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Minimal Sex-Differential Modulation of Reactivity to Pathogens and Toll-Like Receptor Ligands following Infant Bacillus Calmette–Guérin Russia Vaccination

Fatoumatta Darboe¹, Jane U. Adetifa¹, John Reynolds², Safayet Hossin¹, Magdalena Plebanski³,⁴, Mihai G. Netea⁵, Sarah L. Rowland-Jones⁵,⁶, Jayne S. Sutherland¹ and Katie L. Flanagan¹,³,⁷*¹

1Vaccines and Immunity Theme, MRC Unit, Fajara, Gambia, 2Biostatistics Consulting Platform, Faculty of Medicine, Nursing and Health Sciences, Monash University, Prahran, VIC, Australia, 3Department of Immunology and Pathology, Monash University, Prahran, VIC, Australia, 4Monash Institute of Medical Engineering, Monash University, Prahran, VIC, Australia, 5Department of Internal Medicine, Radboud Center for Infectious Diseases, Nijmegen University Nijmegen Medical Center, Nijmegen, Netherlands, 6Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom, 7School of Medicine, University of Tasmania, Hobart, TAS, Australia

Bacillus Calmette–Guérin (BCG), the only licensed vaccine against tuberculosis, has been shown to provide heterologous protection against unrelated pathogens and enhance antibody responses to several routine expanded program on immunization (EPI) vaccines. Understanding these heterologous effects is important for the development of optimal vaccination strategies. We set out to assess the effect of vaccination with BCG Russia of 6-week-old infants on in vitro reactivity to a panel of toll-like receptor (TLR) agonists (TLR2, 4, and 7/8) and heat-killed pathogens [Streptococcus pneumoniae, Candida albicans (CA), and Escherichia coli], and antibody responses to other EPI vaccines compared to BCG naïve infants. We observed no effect of BCG vaccination on innate (TNF-α) or Th2 (IL-4) cytokine responses, but found enhanced CA-specific CD8+IFN-γ+ responses in BCG vaccinated males and females 1 week after vaccination and decreased IFN-γ:IL4 ratio to SP in females. By 12 weeks (but not 1 week) of post-vaccination, there was significant downmodulation of Th1 cytokine responses in BCG vaccinated infants; and TLR-stimulated IL-10 and IL-17 responses declined in BCG vaccinated females but not males. Significant changes also occurred in the BCG naïve group, mainly at 18 weeks, including decreased Th1 and increased IL-10 responses. The effects at 18 weeks were most likely a result of immune modulation by the intervening EPI vaccines given at 8, 12, and 16 weeks of age. There was no effect of BCG vaccination on EPI antibody levels at either time point. Taken together, our results support minimal early heterologous immune modulation by BCG Russia vaccination that did not persist 12 weeks after vaccination.

Keywords: vaccine, toll-like receptors, non-specific effects, heterologous effects, cytokines, innate immunity, adaptive immunity
INTRODUCTION

Tuberculosis (TB) is a significant public health problem with approximately two billion people infected globally (1). Bacillus Calmette–Guérin (BCG) is the only licensed vaccine against TB, and while it provides good protection against disseminated TB in childhood, it has poor efficacy against adult pulmonary TB (2, 3). Several reasons have been given for the variability in efficacy throughout the world, including, but not limited to, different strains of BCG vaccine (4–6), geographical location (7), and age at vaccination (8, 9).

Neonates have Th2-biased immunity and are thus susceptible to microbial infections requiring Th1 immunity for clearance, such as TB (10–12). Despite this, BCG vaccination at birth can induce a robust adaptive immune response, inducing poly-functional Th1 cells (13–15) and Th17 cells (8, 16–18). Besides inducing a classical antigen-specific T cell response, BCG vaccination also induces an innate immune response involving dendritic cell activation, natural killer (NK) and NK T cells, and neutrophils (19–22). The innate immune system plays a critical protective role against infectious diseases throughout life, inducing a rapid immunological response against invading pathogens prior to the generation of adaptive immunity. This is particularly important in early life when adaptive immunity is poorly developed.

Toll-like receptors (TLRs) expressed by host immune cells recognize pathogen-associated molecular patterns (PAMPS), required for microbial recognition by macrophages and dendritic cells (23, 24). TLR ligands have been used in murine models as vaccine adjuvants, leading to enhanced Th1 responses (25). Certain TLR polymorphisms have been associated with an increase in BCG induced Th-1 type responses in whole blood 10 weeks after BCG vaccination in South African infants (26). Furthermore, BCG has been shown to induce epigenetic reprogramming of innate immune cells toward a pro-inflammatory profile characterized by increased IFN-γ, TNF-α, and IL-1β in both murine models and adult European individuals (27). These studies showed that innate cells exhibit characteristics of adaptive immunity or adaptation to secondary infection in a process termed “trained immunity.” This immune training by BCG vaccination was further able to enhance innate immunity to other unrelated pathogens including Candida albicans (CA), Staphylococcus aureus, and Streptococcus pneumoniae (SP) (27).

Epidemiological studies dating back to the 1940s have suggested that BCG vaccination provides protection against unrelated infections, and vaccination is associated with a decrease in all-cause mortality in certain settings (28). A meta-analysis of clinical trial data from the US and UK in the 1940s and 1950s estimated a 25% reduction from diseases other than TB when BCG was given (29). Randomized controlled trials in Guinea Bissau have shown that BCG vaccination of low birth weight neonates is associated with an almost 50% reduction in all-cause mortality in the first few weeks of life, mainly from unrelated infections (30). In an analysis of almost half a million childhood hospitalization episodes in the official Spanish registry, BCG vaccination was associated with a 32.4–66.6% reduction in hospitalization due to respiratory infection, and a 52% reduction in sepsis in <1-year olds (31). Similarly, data from two large cohorts of children (n = 58,021 and 93,301) from 19 countries found a 17–37% reduction in acute lower respiratory tract infection in BCG vaccinated children (32). Other evidence for non-specific effects of BCG include its widespread use as a therapeutic agent against bladder cancer (33) and as an adjunctive therapy for malignant melanoma (34). BCG has also been shown to boost antibody responses to other expanded programs on immunization (EPI) vaccines supporting an adjuvant effect in humans (15, 35).

Interestingly, non-specific effects of vaccines have been shown to manifest differently in the sexes, with females generally being more susceptible than males to the non-specific effects of vaccination (36–38). Indeed, the protection against death in the low birth weight studies occurred in the first 3 days of life in males and after 1 week in females, suggesting different mechanisms (30). Despite these important findings, the underlying immunological mechanisms are still not understood.

Herein, we conducted a randomized trial aimed at assessing the effect of BCG vaccination of 6-week-old Gambian infants on in vitro responses to toll-like receptor (TLR) ligands, unrelated pathogens, and antibody responses to EPI vaccines compared to a BCG naïve control group. Our results support early enhanced CA-specific IFN-γ responses in BCG vaccinated infants but no effect on TNF-α or EPI vaccine antibody responses. Our findings contribute to the relatively scarce evidence for heterologous immunological effects of BCG vaccination in humans.

MATERIALS AND METHODS

Study Site and Subject Recruitment

Expectant mothers, presenting at the Sukuta Health Centre (a peri-urban setting in The Gambia) for antenatal care were sensitized about the study. Their children were recruited into the study upon birth or when they presented for BCG vaccination (within 48 h of birth). BCG vaccination was withheld for those that agreed to participate (Table 1). Exclusion criteria included low birth weight (<2.5 kg), congenital defects, and multiple births.

### Table 1 | Vaccines given throughout the study.

<table>
<thead>
<tr>
<th>BCG group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
<td>OPV, HepB</td>
</tr>
<tr>
<td>6 weeks</td>
<td>BCG</td>
</tr>
<tr>
<td>8 weeks</td>
<td>Penta 1 (Hib, HepB, DTwP), PCV-13, OPV</td>
</tr>
<tr>
<td>12 weeks</td>
<td>Penta 2 (Hib, HepB, DTwP), PCV-13, OPV</td>
</tr>
<tr>
<td>16 weeks</td>
<td>Penta 3 (Hib, HepB, DTwP), PCV-13, OPV</td>
</tr>
<tr>
<td>18 weeks</td>
<td>TST</td>
</tr>
</tbody>
</table>

All vaccines given to study infants from birth to the end of the study at 18 weeks of age. OPV, oral polio vaccine; HBV, hepatitis B vaccine; BCG, bacillus Calmette–Guérin vaccine; Pentax, pentavalent vaccine; PCV-13, 13-valent pneumococcal conjugate vaccine; DTwP, diphtheria-tetanus-whole cell pertussis vaccine; Hib, Haemophilus influenzae group b vaccine; TST, tuberculin skin test.
Ethics Statement
The Scientific Coordinating Committee (SCC) at the Medical Research Council (MRC) Unit, The Gambia and the MRC-Gambian Government Joint Ethics Committee approved this study (study number SCC 1233). Written informed consent was provided by the parent/guardian of participating infants in accordance with the Declaration of Helsinki.

Blood Sampling and Vaccination
Infants were randomized at 6 weeks of age into one of two groups: 0.1 mL intradermal vaccination with BCG vaccine (Russian strain, SSI India) (BCG group) or no vaccination (Control group) to be given at 6 weeks of age. All infants received hepatitis B vaccine (HepB) (Serum Institute of India) and oral polio vaccine (OPV) (Sanofi Pasteur, France) at birth in accordance with the EPI schedule of The Gambia. At 8, 12, and 16 weeks, all infants received the pentavalent [diphtheria–tetanus–whole cell pertussis, Haemophilus influenzae B (Hib), HepB] vaccine (Panacea biotech, India), OPV, and the 13-valent pneumococcal conjugate vaccine (PCV-13) (Table 1). At 18 weeks of age, all infants were tested by standard tuberculin skin test (TST) for mycobacterial reactivity by injecting 0.1 mL containing 2 U of tuberculin (Serum Staten Institut, Denmark) intradermally. Induration was read longitudinally and transversely by a trained nurse at 48–72 h to determine the average induration. The infants in the BCG naïve control group were then vaccinated with BCG at 18 weeks of age. A maximum of 5 mL of venous whole blood was collected into EDTA tubes at 6 weeks (prior to BCG vaccination of group 1) [Visit 1 (V1)], 7 weeks (1 week post-BCG vaccination) [Visit 2 (V2)], and 18 weeks (12 weeks post-BCG vaccination) [Visit 3 (V3)] (Figure 1).

Peripheral Blood Mononuclear Cell (PBMC) Separation and Cryopreservation
EDTA blood was spun at 1,500 rpm for 10 min and 1 mL of plasma collected and stored at −20°C until use. The remaining whole blood was diluted 1:1 with RPMI (Sigma, UK) and PBMC separation was performed via density gradient centrifugation

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**FIGURE 1** | Participant numbers and drop outs. Numbers of study infants throughout the study from recruitment at birth, to enrollment and randomization, and follow-up until 18 weeks of age. Infants were bled at three time points—Visit 1 (V1) at 6 weeks of age (baseline), visit 2 (V2) at 7 weeks (+1 week) and visit 3 (V3) at 18 weeks of age (+12 weeks).
on a Lymphoprep (Fresenius Kabi Norge AS, Norway) cushion. Harvested PBMCs were washed twice in RPMI and cells counted using Trypan blue exclusion. For every 10 million live cells, 500 µL of fetal calf serum (FCS) (Gibco, Life technologies, USA) was added, cells were left on ice for 30 min and 500 µL of 20% dimethyl sulfoxide (Sigma-Aldrich, UK) in FCS was added before cells were transferred into a Nalgene® Mr Frosty container (Sigma-Aldrich) and stored at −70°C then transferred to liquid nitrogen within 24 h.

**Peripheral Blood Mononuclear Cells**

Peripheral Blood Mononuclear Cells were thawed rapidly at 37°C in a water bath. Cells were then resuspended slowly in warm 10% FCS in RPMI (R10) prior to washing twice. Viability counts were done on the samples before being resuspended in R10 and 25 U/mL of benzonuclease (Sigma, UK). PBMCs were then resuspended at 37°C 5% CO₂ for 6 h.

**In Vitro Culture Conditions**

0.1 × 10^6 PBMCs in 100 µL of culture medium (2% penicillin/streptomycin, 1% l-glutamine, and 10% FCS in RPMI) were stimulated in 96-well round bottom plates for 16 h with the following antigens: purified protein derivative (PPD) (10 µg/mL, Serum Staten Institute, Denmark), heat-killed *Listeria monocytogenes* (HKLM) (TLR2 agonist; 10^6/mL, Invivogen), lipopolysaccharide (LPS) (TLR4 ligand; 1 µg/mL, Invivogen), CLO-75 (TLR7/8 agonist; 10 µg/mL, Invivogen); and heat-killed *Streptococcus pneumonia* (SP) (10^6 cells/mL), *C. albicans* (10^6 cells/mL), or *E. coli* (10^6 cells/mL) per test. Cells were incubated at 37°C 5% CO₂ for 18 h with Brefeldin A (10 µg/mL) added after the first 2 h of incubation. Cells were stained with a live/dead aqua yellow stain (Invitrogen, USA), incubated for 10 min at room temperature before staining with a surface marker cocktail consisting of either CD3-PerCP Cy5.5 or CD3 APC efluor 750 and CD8 APC efluor 780. Cells were incubated for 30 min at room temperature and washed with 1 mL of PBS/1% FCS/0.2% Sodium Azide (FACS buffer). Supernatant was removed and cells washed in perm/wash solution (Ebioscience, UK). Permeabilization was then performed using fix/perm solution (Ebioscience) and cells incubated for 20 min at 4°C before centrifugation at 1,800 rpm for 5 min. A cocktail of intracellular cytokines IL-2 FITC, IL-17 PE, TNF-α PE-Cy7, IL-10 efluor-450, and IFN-γ APC (all from ebioscience) was added, and cells incubated for 30 min at room temperature. Cells were washed with perm/wash solution and 300 µL FACS buffer was added before acquisition. At least 100,000 lymphocytes were acquired for each sample on a CyanADP (Beckman Coulter, USA) flow cytometer using Summit v4 software and analyzed using FlowJo v10.0.2 (Tree Star, USA) according to the gating strategy shown (Figures S1 and S2 in Supplementary Material).

**Intracellular Cytokine Staining**

0.5 × 10^6 PBMCs were stimulated with RPMI, PMA/ionomycin, PPD, a peptide pool of the TB-specific antigens ESAT-6/CFP-10 (2.5 µg/mL, Proimmune, UK), heat-killed SP (10^6 cells/mL), *C. albicans* (10^6 cells/mL), or *E. coli* (10^6 cells/mL) per test. Cells were incubated at 37°C 5% CO₂ for 18 h with Brefeldin A (10 µg/mL) added after the first 2 h of incubation. Cells were stained with a live/dead aqua yellow stain (Invitrogen, USA), incubated for 10 min at room temperature before staining with a surface marker cocktail consisting of either CD3-PerCP Cy5.5 or CD3 APC efluor 750 and CD8 APC efluor 780. Cells were incubated for 30 min at room temperature and washed with 1 mL of PBS/1% FCS/0.2% Sodium Azide (FACS buffer). Supernatant was removed and cells washed in perm/wash solution (Ebioscience, UK). Permeabilization was then performed using fix/perm solution (Ebioscience) and cells incubated for 20 min at 4°C before centrifugation at 1,800 rpm for 5 min. A cocktail of intracellular cytokines IL-2 FITC, IL-17 PE, TNF-α PE-Cy7, IL-10 efluor-450, and IFN-γ APC (all from ebioscience) was added, and cells incubated for 30 min at room temperature. Cells were washed with perm/wash solution and 300 µL FACS buffer was added before acquisition. At least 100,000 lymphocytes were acquired for each sample on a CyanADP (Beckman Coulter, USA) flow cytometer using Summit v4 software and analyzed using FlowJo v10.0.2 (Tree Star, USA) according to the gating strategy shown (Figures S1 and S2 in Supplementary Material).**
two-way group-by-bleed and three-way sex-by-group-by-bleed interactions. Comparisons between treatment groups, and sexes within treatment groups, at each time point, were based on t-tests utilizing the predicted means and SEs of differences recovered from the fitted mixed model. Comparisons were conducted at the 5% significance level with no adjustments for multiplicity of outcome variables or comparisons. Ratios of cytokines were intrinsically skewed and required logarithmic transformation prior to analysis, whereas diagnostic plots of residuals indicated that the cytokine data did not require log transformation. Logarithmic transformations were also required to overcome heterogeneity of variance in the antibody data. Data were analyzed using GenStat 17 (VSN International), R 3.1.2 (www.r-project.org), and Stata version 12.1 (StataCorp LP, USA).

RESULTS

Infant Characteristics
137 infants were recruited into the study at birth with males and females randomized separately (Figure 1). The groups were of equivalent weight-for-age z scores at recruitment (data not shown). At 6 weeks (range 5–7 weeks), 131 babies remained in the study and were randomized to one of the two study groups: 67 infants (34 males and 33 females) received BCG and 64 (35 males and 29 females) were randomized to the control group and received BCG at 18 weeks upon study completion (Figure 1).

Lack of Enhanced In Vitro PPD Reactivity, but Greater TST Responses, in BCG Vaccinated Infants
Tuberculin skin test reactivity and in vitro PPD assays were used to assess mycobacterial responses post-vaccination. The BCG vaccinated group had greater TST reactivity than the naïve control group (Table 2). Thus, while only 7 of 52 (13.5%) BCG naïve infants had a positive TST response (any induration >1 mm), 32 of 53 (60.4%) BCG vaccinated infants were TST positive. The median induration in the unvaccinated infants was 0 (IQ range 0, 0) further indicating low level TST activity in this group, as compared to the BCG vaccinated group who had a median induration of 4.5 mm (IQ range 0, 10.5) (Control group vs BCG group p < 0.0001). The BCG vaccinated males had greater induration than females (M 8.5 mm, F 1.25 mm, M vs F p = 0.014) and responded more frequently than females (M = 69%, F = 50%).

Surprisingly, there was no evidence of a boosting of PPD reactivity in the BCG vaccinated group at V2 or V3 for any of the cytokines tested in culture supernatants (Table S1 in Supplementary Material). On the contrary, in BCG vaccinated females but not males the PPD-specific IL-12(p70) (p = 0.0025) (Figure 2A), IFN-γ (p = 0.0003) (Figure 2B), IL-4 (p = 0.0018) (Figure 2C), IL-10 (p = 0.004) (Figure 2D), and IL-17 (p = 0.0003) (Figure 2E) had all declined by V3 compared to V2. By contrast, the BCG naïve group had an increase in IFN-γ (p = 0.0164) (Figure 2A), IL-4 (p = 0.0018) (Figure 2C), IL-10 (p = 0.004) (Figure 2D), and IL-17 (p = 0.0003) (Figure 2E) at V2 compared to baseline.

Intracellular staining by flow cytometry was performed to characterize CD4 and CD8 T cells, and innate cells (CD3− cells) expressing IL-2, IL-17, TNF-α, IL-10, IFN-γ, or combinations of these cytokines intracellularly (Figures S1 and S2 in Supplementary Material). This failed to show any significant sex, group, or time interactions for PPD-specific CD4+, CD8+, or CD3− ICS responses for any of the cytokines in BCG vaccinated or control infants (data not shown).

No Effects of BCG Vaccination on Innate Cytokine Responses
Levels of the innate pro-inflammatory cytokine TNF-α were analyzed in the TLR ligand and pathogen culture supernatants after overnight stimulation. While robust TNF-α responses were detected to the TLR ligands (Table S2 in Supplementary Material) and heat-killed pathogens (Table S3 in Supplementary Material), there was no difference in TNF-α responses between BCG vaccinated and naïve infants nor between males and females. In keeping with the soluble cytokine data, there was no effect of vaccine group, bleeding time point or sex on TNF-α responses by ICS of CD4+, CD8+, or CD3− cells (data not shown).

Sex-Differential Effects of BCG Vaccination on Th1/Th2 Polarizing Cytokines
IL-12(p70) (V1 vs V3 p = 0.0355) and IFN-γ (V1 vs V3 p = 0.0018; V2 vs V3 p = 0.0232) responses declined to the TLR4 ligand LPS in BCG vaccinated females, and IL-12(p70) (V1 vs V3 p = 0.0129) but not IFN-γ declined in the BCG naïve control females too (Figures 3A,B). BCG naïve females showed an increase in IFN-γ production to PMA/ionomycin by V3 (V1 to V3: p = 0.0019; and V2 to V3: p = 0.0334), while the BCG vaccinated females did not (Figure 3C). BCG naïve, but not vaccinated, males had increased IL-2 production to both the TLR2 ligand HKLM (V1 vs V2 p = 0.0057) (Figure 3D) and CA (V1 vs V2 p = 0.0207) (Figure 3E) at V2, which declined by V3 (HKLM V2 vs V3 p = 0.0005; CA V2 vs V3 p = 0.026). The BCG vaccinated males also had an increase in IL-2 to CA at V2, which did not quite reach significance (p = 0.0512), but declined significantly by V3 (V2 vs V3 p = 0.0498) (Figure 3E). The only effect on intracellular cytokine production by flow cytometry after stimulating with the TLR ligands and heat-killed pathogens was an increase in IFN-γ from CD8+ T cells after CA stimulation in the BCG vaccinated females at V2 compared to V1 (p = 0.0316) (Figure 3F).
Sex-differential changes in purified protein derivative (PPD) reactivity. Figure shows comparisons of means within sexes for analyses with significant effects (main effect of interactions) involving bleed for the cytokine responses to stimulation with PPD. Significant effects were only observed in females and not male infants. The bacillus Calmette–Guérin (BCG) vaccinated females had detectable responses at baseline, in particular IFN-γ and IL-10, but these were not boosted further 1 (V2) or 12 weeks (V3) post-vaccination. Indeed, by V3, there was a significant decline in IL-12(p70) (A), IFN-γ (B), IL-4 (C), IL-10 (D), and IL-17 (E) at V3 in the BCG vaccinated females. Mean baseline levels were low at baseline for the BCG naïve group, but increased significantly for IFN-γ (B), IL-4 (C), and IL-17 (E) by V2 (+1 week). A fitted linear mixed model of cytokine values was used to determine significant differences by vaccine group, bleed, and sex. *p < 0.05, **p < 0.01, and ***p < 0.001. The figures show the arithmetic mean and SEM for cytokines in picograms per milliliter. Data shown for n = 93 infants (26 BCG vaccinated females, 18 BCG vaccinated males, 24 control females, and 25 control males).
Similar increase in CA-specific CD8+IFN-γ+ responses from V1 to V2 was not significant in the males, but the level at V2 was significantly higher than at V3 ($p = 0.0151$) (Figure 3G).

While the Th2 cytokine IL-4 was not affected by vaccine group or sex in culture supernatants, the ratio of IFN-γ to IL-4, indicative of the Th1:Th2 cytokine balance, was affected. Thus, BCG naïve males had decreased IFN-γ:IL-4 at V3 to TLR4 (LPS) (V1 vs V3 $p = 0.0393$) (Figure 4A) and TLR7/8 (CLO-75) stimulation (V2 vs V3 $p = 0.0194$) (Figure 4B); and BCG vaccinated females had decreased IFN-γ:IL-4 to SP at V2 compared to baseline (V1 vs V2 $p = 0.0128$) which increased again by V3 (V2 vs V3 $p = 0.0227$) (Figure 4C; Table 3; Tables S2 and S3 in Supplementary Material).

**Sex-Differential Effects on the Anti-inflammatory Cytokine IL-10**

IL-10 is an anti-inflammatory cytokine that can be produced by T helper 1 (Th1), Th2, and regulatory T cells (41). IL-10 responses...
Effects on the anti-inflammatory cytokine IL-10. The modeling analysis showed differences by vaccine group, bleed, and sex for female IL-10 responses to LPS (a) and CA (B). IFN-γ:IL-10 ratios declined in SP cultures in BCG naïve females (c); and declined in SP (D), HKLM (E), and LPS (F) cultures in BCG naïve males. TNF-α:IL-10 levels decreased by V3 (+12 weeks) in EC (G) and SP (H) cultures in BCG naïve females. *p < 0.05, **p < 0.01, and ***p < 0.001. The figures show the arithmetic mean and SEM for individual cytokines in picograms per milliliter, and geometric mean and 95% CI for log transformed cytokine ratios. CA, Candida albicans; EC, Escherichia coli; HKLM, heat-killed Listeria monocytogenes; LPS, lipopolysaccharide; SP, Streptococcus pneumoniae.

Data shown for n = 79 infants (23 BCG vaccinated females, 15 BCG vaccinated males, 21 control females, and 20 control males).

**Figure 5** | Effects on the anti-inflammatory cytokine IL-10. The modeling analysis showed differences by vaccine group, bleed, and sex for female IL-10 responses to LPS (A) and CA (B). IFN-γ:IL-10 ratios declined in SP cultures in bacillus Calmette–Guérin (BCG) naïve females (C), and declined in SP (D), HKLM (E), and LPS (F) cultures in BCG naïve males. TNF-α:IL-10 levels decreased by V3 (+12 weeks) in EC (G) and SP (H) cultures in BCG naïve females. *p < 0.05, **p < 0.01, and ***p < 0.001. The figures show the arithmetic mean and SEM for individual cytokines in picograms per milliliter, and geometric mean and 95% CI for log transformed cytokine ratios. CA, Candida albicans; EC, Escherichia coli; HKLM, heat-killed Listeria monocytogenes; LPS, lipopolysaccharide; SP, Streptococcus pneumoniae. Data shown for n = 79 infants (23 BCG vaccinated females, 15 BCG vaccinated males, 21 control females, and 20 control males).
Sex-Differential Effects of BCG Vaccination on IL-17 Responses to TLR2 Ligand Stimulation

IL-17 production following TLR2 ligand (HKLM) stimulation increased at V2 in the BCG naïve (V1 vs V2 \( p = 0.0407 \)), but not vaccinated, males (Figure 6A); and HKLM-specific IL-17 declined at V3 in BCG vaccinated (V1 vs V3 \( p = 0.0165 \)), but not naïve, females (Figure 6B). There was no effect of vaccine group or sex on IL-17 responses for any of the other stimuli (Tables S2 and S3 in Supplementary Material). IL-17 reactivity in CD4^+, CD8^+, or CD3^- cells by ICS was not affected by BCG vaccination or sex at any time point (Table 3).

No Effect of BCG Vaccination at 6 Weeks on Antibody Responses to Other EPI Vaccines

Since BCG has been shown to boost antibody responses to other EPI vaccines, even when given before the vaccine in question (35), we measured antibody levels to hepatitis B vaccine, poliovirus serotypes 1 and 3, diphtheria toxoid, tetanus toxoid (TT), and pertussis toxoid. There was significant induction of antibody responses to all vaccines by V3, but not V2, compared to V1 (Table 4). All infants except for one had protective antibody levels to TT at V1, confirming a benefit of maternal vaccination, as expectant mothers in The Gambia receive two doses of TT vaccine during the second and third trimesters. No additional boosting effect was observed in the BCG vaccinated group compared to the naïve group for any of the vaccines. The only sex difference was higher HepB IgG levels in control males compared to control females at V3 (\( p = 0.040 \)), while no such difference was observed in the BCG vaccinated group.

DISCUSSION

The lack of consistent and reliable protection by BCG has galvanized the search for new vaccination strategies using recombinant technology or heterologous vector-based vaccines (42, 43), as a prime-boost to neonatal BCG vaccination. To develop optimal vaccination strategies, it is important to understand the role that the heterologous effects of BCG vaccination play in TB endemic settings. The aim of this study was to analyze in vitro reactivity to innate ligands and a panel of common pathogens to see if BCG vaccination of 6-week-old Gambian infants enhanced reactivity to these antigens compared to BCG naïve infants.

One of the surprising findings in this study was a lack of induced in vitro reactivity to PPD in the BCG vaccinated infants. However, the BCG vaccinated group had good induction of TST reactivity compared to the naïve group suggesting that the vaccine was effective. In this study, we performed overnight cultures only, whereas we have previously shown that T cell responses to PPD generally peak at 5–7 days of culture (13), thus we probably measured predominantly innate responses to intrinsic PAMPs expressed by mycobacteria in the BCG vaccine (44), which were not boosted by vaccination. Indeed, paradoxically, the BCG vaccinated females had a decline in Th1, Th2, and IL-17 cytokine reactivity to PPD at V3, while the BCG naïve infants experienced an increase in reactivity. This decline may be due to the intervening vaccines given including DTwP that has specifically been shown to suppress innate and adaptive immunity in Gambian females but not males (45). One week is very early for detection of memory T cell responses following BCG vaccination, so this early time point also likely contributed to lack of induced PPD responses. It is also possible that the PPD itself was not immunogenic; however, the same batch was effective in previous studies. We used BCG Russia in this study which has been shown to have poor immunogenicity compared to other strains such as BCG Japan and BCG Denmark (6, 46, 47), which would further contribute to low PPD reactivity in our study. Another explanation is that the mycobacterial response may have been negatively attenuated by environmental bacteria, although our previous studies in infants from the same study area do not support this conclusion (13).

Previous studies have shown that BCG can “train” the innate immune system of healthy European adults via epigenetic

![Figure 6](https://example.com/image6.png)

**FIGURE 6** IL-17 responses. IL-17 responses in heat-killed *Listeria monocytogenes* (HKLM) cultures increased in bacillus Calmette–Guérin (BCG) naïve males at V2 (+1 week) (A), and decreased in HKLM cultures in BCG vaccinated males (B). \( * p < 0.05 \) and \( ** p < 0.01 \). The figures show the arithmetic mean and SEM for IL-17 in picograms per milliliter. Data shown for \( n = 79 \) infants (23 BCG vaccinated females, 15 BCG vaccinated males, 21 control females, and 20 control males).
modification of innate immune cells leading to enhanced pro-
inflammatory responses to a range of stimuli (27, 48–51).
Furthermore, we have previously found that neonatal BCG
vaccination enhanced reactivity to certain TLR ligands in a
sex-differential manner, with greater enhancement in females
than males (49). However, in this study, we found no evidence
of enhanced innate responses (analyzed by TNF-α production)
in BCG vaccinated infants in culture supernatants or by ICS.
The reason for the discrepancy with previous studies could be
due to a number of factors including differences in experimental
techniques, the age group at which BCG was given, key genetic
differences, and exposure to environmental mycobacteria.
Another likely contributory factor is our use of BCG Russia since
the previous immune-enhancing studies used BCG Denmark.
BCG scar rates predict non-specific beneficial effects, and those
immunized with BCG Russia have lower scar rates (approximately
50%) compared to BCG Denmark (72–97%) (6). By implication,
this would suggest that BCG Russia is less likely to have beneficial
non-specific effects that BCG Denmark (5). This was confirmed
in a study conducted in more than 1,000 infants in Uganda in
which BCG Denmark was found to have stronger non-specific
immunological effects than BCG Russia and BCG Bulgaria (4).
Ongoing studies are hoping to confirm this critical issue.

The Th1 polarizing cytokines IL-2, IL-12( p70), and IFN-γ
were all affected by vaccine group and sex in this study. The
increased CD8+ T cell IFN-γ production to C. albicans in males
and females 1 week after BCG vaccination supports early immune
enhancement by the vaccine. There were no other enhanced Th1
type cytokine responses in BCG vaccinated males or females.
On the contrary, BCG vaccinated females had decreased IFN-γ:IL-4 to SP 1 week after vaccination.

Many of the significant changes in cytokine reactivity occurred
at V3 (+12 weeks) rather than V2 (+1 week). For example, at V3,
both BCG vaccinated and naive infants had decreased Th1 cytokine
reactivity and decreased pro- to anti-inflammatory cytokine ratios
to certain stimuli. Between V2 and V3 all infants received three
doses of OPV, pentavalent vaccine (diphtheria, tetanus, whole
cell pertussis, Haemophilus influenzae type b, hepatitis B), and
PCV-13 at 8, 12, and 16 weeks of age, all of which are likely to have heterologous effects on innate and adaptive immunity. Indeed,
we have shown that DTwP vaccination leads to decreased innate
and T cell responses in infant females but not males (45), and
OPV has also been shown to attenuate immune responses to BCG
vaccination (49). Thus, changes in immune reactivity observed in
this study may also reflect heterologous effects of these vaccines,
and differences between the BCG vaccinated and naive groups
are likely reflect the additional immune modifying effects of
BCG. In particular, the negative heterologous effects of DTwP
(45) could explain the decline in PPD reactivity for multiple
cytokines in BCG vaccinated females but not males at V3.

IL-10 is a key anti-inflammatory cytokine, implicated in the
homeostatic regulation of inflammatory responses (41). TLR4-
specific IL-10 responses declined in BCG vaccinated females, and
IL-10 reactivity to C. albicans increased in BCG naïve females at
both V2 and V3. BCG naïve males and females also had decreased
IFN-γ:IL-10 and TNF-α:IL-10 ratios to a range of stimuli. These

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**TABLE 4** | Antibody titers to EPI vaccines in the bacillus Calmette–Guérin (BCG) vaccinated and naïve infants.

| Vaccine type (unit) | Visit | Boost from baseline | | Females Boost from baseline | | Males vs females | | BCG vaccinated group | | Median (IQR) | | p-Value | | Median (IQR) | | p-Value | | BCG naïve control group | | Median (IQR) | | p-Value | | Median (IQR) | | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polio 1 (titers) | 1 | 24 (8, 160) | | 32 (8, 112) | | NS | | 16 (8, 112) | | 0.003 | | <0.0001 | | 16 (8, 48) | | NS | | 24 (16, 256) | | NS | | 32 (16, 64) | | NS | | 256 (128, 1024) | | <0.0001 | | 192 (64, 1024) | | <0.0001 | | 256 (10, 512) | | NS |
| Polio 3 (titers) | 1 | 8 (8, 80) | | 8 (8, 14) | | NS | | 8 (8, 80) | | 0.003 | | <0.0001 | | 8 (8, 12) | | NS | | 24 (8, 128) | | NS | | 8 (8, 16) | | NS | | 256 (32, 1024) | | <0.0001 | | 256 (10, 512) | | NS | | 256 (128, 512) | | NS |
| HepB (IU/mL) | 1 | 10 (10, 10) | | 10 (10, 10) | | NS | | 10 (10, 10) | | 0.003 | | <0.0001 | | 10 (10, 10) | | NS | | 10 (10, 10) | | NS | | 10 (10, 10) | | NS | | 256 (32, 1024) | | <0.0001 | | 256 (10, 512) | | NS | | 483 (384, 1000) | | <0.0001 | | 1000 (393, 1000) | | <0.0001 | | 353 (241, 1000) | | NS | | 0.04 |
| Diphtheria toxoid (IU/mL) | 1 | 0.01 (0.01, 0.02) | | 0.01 (0.01, 0.02) | | NS | | 0.01 (0.00, 0.03) | | <0.0001 | | <0.0001 | | 0.01 (0.00, 0.01) | | NS | | 0.01 (0.00, 0.02) | | <0.0001 | | <0.0001 | | 0.01 (0.00, 0.01) | | NS | | 2.6 (1.4, 3.7) | | <0.0001 | | 2.7 (1.9, 4.1) | | <0.0001 | | 2.8 (1.7, 4.4) | | <0.0001 | |
| Pertussis toxoid (EU/mL) | 1 | 3.0 (1.5, 9.6) | | 5.0 (1.2, 9.5) | | <0.0001 | | 5.0 (1.2, 9.5) | | <0.0001 | | <0.0001 | | 4.6 (1.8, 8.8) | | <0.0001 | | 4.6 (1.8, 9.7) | | <0.0001 | | 4.1 (1.6, 9.7) | | <0.0001 | | 252 (112, 572) | | <0.0001 | | 225 (123, 568) | | <0.0001 | | 240 (61, 582) | | <0.0001 | |
| Tetanus toxoid (TT) (IU/mL) | 1 | 1.7 (0.7, 3.2) | | 1.9 (0.8, 3.0) | | NS | | 1.9 (0.8, 3.0) | | <0.0001 | | <0.0001 | | 1.3 (0.6, 3.2) | | <0.0001 | | 1.3 (0.6, 3.2) | | <0.0001 | | 1.0 (0.4, 2.6) | | <0.0001 | | 7.5 (3.2, 10.2) | | <0.0001 | | 5.5 (3.8, 15.6) | | <0.0001 | | 6.3 (3.4, 17.8) | | <0.0001 | |

Summary of antibody levels at each visit for polio 1 and 3, hepatitis B, diphtheria toxoid, pertussis toxoid, and TT showing significant boosting of levels in both vaccine groups by visit 3 (+12 weeks). BCG naïve males had higher HepB antibody titers at visit 3 compared to BCG naïve females; this was not the case for the BCG vaccinated males and females. A fitted linear mixed model of log transformed antibody values was used to determine significant differences by vaccine group, bleed and sex. Data shown for n = 126 infants.

(30 BCG vaccinated females, 33 BCG vaccinated males, 28 control females, and 35 control males).
results suggest a skewing of immunity toward a more anti-
inflammatory profile in those infants who had not received BCG, 
which was prevented by the prior administration of BCG. Greater 
anti-inflammatory responses may be detrimental by resulting in 
less efficient pathogen clearance, and prevention of it by prior 
administration of BCG could provide an immunological benefit.

Th17 cells have been implicated in protective mycobacterial 
responses after BCG vaccination (16, 52) and in clearing a range 
of other pathogens (53). However, we observed high levels of 
IL-17 production from non-T cells after stimulation with PPD, 
and thus not a Th17 response; and further found no difference 
between BCG vaccinated and naïve infants following stimulation 
with antigens (both vaccine-specific and unrelated) suggesting 
no effect of BCG vaccination. However, BCG naïve males had 
evidence of increased IL-17 reactivity to TLR2, while BCG 
vaccinated females had a decrease, supporting sex-differential 
enhanced IL-17 immunity in the naïve group.

In summary, we found no evidence that BCG vaccination of 
6-week-old Gambian infants alters innate immunity and have 
shown that Th1 and IL-17 cytokine responses were more likely 
to increase over time in the BCG naïve individuals. However, the 
BCG naïve group also had decreased pro- to anti-inflammatory 
cytokine ratios (IFN-γ:IL-4, IFN-γ:IL-10, and TNF-α:IL-10) and 
increased IL-10 to certain stimuli, indicating a bias toward an 
anti-inflammatory response. All of these effects were sex differ-
tential, with males and females often showing opposite patterns. 
This is in keeping with the growing body of literature reporting 
sex differences in immunity (54). These sex differences are likely 
caused by a combination of factors including differences in sex 
hormone levels and expression of X-linked immune response 
genes. Estrogens are generally immune enhancing and higher in 
females most of the first year of life; and males undergo a tes-
tosterone surge at about 6 weeks of age, which is known to have 
immunosuppressive effects on immunity (55).

It has been shown that concomitant administration of BCG 
can enhance antibody responses to other EPI vaccines, including 
hepatitis B, TT, and OPV (15). Furthermore, certain pneumococ-
cal vaccine serotypes were boosted in infants immunized with 
BCG at birth indicating that BCG can affect responses to vaccines 
given later in infancy (35). We did not find a boosting effect of 
BCG vaccine on the any of the vaccine antibody levels measured. 
This might be due to the different vaccine strain used in our 
study as discussed earlier; or the timing of BCG vaccination at 
6 weeks of age and thus 6 weeks after their birth HBV and OPV, 
and 2 weeks before their first pentavalent vaccine (DTP, Hib, and 
HBV). Importantly, however, protective antibody levels to polio, 
TT, and hepatitis B were achieved in all subjects regardless of 
vaccine group, showing that the delay in BCG vaccination was 
not detrimental to antibody levels in our study. Control males had 
higher hepatitis B titers than females, which contradicts previous 
studies showing higher responses among females (54).

Several limitations to this study should be borne in mind. 
First, the vaccine was administered at 6 weeks of age rather than 
at birth, yet it is recommended that the vaccine be given at the 
earliest opportunity after birth. However, in the real life setting, 
it is estimated that less than half receive BCG in the first month 
of life, often because BCG comes in multi-dose vials that are not

ETHICS STATEMENT

The Scientific Coordinating Committee (SCC) at the Medical 
Research Council (MRC) Unit, The Gambia and the MRC-
Gambian Government Joint Ethics Committee approved this 
study (study number SCC 1233). Written informed consent was 
given by those who agreed to participate in accordance with the 
Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FD, JS, and KF designed the study; JA oversaw infant recruit-
ment/vaccination/bleeding/clinical assessment; FD and JS did 
the laboratory assays; FD, JR, MP, MN, SR-J, and KF did the 
statistical analysis and/or interpreted the data; SH designed the 
study database and oversaw data entry; all authors critically 
revised and approved the manuscript and are accountable for the 
accuracy and integrity of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01092/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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