen from persistent viral or bacterial infection is known to drive continuous T-cell activation which could lead to dysfunction of antigen specific T-cells [19]. Further to the findings of the infant study, it was identified that cytomegalovirus (CMV)-specific IFN-γ responses were associated with T-cell activation and could have contributed to increased risk of developing TB disease [20].

The mycobacterial growth inhibition assay (MGIA) has been developed as a measure of vaccine immunogenicity *ex vivo*. Following optimisation works in the past few years [21-23], the assay has gained attention for its potential ability to detect vaccine-mediated inhibition of growth following BCG vaccination in adults and infants [24-26]. The assay described in the present study involves direct co-culture of peripheral blood mononuclear cells (PBMCs) with mycobacteria, and subsequent measurement of mycobacterial growth inhibition as a functional assessment of vaccine response. Several studies have demonstrated the ability of the MGIA to detect changes in the innate and adaptive compartment following vaccination [25, 27-30]. Recently, Joosten and colleagues (2018) found that the capacity to control mycobacterial growth following recent *Mtb* exposure or BCG vaccination is associated with nonclassical monocytes, and this observation is reflective of the trained innate immune mechanism [26]. In a study by Jensen *et al.*, IFN-γ was associated with reduction of mycobacterial growth *ex vivo* following immunisation with a TB vaccine candidate in mice [31]. However, in that study the source IFN-γ was not found among the investigated vaccine-specific T-cells, suggesting potential contribution from other cell types, such as NK cells.

In this study, we demonstrated the impact of immune cell phenotype, CMV-specific response and sex on vaccine-specific mycobacterial growth inhibition following historical BCG vaccination in adult healthy volunteers.

Materials and Methods

Study participants and ethics statement

We recruited 100 healthy adult participants with (i) no history of BCG vaccination or (ii) a history of BCG vaccination more than 6 months before study enrolment. Verbal interviews

were conducted to determine eligibility based on the absence of any major chronic illness, current medication administration or symptoms of infection. Participants were aged 18 to 80 years with no evidence of exposure or infection with TB. Participants were excluded if they were suffering from any persistent medical condition or infection. Sample size was calculated based on the assumption of effect size 0.70, with power 0.8 and significance level 0.05. Written informed consent was obtained from all participants prior to enrolment in the study. Individuals were recruited under protocols approved by the LSHTM Observational Research Ethics Committee (ref 8762 and 10485). All procedures were conducted in accordance with the Declaration of Helsinki, as agreed by the World Medical Association General Assembly (Washington, 2002) and ICH Good Clinical Practice (GCP).

PBMCs isolation and IFN-γ Enzyme-linked immunospot (ELISpot) assay

Peripheral blood (50ml) was collected and processed within 6 hours. PBMCs isolation and IFN-γ ELISpot assay were performed as previously described [32]. PBMCs were cryopreserved in FBS (Labtech International Ltd, Uckfield, UK) containing 10% DMSO (Sigma-Aldrich) and stored in -80 ^{o}C freezer using CoolCell containers (VWR International, Lutterworth, UK). PBMCs were thawed and an *ex vivo* IFN-γ ELISpot assay was performed to assess antigen-specific response. PBMCs were incubated overnight for 18 hours with 20 μg/ml purified protein derivative (PPD) (Oxford Biosystem, Oxfordshire, UK). Positive control Phytohemagglutinin (PHA) (10 μg/ml, Sigma-Aldrich) and negative control (medium-only) wells were included for each participant samples. Results are reported as spot forming cells (SFC) per million PBMCs, 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. Spots were quantified using an automated plate reader with ELISpot 5.0 software as well as checked visually.

***Ex vivo* Mycobacterial Growth Inhibition Assay**

The growth inhibition assay was performed using cryopreserved PBMCs of the study participants, as previously described [32]. In brief, a 2-ml screw-cap tubes containing 3 x 10^6 PBMCs in 600 ml of medium were rotated at 37^{o}C with ~100 Colony Forming Units (CFU) of BCG Pasteur Aeras strain (Rockville, MD, USA) 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 (all from Becton Dickinson, Oxford, UK). The tube

was placed in a Bactec MGIT 960 and incubated until growth was detected (measured as time to positivity [TTP]). Use of a standard curve enables conversion of the TTP of a sample tube into bacterial numbers (log CFU) (Supplementary Fig. S1). All work with cells pre-BCG infection and involving BCG infected samples was done in Biosafety Level (BSL) 2 laboratory.

Enzyme-linked immunosorbent assay (ELISA)

MGIA supernatants were analysed to assess cytokine concentrations by ELISA. The levels of following cytokines were measured: IFN-γ, interleukin (IL)-12p40, IL-6 [BD OptiEIA kits, Becton Dickinson, UK], tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma-induced protein 10 (IP-10), granzyme B, IL-32, IL-22 [R&D Systems, Abingdon, UK], IL-10, IL-17 [BioLegend, London, UK] and perforin [Abcam, Cambridge, UK]. Assays were performed according to the manufacturers' instruction.

Flow cytometric immune phenotyping

PBMC were washed and stained with 1 µl/ml Live Dead Blue Stain (Invitrogen), followed by staining with the following titrated antibody for the Lymphocyte panel: 2.5 µl CD3-AF700 (clone UCHT1, Ebioscience, Loughborough, UK), 1.25 µl CD4-APC/Cy7 (clone RPA-T4, BioLegend), 1.25 µl CD8-Superbright645 (clone RPA-T8, Ebioscience), 2.5 µl CD19-FITC (clone HIB19, BioLegend), 2.5 µl CD56-APC (clone HCD56, BioLegend), 2.5 µl CD16-BV510 (clone 3G8, BioLegend), 5 µl HLA-DR-PE (clone L243, BioLegend), 5 µl LAG3-PE/Cy7 (clone 11C3C65, BioLegend) and 1.25 µl PD1-BV421 (clone EH12.2H7, BioLegend). For the Monocyte panel, the cells were stained with the following titrated antibodies: 2.5 µl CD3-AF700 (clone UCHT1, Ebioscience), 2.5 µl CD19-FITC (clone HIB19, BioLegend), 2.5 µl CD14-BV421 (clone HCD14, BioLegend), 2.5 µl CD16-BV510 (clone 3G8, BioLegend), 1.25 µl CD86-APC/Cy7 (clone IT2.2, BioLegend), 5 µl HLA-DR-PE (clone L243, BioLegend), 5 µl CD206-APC (clone 15-2, BioLegend), 5 µl CD163-BV605 (clone GHI/61, BioLegend), 2.5 µl CD64-APC/Cy7 (clone 10.1, BioLegend) and 5 µl CD123-BV650 (clone 6H6, BioLegend). Fluorescence minus one (FMO) controls were set using cells for each antibody and used to guide gating. Cells were acquired on a BD LSR II flow cytometer. Data was analysed with FlowJo software version 10.4 (Treestar Inc., USA).

Results are presented as percentages of cells after gating out of dead cells and doublets. Gating strategies for the lymphocyte and monocyte panels are described in Supplementary Fig. S2A and S2B.

Intracellular cytokine staining (ICS) flow cytometry

The ICS flow cytometry was performed as previously described [32]. In brief, PBMCs were then incubated alone (medium only) as a negative control, with 5 μg/ml *Staphylococcus* enterotoxin B (SEB; Sigma, UK) as a positive control, with ~100 CFU BCG (as per the MGIA protocol) and with 10 μg/ml CMV peptide pool (5 peptides, 2 μg/ml/peptides, ANASPEC, Fremont, CA, USA). The CMV peptide pool used is the same as the Fletcher *et al.* study [20]. The incubation with BCG was performed for 4 days and the addition of SEB and CMV was performed on Day 3. Two hours after the addition of SEB and CMV to the respective tubes, brefeldin A (Sigma, UK) was added to all tubes which were then incubated for 18 hours at 37^{o}C until Day 4. Data was acquired using an LSRII flow cytometer (BD Biosciences) and FACSDiva acquisition software (BD Biosciences). ICS flow cytometry data was analysed using FlowJo software version 10.4 (TreeStar Inc., Ashland, OR, USA). Samples were gated sequentially on singlet, live, $CD14^{-}CD19^{-}$, $CD3^{+}$ (lymphoid), $CD4^{+}$, $CD8^{+}$ cells and negative control stimulation tubes were used to set cytokine gates (see Supplementary Fig. S3, ICS gating).

Statistical analysis

To identify statistical significance of *ex vivo* growth inhibition (log CFU values) and ELISA responses, students *t*-test were used. Mann-Whitney *U* Test was performed to identify significant differences of the ELISpot, cell surface flow cytometry and ICS responses between groups. Spearman's correlation coefficient was used to test for correlations between growth inhibition and immune responses. A multiple comparison correction was included (Bonferroni), as indicated in each figure legend. Statistical analyses were performed in Graphad Prism 7 (GraphPad, La Jolla, CA, USA).

Results

Demographics of enrolled participants

One hundred participants were enrolled in the study; 37 vaccine-naïve volunteers with no history of BCG vaccination and 63 volunteers previously-vaccinated with BCG (average time since vaccination 29.4 years prior to enrolment). Table 1 summarises the characteristics of the study participants. Almost 70% of the BCG-vaccinated participants were from the UK.

Assessment of *ex vivo* growth inhibition and mycobacterial antigen-specific cytokine responses

The growth inhibition assay was performed to assess impact of historical BCG vaccination on *ex vivo* mycobacterial growth control. Using cryopreserved PBMCs, enhanced growth inhibition in PBMCs from BCG-vaccinated individuals was observed compared to vaccine-naïve individuals (median log CFU 1.680 and 2.027, $p<0.0001$, Figure 1A). The IFN-γ ELISpot assay was performed to measure the magnitude of the mycobacteria-specific response. The secretion of IFN-γ in response to PPD was elevated in samples from vaccinated individuals in comparison to unvaccinated individuals (median SFC 109.5 and 48, $p<0.0001$, Figure 1B). There was a significant inverse correlation between higher IFN-γ ELISpot response and lower mycobacterial growth ($p=0.022$, Spearman $r = -0.23$, Figure 1C).

Trends for higher production of Th1-type cytokines (IFN-γ, IP-10, TNF-α, IL-12) as well as GM-CSF were observed in the BCG-vaccinated group compared to the vaccine-naïve group (Table 2). There was a statistically significant correlation between higher IL-10 production and higher mycobacterial growth (Spearman $r = 0.37$, $p=0.0003$, Table 2). Meanwhile, historical BCG-vaccination was associated with significantly increased frequency of mycobacterial antigen specific IL-2^+ CD4 T-cells in the BCG-vaccinated group upon 4 days of stimulation with BCG ($p=0.008$, Supplementary Figure S4). Similar trends were observed with the frequencies of IFN-γ^+ as well as TNF-α^+ CD4 T-cells (Supplementary Figure S4). There were no significant correlations between the frequencies of BCG-specific CD4 and CD8 T-cells and mycobacterial growth, although the observed trends suggest that these cells may contribute to control of growth (Supplementary Table S1).

Associations between historical BCG vaccination and the frequency of circulating leukocyte subsets

Historical BCG vaccination was not associated with frequencies of circulating leukocytes in T-cell, NK cell and monocyte compartments (Supplementary Table S2). However, significant correlations were observed between the frequencies of NK cells and enhanced control of mycobacterial growth *ex vivo* in the naïve and BCG-vaccinated groups ($p<0.05$, Spearman's correlations, Table 3). In the BCG-vaccinated group, higher frequency of cytokine-producing NK cells was associated with reduced mycobacterial growth (Spearman r = -0.41, p=0.015, Figure 2A). A higher production of perforin was observed from the cells of BCG-vaccinated participants compared to naïve (p=0.018, Figure 2B). The production of perforin significantly correlated with enhanced growth inhibition (Spearman r = -0.44, p=0.013, Figure 2C and Supplementary Table S3), and the association was still significant when the correlation was performed in the BCG-vaccinated group only (Spearman r = -0.36, p=0.037, data not shown). Correlations with other measured NK cell associated markers (granzyme, IL-32, IL-22) did not reach significance (Supplementary Table S3).

Impacts of CMV-specific T-cell response and T-cell activation on *ex vivo* mycobacterial growth inhibition

CMV-specific T-cells producing IFN-γ^+ and TNF-α^+, notably in the CD8 compartment, were significantly associated with the frequency of T-cells expressing LAG3 and PD1 markers ($p<0.05$, Spearman's correlations, Table 4 and Figure 3 A-D). Historical BCG-vaccination was not associated with differences in CMV-specific response nor T-cell activation (Supplementary Table S2 and S4). However, T-cell activation was shown to correlate with higher growth of mycobacteria *ex vivo*, particularly in the naïve group (Figure 3 and Supplementary Table S5). $LAG3^+$ CD4 T-cells were significantly associated with growth of mycobacteria (p=0.047), with a similar trend for $LAG3^+$ CD8 T-cells (p=0.072) (Figure 3 F and I).

Impact of sex on *ex vivo* mycobacterial growth inhibition and cytokine responses, and its association with immune cell phenotype

In this study, we demonstrated that sex was associated with differences in immune response following historical BCG vaccination. First, BCG-vaccinated females exhibited a superior capacity to control mycobacterial growth when compared to males (p=0.029, Figure 4B). In contrast, males showed a trend towards higher IFN-γ response from PPD-stimulated PBMCs

as well as higher IP-10 production in the MGIA supernatant, both in naïve and BCG-vaccinated groups (Figure 4 C-F). Supplementary Table S6 summarises the sex comparisons of all measured cytokines from the MGIA supernatants.

In the BCG-vaccinated group, females had a higher frequency of cytokine-producing NK cells (p=0.018, Figure 5A). There was also a higher CD4/CD8 ratio in females compared to males in the naïve group (p=0.028, Figure 5B). Interestingly, there was a higher frequency of monocytes in males in the BCG-vaccinated group (p=0.049, Figure 5C), with a trend of higher monocyte-to-lymphocyte (ML) ratio in BCG-vaccinated males compared to females (p=0.08, Supplementary Table S7). In terms of T-cell activation, BCG-vaccinated females exhibited a lower frequency of $LAG3^{+}$ CD8 T-cells (p=0.0297, Figure 5D). While in the naïve group, females also had lower frequencies of activated CD8 T-cells expressing HLA-DR, LAG3 and PD1 ($p<0.05$, Supplementary Table S7). The lower frequencies of activated T-cells in females may be a consequence of lower CMV-specific CD8 T-cells response (Figure 5E and Supplementary Table S7).

Discussion

The present study reports that mycobacterial growth inhibition *ex vivo* is enhanced following historical BCG vaccination in adult healthy volunteers. In this study, the average time since BCG vaccination was 29.4 years prior to enrolment. Our results are in line with previous studies such as Fletcher *et al*. [24] and Prabowo *et al.* [32] which detected the impact of historical BCG vaccination after more than 20 years using the same PBMC-based MGIA. Most vaccinated individuals enrolled in our study are UK participants in which BCG vaccination is known to be effective [9]. A higher IFN-γ response was also observed in the BCG-vaccinated group compared to the naïve group using the ELISpot assay, reflecting the presence of mycobacterial-specific memory cells. Moreover, there was a significant correlation between IFN-γ response and lower mycobacterial growth. Several published MGIA studies reported increased IFN-γ production following BCG vaccination [24, 33, 34], and BCG-specific IFN-γ response measured by ELISpot assay is known to be associated with reduced TB disease risk following BCG vaccination in infants [18]. The ELISpot assay measures all cells that secrete IFN-γ in response to antigen stimulation, including NK cells and γδ T-cells in addition to conventional T-cells. Focusing on the conventional T-cells response, in our study, we did not observe a significant association between Th1-type

cytokine-expressing T-cells and *ex vivo* mycobacterial growth inhibition. This finding was in contrast with the study of Smith *et al*. [25] which showed an association between MGIA control capacity and the frequency of polyfunctional CD4 T-cells using studying a small cohort of BCG-vaccinated infants. However, our results were consistent with the finding of Joosten *et al.* [26] using the same PBMC-based MGIA, as well as with a study by Kagina *et al*. [35] which showed no association between polyfunctional T-cells and the risk to develop TB disease following BCG vaccination.

We also observed trends of higher Th1-type cytokines in the MGIA supernatants from BCG-vaccinated participants compared to the naïve. Interestingly, there was a strong significant correlation between IL-10 production and reduced control of mycobacterial growth. This observation replicates earlier findings, in which IL-10 was associated with reduced *ex vivo* growth inhibition, and was significantly predictive of mycobacterial growth through inhibition of other pro-inflammatory cytokines [36, 37]. IL-10 is known to have immunosuppressive activity by inhibiting T-cell proliferation and IFN-γ production, leading to reduced macrophage activation [38]. The capacity of individuals to produce IL-10 may need to be considered when assessing TB vaccine effects in clinical trials.

In this study, the frequency of NK cells – in particular cytokine-producing NK cells – is associated with enhanced *ex vivo* mycobacterial growth inhibition following historical BCG vaccination. This may account for our correlation between IFN-γ ELISpot response and control of mycobacterial growth as IFN-γ secreting NK cells will be measured in addition to CD4 and CD8 positive T-cells. Our results again support a recent finding, in which a greater frequency of putative cytokine-producing $CD16^-$ NK cells was associated with reduced mycobacterial growth in the multiple regression analysis of MVA85A correlate of risk study [36, 39]. Cytokine-producing NK cells are the main source of NK-cell derived cytokines such as IFN-γ, TNF-α and GM-CSF [40], which were modestly increased in the MGIA supernatants of the BCG-vaccinated group in our study. Initially, cytotoxicity and cytokine-producing functions of NK cells were regarded as two distinct functions with little synergy between them [40, 41]. However, it was recently shown that IFN-γ and TNF-α could synergistically enhance NK cell cytotoxicity [42]. In our study, cells obtained from BCG-vaccinated participants produced a higher level of perforin and the secretion of this lytic granules was associated with enhanced growth inhibition.

Although considered a component of innate immune system, an emerging body of evidence has revealed that NK cells can also behave in a memory-like manner following infection or vaccination [reviewed in [43, 44]]. NK cells isolated from pleural fluid express the memory marker CD45RO and produce higher amounts of IFN-γ and IL-22 in response to stimulation with IL-12, IL-15 and BCG when compared with $CD45RO^-$ cells [45, 46]. Even though NK cells do not have antigen receptors generated by genetic rearrangement, they possess receptors which allow direct antigenic contact, resulting in subsequent cellular activation [44]. This process will generate antigen-specific NK cells, which lead to enhanced response following re-exposure with the same stimulus [47, 48]. In addition, work by Kleinnijenhuis *et al.* reveals that BCG vaccination promotes augmented secondary responses towards the same and unrelated stimulus through trained innate immunity mechanism [49]. The growth inhibition assay has recently been shown to be able to detect contribution from the trained innate immune compartment, following *Mtb* exposure or BCG vaccination, by the role of nonclassical monocytes [26]. Our present study has shown the additional contribution of NK cells to *ex vivo* mycobacterial growth control, and in line with this, recent clinical trials also reported that immune cells associated with protection from TB disease and after BCG vaccination were not T-cells, but IFN-γ–producing NK cells [50, 51].

Furthermore, we have demonstrated that a CMV-specific response may be associated with T-cell activation, in particular in the CD8 compartment, and this activation is correlated with mycobacterial growth *ex vivo*. In HIV, T-cell activation has been established as a risk factor for acquisition of infection as well as progression from infection to disease [52-54]. In TB, evidence has emerged denoting the role of CMV and T-cell activation on TB disease risk [18, 20], and our study is the first the show such association with *ex vivo* mycobacterial growth. In this study, we chose to measure CMV-specific T-cell cytokine response with ICS flow cytometry rather than with serology, as evidence in the literature showed that CMV-antibody levels do not correlate with the size of the T-cell response against CMV and the ICS method is more sensitive for detection of CMV-specific cytokine-producing T-cells [55, 56]. Unfortunately in this study due to a technical limitation, we were unable to perform CMV serology in our cohort samples. CMV infection is recognised to drive the expansion of $NKG2C^+$ NK cells [57], which do not respond well to cytokine stimulation discussed above [58, 59]. Further studies are required to better understand the interplay between CMV-specific response, T-cell activation and NK cells in the context of BCG vaccination.

Differences in TB disease notification rates between the sexes are well documented and thought to be a result of biological factors, in addition to social factors [11, 14, 60]. Therefore, it is of interest that our study demonstrated a higher capacity of BCG-vaccinated females to control mycobacterial growth *ex vivo* compared to males. In conjunction to this data, we found that females had a higher frequency of cytokine-producing NK cells, and lower frequency of activated T-cells as well CMV-specific response. In addition, females also had a lower monocyte frequency, with a trend of a lower ML ratio compared to males. Altogether, these individual-level factors appear to contribute to the enhanced growth inhibition in females following BCG vaccination. Such sex specific effect has also been observed with measles and smallpox vaccines, where females are more protected than males following vaccination [61, 62]. The epidemiological observation that the sex bias in TB does not arise until puberty has suggested the important role of sex hormones [11]. In general, testosterone is considered to downregulate the Th1 response, whereas estrogen is believed to enhance it [14]. Moreover, genetic or epigenetic differences between sex may also play a role as well in the observed sex-differential protective effect [13].

In summary, we have demonstrated the impact of individual-level factors on *ex vivo* mycobacterial growth inhibition in a cohort of healthy, adult volunteers. Our results indicate that immune cell phenotype, cytomegalovirus-specific response and sex have impacts on immunity following BCG vaccination. These *ex vivo* observations are reflective of epidemiological data and published human studies, and such impacts may need to be considered when testing TB vaccine candidates in trial populations. Importantly, researchers should consider the impact of sex in clinical vaccine studies, as the impact of sex in infectious diseases is common but often neglected [63]. The MGIA assay offers an *ex vivo* testing platform for assessment of a wide range of candidate TB vaccines, either using BCG or virulent *Mtb* as the immune target, with the ability to reflect inter-individual variation which may be important for vaccine effectiveness. The *ex vivo* MGIA is therefore an important additional tool for the TB vaccine community and should continue to be assessed for its ability to act as a correlate of vaccine-induced protection.

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Author Contributions

SP conceived and planned the experiments, supervised by HF. SP performed laboratory work and analysed the results supervised by HF, KS and SS. SP wrote the first draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interest: All authors declare no competing interests.

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583 **Tables**

584
585
586 **Table 1.** Characteristics of study participants.
587

Characteristic	Total Participants : 100	
	Naïve (n = 37)	BCG Vaccinated (n = 63)
Female [no. (%)]	28 (75.7 %)	42 (66.7 %)
Median age [yr (range)]	31 (23 – 70)	39 (24 – 80)
Average time since BCG vaccination [yr (range)]	-	29.4 (10 – 58)
Country of Origin UK [no. (%)]	8 (21.6 %)	44 (69.8 %)

588

589 **Table 2. Summary of mean cytokine responses measured with ELISA assays,** assessed
590 from MGIA supernatant samples after 4 days of co-culture. Comparisons were made between
591 naïve and BCG-vaccinated groups (unpaired t-test), the values indicate mean of concentration
592 in pg/ml [95% CI]. Correlations were assessed with *ex vivo* mycobacterial growth among
593 responders (Spearman's correlation). A p value <0.05 was considered statistically significant
594 (in bold), and after a multiple testing correction only values with p <0.0063 were considered
595 significant (underlined). n= 37 BCG-naïve and n=63 BCG-vaccinated participants.
596 ***p<0.001.

Cytokine (pg/ml)	Comparison			Correlation with *ex vivo* mycobacterial growth	
	Naïve	BCG-vaccinated	p-value	r	p-value
IFN-γ	12.47 [8.245-16.69]	23.37 [10.8-35.94]	0.1962	-0.027	0.8432
IP-10	111.7 [55.42-168]	204.5 [112.1-297]	0.1505	0.19	0.1158
TNF-α	37.97 [2.547-73.4]	97.98 [38.61-157.4]	0.1471	-0.35	0.0558
IL-12	27.6 [3.033-52.17]	63.61 [20.83-106.4]	0.2299	-0.23	0.3158
IL-10	52.55 [31.07-74.03]	59.99 [36.56-83.41]	0.6688	**0.37*** **	**0.0003**
GM-CSF	7.729 [-1.688-17.15]	88.54 [26.19-150.9]	0.0512	-0.37	0.1552
IL-6	356.7 [246.1-467.3]	315 [236.5-393.4]	0.5293	0.071	0.5449
IL-17	0.00 [0.00-0.00]	0.1596 [0.00-0.4083]	0.3291	-0.13	0.2141

597

598 **Table 3.** Correlation of immune cell frequencies in peripheral blood and *ex vivo*
599 mycobacterial growth inhibition. Assessment was performed from 16 BCG-naïve and 34
600 BCG-vaccinated participants. Correlations were performed from a total of 50 participants, as
601 well as from each naïve and BCG-vaccinated groups respectively (Spearman's correlation). A
602 p value <0.05 was considered statistically significant (in bold), and after a multiple testing
603 correction only values with p <0.0031 were considered significant (underlined). Note: The
604 ML ratio was obtained by dividing the percentage of monocytes by the sum of the
605 percentages of T- and B-cells. The NK cell ratio was obtained by dividing the percentage of
606 cytokine-producing by cytotoxic NK cells. *p<0.05, **p<0.01.

Leukocyte subsets	Correlation with *ex vivo* mycobacterial growth					
	All participants		Naïve		BCG-vaccinated	
	r	p-value	r	p-value	r	p-value
T-cells	-0.068	0.6367	0.29	0.2708	-0.30	0.0866
CD4 T-cells	-0.041	0.7764	0.17	0.5172	-0.091	0.6080
CD8 T-cells	0.24	0.0938	0.36	0.1714	0.093	0.6011
CD4/CD8 ratio	-0.16	0.2718	-0.20	0.4579	-0.058	0.7448
NK cells	-0.27	**0.0593**	**-0.71****	**0.0028**	-0.19	0.2833
Cytokine NK cell	-0.26	0.0702	-0.47	**0.0679**	**-0.41***	**0.0147**
Cytotoxic NK cell	-0.25	0.0814	**-0.64****	**0.0093**	-0.19	0.2699
NK cell ratio	-0.2	0.1602	-0.35	0.1866	-0.087	0.6241
Monocytes	0.12	0.4244	-0.0088	0.9758	0.13	0.4638
ML ratio	0.064	0.6609	-0.044	0.8714	0.083	0.6390
M1 monocytes	-0.076	0.5993	-0.28	0.2867	-0.031	0.8610
M2 monocytes	-0.16	0.2784	-0.16	0.5458	-0.12	0.4978
M1/M2 ratio	0.059	0.6831	-0.17	0.5283	0.15	0.3939
$CD64^+$ monocytes	-0.063	0.6659	-0.29	0.2664	0.028	0.8759
$CD123^+$ monocytes	-0.072	0.6169	-0.27	0.3025	0.015	0.9313
Suppressor monocytes	0.21	0.1414	0.31	0.2381	0.089	0.6149

Table 4. Correlation of CMV-specific T-cell responses and T-cell activation. Associations were investigated from 3 different subsets of CMV-specific $cytokine^+$ T-cells producing IFN-γ^+, IL-2^+ or TNF-α^+, respectively. Three markers were used for T-cell activation: HLA-DR, LAG3 and PD1. A p value <0.05 was considered statistically significant (in bold), and after a multiple testing correction only values with p <0.0083 were considered significant (underlined) (Spearman's correlation). n=50 participants, consisted of 16 BCG-naïve and n=34 BCG-vaccinated participants. *p<0.05, **p<0.01.

CMV-specific $cytokine^+$ T-cells	Correlation with activated T-cells					
	$HLA\text{-}DR^+$ CD4 T-cells		$LAG3^+$ CD4 T-cells		$PD1^+$ CD4 T-cells	
	r	p-value	r	p-value	r	p-value
IFN-γ^+ CD4 T-cells	0.026	0.8748	-0.004	0.9805	0.20	0.2112
IL-2^+ CD4 T-cells	-0.045	0.7823	-0.056	0.7310	-0.0082	0.9601
TNF-α^+ CD4 T-cells	0.054	0.7401	0.058	0.7239	0.091	0.5757
	$HLA\text{-}DR^+$ CD8 T-cells		$LAG3^+$ CD8 T-cells		$PD1^+$ CD8 T-cells	
IFN-γ^+ CD8 T-cells	0.31	0.0552	**0.39***	**0.0140**	**0.44****	**0.0049**
IL-2^+ CD8 T-cells	-0.087	0.5917	0.0024	0.9885	-0.15	0.3609
TNF-α^+ CD8 T-cells	0.28	0.0799	**0.35***	**0.0281**	**0.33***	**0.0375**

Figure Legends

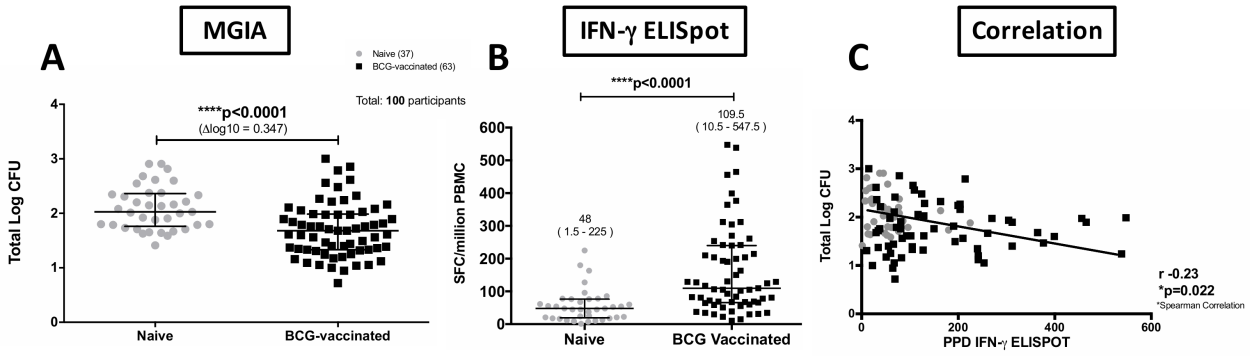
Figure 1. Growth inhibition and immune responses following historical BCG vaccination. Assessment was performed from 37 BCG-naïve and 63 BCG-vaccinated participants. **(A)** Growth inhibition was compared using BCG input ~ 100 Colony Forming Unit (CFU) as immune target (unpaired t-test). Data is presented as total number of log CFUs per sample, which was determined by use of a standard curve. **(B)** IFN-γ production from PBMC following stimulation with PPD was compared (Mann-Whitney test). Numbers above each group represent median (range). SFC, spot forming cells. **(C)** The correlation between *ex vivo* growth inhibition and PPD-specific IFN-γ response was assessed (Spearman's correlation). A p value <0.05 was considered statistically significant. Dots and squares represent individual data points, and the central lines indicate the median response with inter-quartile range (IQR). ****p<0.0001.

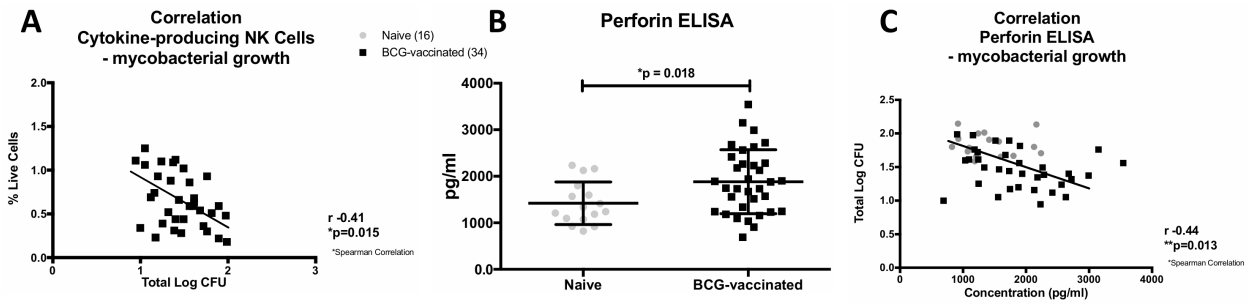
Figure 2. NK cells correlations. A higher frequency of cytokine-producing NK cells ($CD56^{bright}$ $CD16^{+/-}$) correlated with enhanced *ex vivo* mycobacterial growth inhibition (Spearman's correlation) **(A)**. A perforin ELISA was performed from MGIA supernatants and the response was compared between vaccination groups (unpaired t-test) **(B)**. The production of perforin was associated with enhanced *ex vivo* growth inhibition (Spearman's) **(C)**. A p value <0.05 was considered statistically significant. *p<0.05, **p<0.01.

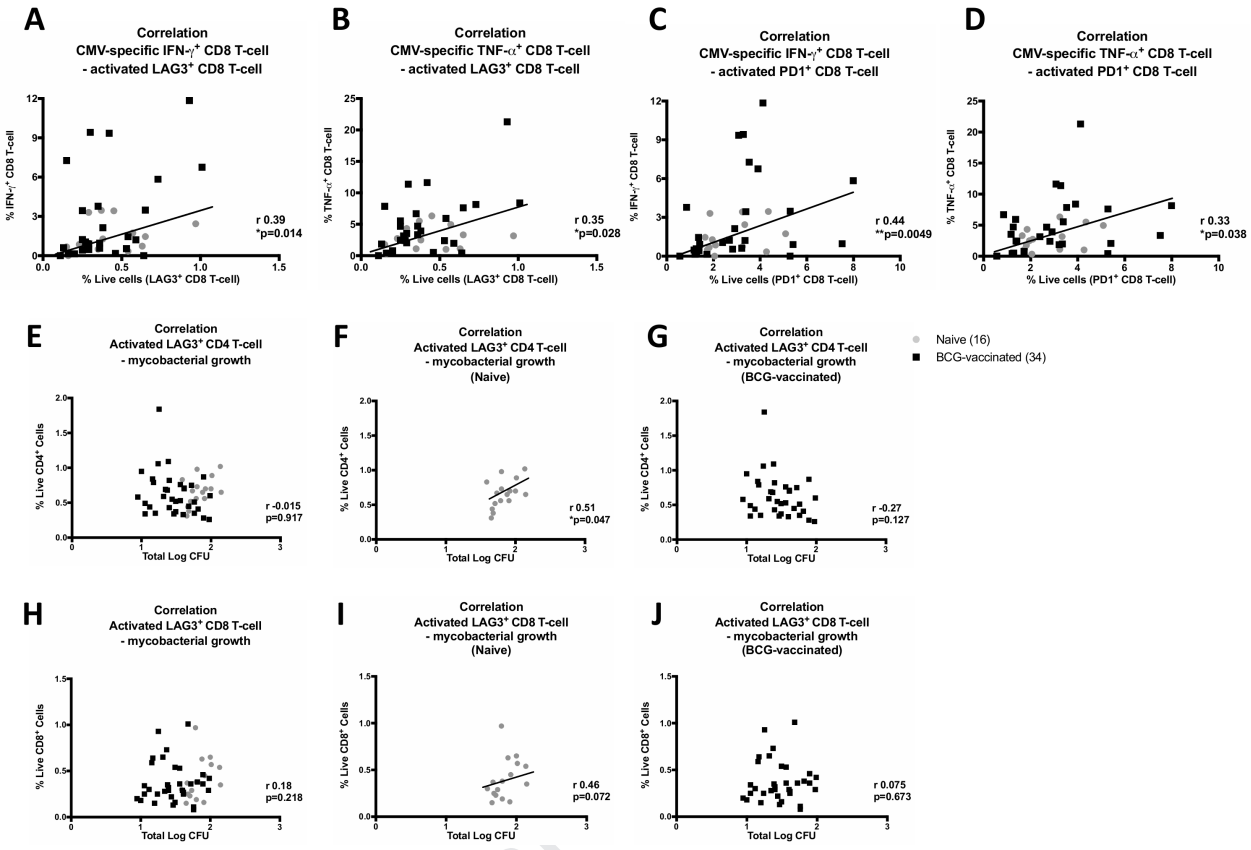
Figure 3. CMV-specific responses were associated with higher CD8 T-cell activation, expressing markers LAG3 (**A-B**) and PD1 (**C-D**) respectively. Activated CD4 and CD8 T-cells (**E-J**) were correlated with higher growth of mycobacteria, notably in the naïve groups (**F, I**). A p value <0.05 was considered statistically significant (Spearman's correlation). n=50 participants, consisted of 16 BCG-naïve and 34 BCG-vaccinated participants. *p<0.05, **p<0.01.

Figure 4. Sex impact on growth inhibition and immune responses following historical BCG vaccination. Assessment was performed from 37 BCG-naïve **(A,C,E)** and 63 BCG-vaccinated participants **(B,D,F)**. (A-B) Growth inhibition was compared between sex and data was presented as total number of log CFUs per sample (unpaired t-test). (C-D) IFN-γ production from PBMC following stimulation with PPD was compared (Mann-Whitney test). Numbers above each group represent median (range). SFC, spot forming cells. (E-F) IP-10 was measured from MGIA supernatants using ELISA assay (mean, unpaired t-test). Dots and squares represent individual data points, and the central lines indicate the median response with IQR. *p<0.05.

Figure 5. Comparison by sex of immune cells phenotype (A-C), T-cell activation (D) and CMV-specific T-cell response (E). Assessment was performed from 16 BCG-naïve and 34 BCG-vaccinated participants. The box plots show the minimum and maximum values (ends of the whiskers), the median (band near the middle of the box) and interquartile ranges. Blue and red colour represent males and females, respectively. A p value <0.05 was considered statistically significant (Mann-Whitney). *p<0.05, **p<0.01, ****p<0.0001.



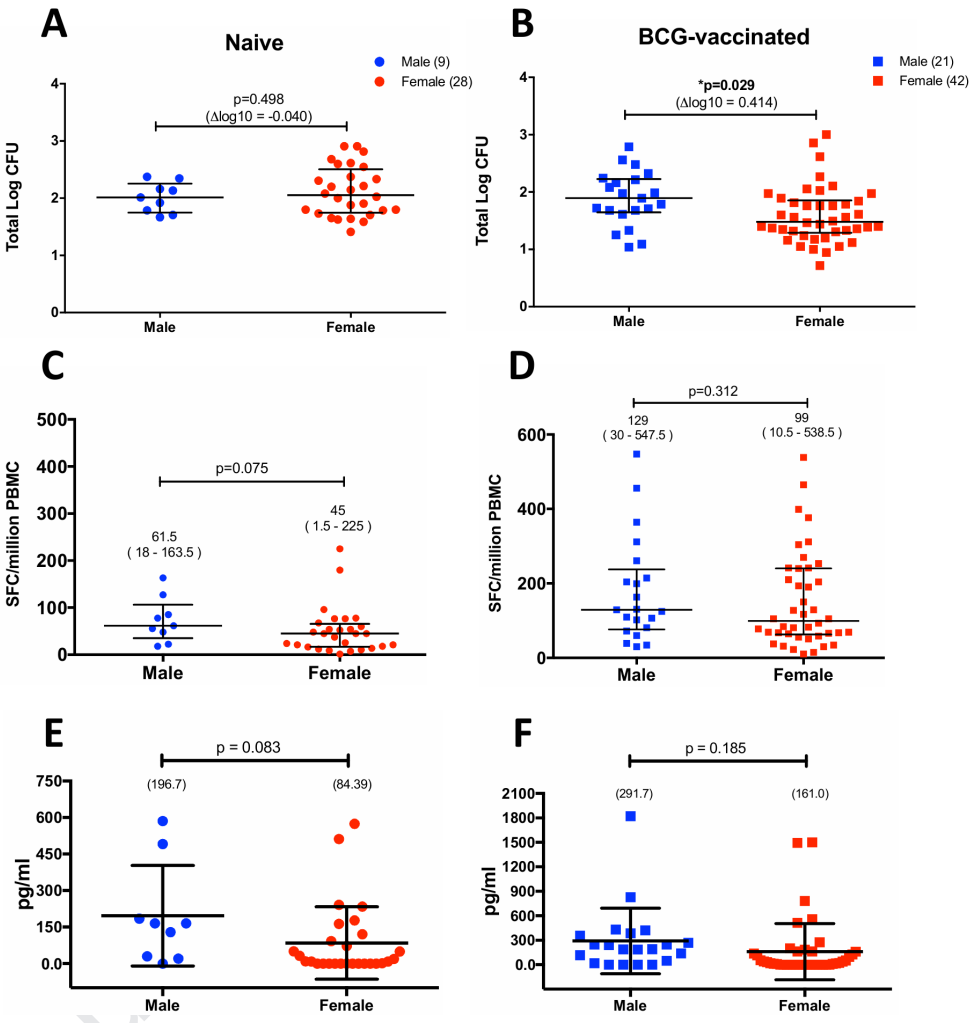


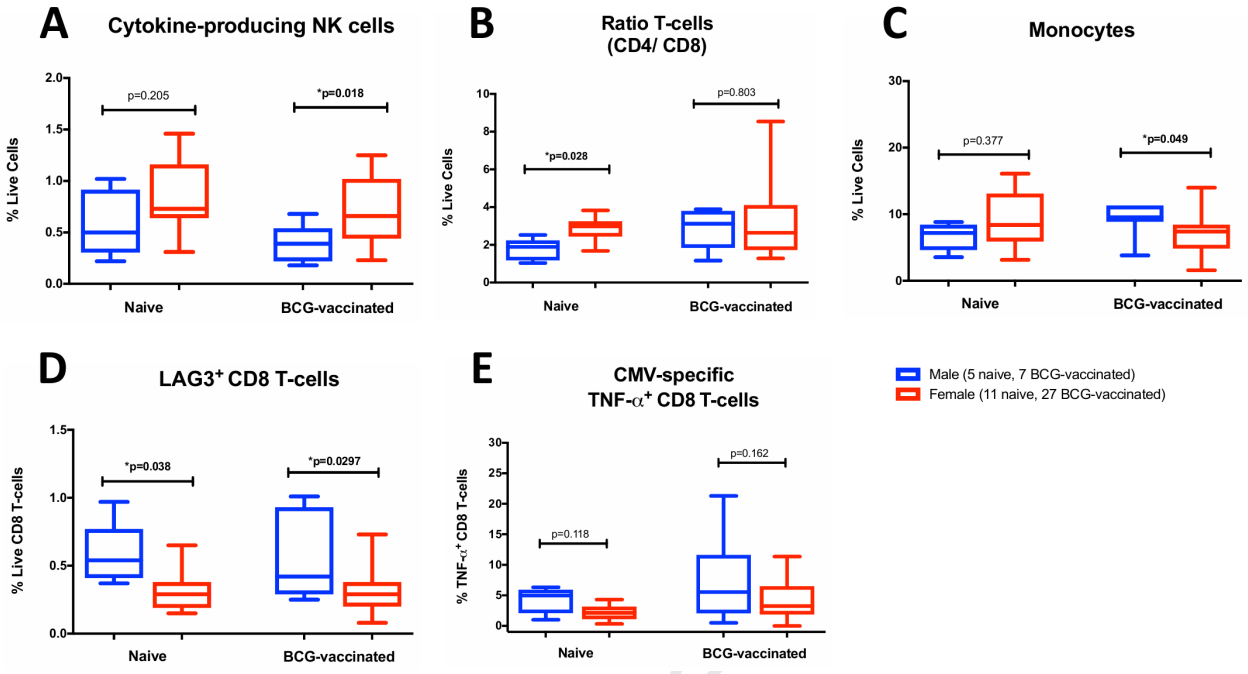

A
Correlation
CMV-specific IFN-γ+ CD8 T-cell
- activated LAG3+ CD8 T-cell
% IFN-γ+ CD8 T-cell
% Live cells (LAG3+ CD8 T-cell)
r 0.39
*p=0.014
B
Correlation
CMV-specific TNF-α+ CD8 T-cell
- activated LAG3+ CD8 T-cell
% TNF-α+ CD8 T-cell
% Live cells (LAG3+ CD8 T-cell)
r 0.35
*p=0.028
C
Correlation
CMV-specific IFN-γ+ CD8 T-cell
- activated PD1+ CD8 T-cell
% IFN-γ+ CD8 T-cell
% Live cells (PD1+ CD8 T-cell)
r 0.44
**p=0.0049
D
Correlation
CMV-specific TNF-α+ CD8 T-cell
- activated PD1+ CD8 T-cell
% TNF-α+ CD8 T-cell
% Live cells (PD1+ CD8 T-cell)
r 0.33
*p=0.038
E
Correlation
Activated LAG3+ CD4 T-cell
- mycobacterial growth
% Live CD4+ Cells
Total Log CFU
r -0.015
p=0.917
F
Correlation
Activated LAG3+ CD4 T-cell
- mycobacterial growth
(Naive)
% Live CD4+ Cells
Total Log CFU
r 0.51
*p=0.047
G
Correlation
Activated LAG3+ CD4 T-cell
- mycobacterial growth
(BCG-vaccinated)
% Live CD4+ Cells
Total Log CFU
r -0.27
p=0.127
Naive (16)
BCG-vaccinated (34)
H
Correlation
Activated LAG3+ CD8 T-cell
- mycobacterial growth
% Live CD8+ Cells
Total Log CFU
r 0.18
p=0.218
I
Correlation
Activated LAG3+ CD8 T-cell
- mycobacterial growth
(Naive)
% Live CD8+ Cells
Total Log CFU
r 0.46
p=0.072
J
Correlation
Activated LAG3+ CD8 T-cell
- mycobacterial growth
(BCG-vaccinated)
% Live CD8+ Cells
Total Log CFU
r 0.075
p=0.673

MGIA

IFN-γ ELISpot

IP-10 ELISA




A
Cytokine-producing NK cells
% Live Cells
p=0.205
*p=0.018
Naive
BCG-vaccinated
B
Ratio T-cells
(CD4/ CD8)
% Live Cells
*p=0.028
p=0.803
Naive
BCG-vaccinated
C
Monocytes
% Live Cells
p=0.377
*p=0.049
Naive
BCG-vaccinated
D
LAG3+ CD8 T-cells
% Live CD8 T-cells
*p=0.038
*p=0.0297
Naive
BCG-vaccinated
E
CMV-specific
TNF-α+ CD8 T-cells
% TNF-α+ CD8 T-cells
p=0.118
p=0.162
Naive
BCG-vaccinated
Male (5 naive, 7 BCG-vaccinated)
Female (11 naive, 27 BCG-vaccinated)