MAJOR ARTICLE

Heterologous Immunological Effects of Early BCG Vaccination in Low-Birth-Weight Infants in Guinea-Bissau: A Randomized-controlled Trial

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(See the editorial commentary by Kollmann on pages 859–60.)

Background. Bacillus Calmette–Guérin (BCG) seems to have beneficial nonspecific effects; early BCG vaccination of low-birth-weight (LBW) newborns reduces neonatal mortality by >40% due to prevention of primarily septicemia and pneumonia.

Methods. Within a randomized trial in LBW infants in Guinea-Bissau of early BCG vs the usual postponed BCG, a subgroup was bled 4 weeks after randomization. Levels of interleukin (IL)-1β, IL-5, IL-6, IL-10, IL-17, interferon (IFN)-γ and tumor necrosis factor (TNF)-α were measured from whole-blood assays stimulated with innate agonists to Toll-like receptor (TLR)-2, -4 or -7/8, or purified protein derivative (PPD).

Results. Among 467 infants, BCG significantly increased the in vitro cytokine responses to purified protein derivative of *Mycobacterium tuberculosis* (PPD), as expected. BCG was also associated with increased responses to heterologous innate stimulation, particularly of the cytokines IL-1β, IL-6, TNF-α, and IFN-γ.

Conclusion. Four weeks after immunization, BCG-vaccinated infants have a significantly increased production of cytokines upon heterologous challenge, particularly T helper cell type 1 polarizing and typically monocyte-derived pro-inflammatory cytokines. BCG may accelerate the development of the neonatal immune system, mediating comprehensive protection against infections and mortality.

Keywords. BCG; heterologous immunity; nonspecific effects; cytokines; infants; Africa.

An increasing body of literature supports that vaccines have immune-modulating effects that influence host defense to other diseases than the targeted pathogen, so-called nonspecific effects of vaccines or heterologous immunity [1]. The heterologous immunity may have significant impact on overall health and for certain vaccines may be even more important than the specific protection [2].

An example is the bacillus Calmette–Guérin (BCG) vaccine; many observational studies [2–6] and recently also randomized-controlled trials (RCT) [7, 8] have shown that BCG reduces all-cause mortality more

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than can be ascribed to protection from *Mycobacterium tuberculosis* infections.

Combined, 2 RCTs in Guinea-Bissau in LBW infants have shown that early BCG administration can reduce overall infant mortality up to 20% (mortality rate ratio [MRR] at 12 months of age: 0.79 [95% CI, 0.61–1.02]). Furthermore, BCG almost halved the neonatal mortality (MRR: 0.52 [95% CI, 0.33–0.82]) [7]. This dramatic reduction in the neonatal mortality seems to be mainly due to fewer cases of septicemia and respiratory infections [8].

In Guinea-Bissau, we are now conducting a new BCG trial among LBW infants with neonatal mortality as the main outcome. Nested within this trial, we performed a subgroup study to investigate the immunological effects of BCG on the responsiveness to specific and nonspecific immunological challenge, as measured by the in vitro cytokine production of blood cells after stimulation with innate immunity agonists and recall antigens.

MATERIALS AND METHODS

Setting and Study Population and Enrolment Into the Main RCT

The present study was carried out by the Bandim Health Project (BHP) in Guinea-Bissau, West Africa. The infants were recruited from an RCT of early BCG vaccination in LBW neonates with neonatal mortality as the main outcome (clinicaltrials.gov: NCT00625482). Enrolments into the RCT took place from February 2008 to September 2013. The mortality results of the trial will be reported elsewhere (manuscript). In brief, newborns identified at the maternity ward of the main national hospital right after delivery or at home or at the health centers in the catchment area were eligible for participation in the main RCT if they had a weight <2.50 kg, had no major malformations, and had received no prior BCG vaccination; furthermore, if born at the hospital, they should be ready to be discharged according to local standards; if born at home or at the health centers, they should be due to receive oral polio vaccine (OPV) by local standards.

The mothers/guardians of eligible infants received written and oral information of the study. Provided oral and written consent, the mother/guardian would draw a lot from a bag, allocating the child to receive early BCG (intervention group) or the usual postponed BCG (control group). Same-sex twins were allocated to the same treatment. All infants had their anthropometric measurements recorded (Table 1), and an interview about the pregnancy, the delivery, and socioeconomic status was conducted. Information on human immunodeficiency virus (HIV) infection for mothers delivering at the main hospital was retrieved by cross-checking with the routine HIV registry at the maternity ward.

Infants allocated to early BCG were vaccinated intradermally in the upper deltoid region with 0.05 mL BCG vaccine SSI (Statens Serum Institut, Copenhagen, Denmark) by trained nurses. Infants allocated to the control group were treated according to local practice implying that the vaccination was postponed until they had obtained a weight ≥2.50 kg, most commonly when they came for their first diphtheria-tetanus-pertussis-*Haemophilus influenzae* type b-Hepatitis B (Penta) vaccine recommended at 6 weeks of age. All infants received OPV at birth.

Enrolment Into the Present Immunological Study

Between 18 April 2011 and 12 January 2012, infants in the subgroup immunological study were recruited from the main trial 4 weeks (±7 days) after randomization. Eligible were infants who were not overtly ill (such infants were referred for treatment). Infants living in the Bissau city and closest suburbs were given priority for logistic reasons.

Data and Blood Collection

The infants were visited at home by a BHP field team member. Informed consent to participate in the immunological study was obtained by the same procedure as the main RCT. Provided consent, mid-upper arm circumference, weight, length, and axillary temperature were measured, and information about the health status of the infant including health care use was collected. Capillary blood was collected by heel puncture into a heparinized and an ethylenediaminetetraacetic (EDTA)-coated tube, respectively.

In Vitro Stimulation Assay

The heparinized blood was diluted 1:9 with Roswell Park Memorial Institute medium (RPMI)-1640 void of L-Glutamine (Gibco, Life Technologies Europe BV) supplemented with Pyruvate 1 mM (Na-pyruvate, Lonza, Copenhagen, Denmark) and L-Glutamine–Penicillin–Streptomycin 1X (Gibco, Life Technologies). The following stimulations were performed in 200 μL round-bottom microtiter plates (NUNC, Roskilde, Denmark) in a 37°C humidified incubator with 5% CO_2 for 24 hours (final concentration in parenthesis): phorbol 12-myristate 13-acetate (PMA) (100 ng/mL; Sigma-Aldrich) and ionomycin (1 μg/mL) (Sigma-Aldrich) as a positive control; purified protein derivative (PPD) from *M. tuberculosis* (Statens Serum Institut, Copenhagen, Denmark) (10 μg/mL) to assess the mycobacterial specific response; lipopolysaccharide (LPS) (10 ng/mL) (Sigma-Aldrich) [a Toll-like receptor (TLR) 4 agonist]; (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH,trihydrochloride (Pam3CSK4) (1 μg/mL) [a TLR2/1 agonist] (InvivoGen); Thiazoloquinoline Compound (CL075) (1 μg/mL) [a TLR8/7 agonist] (InvivoGen).

Supernatants were kept at −70°C or lower until analysis. Cytokine concentrations were analyzed at Statens Serum Institut, Copenhagen, by an immunobead-based multiplexed assay as previously described [9]. The cytokines analyzed were interleukin (IL)-1β, IL-5, IL-6, IL-10, IL-17, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. The CL075-stimulated

Table 1. Background Characteristics of the Infants With Measurements of In Vitro Cytokine Production or Differential Counts Obtained at Randomization (Part A) and at Day of Bleeding (Part B)

	All			Male			Female		
Part A. At Randomization	BCG n (%)	Control n (%)	*P* Value	BCG n (%)	Control n (%)	*P* Value	BCG n (%)	Control n (%)	*P* Value
All	261	207		113	92		148	115	
Rainy season	168 (64%)	143 (69%)	.28	72 (64%)	64 (70%)	.38	96 (65%)	79 (69%)	.51
Neonatal OPV	261 (100%)	207 (100%)	. . .	113 (100%)	92 (100%)	. . .	148 (100%)	115 (100%)	. . .
Premature (ballard <32)	38 (17%)	38 (24%)	.13	14 (15%)	21 (30%)	.02	24 (19%)	17 (19%)	.93
Twin	62 (24%)	55 (27%)	.49	26 (23%)	28 (30%)	.23	36 (24%)	27 (23%)	.87
Mother deceased	0 (0%)	1 (0%)	.44	0 (0%)	0 (0%)	. . .	0 (0%)	1 (1%)	.26
House with hard roof	255 (98%)	201 (97%)	.68	109 (96%)	87 (95%)	.51	146 (99%)	114 (99%)	.72
Indoor toilet	52 (20%)	38 (18%)	.67	22 (19%)	18 (20%)	.99	18 (20%)	20 (17%)	.56
Electricity supply at home	90 (34%)	61 (29%)	.25	38 (34%)	28 (30%)	.63	52 (35%)	33 (29%)	.27
Mother HIV positive[a]	4 (4%)	3 (3%)	.82	2 (5%)	2 (5%)	.96	2 (4%)	1 (2%)	.66
	50 (10–90) centile	50 (10–90) centile	*P* Value	50 (10–90) centile	50 (10–90) centile	*P* Value	50 (10–90) centile	50 (10–90) centile	*P* Value
Mother's school, years	6 (0–11)	6 (0–11)	.55	6 (0–11)	6 (0–11)	.82	6 (0–11)	6 (0–11)	.29
Age of infant, days	2 (1–10)	2 (1–10)	.77	2 (1–8)	2 (1–12)	.81	2 (1–10)	2 (1–9)	.89
Age of mother, years	24 (17–33)	24 (18–32)	.47	25 (18–35)	25 (18–32)	.91	23 (17–32)	24 (18–34)	.30
	mean (SD)	mean (SD)	*P* Value	mean (SD)	mean (SD)	*P* Value	mean (SD)	mean (SD)	*P* Value
Height, cm	45 (2)	45 (2)	.08	46 (2)	45 (2)	.02	45 (2)	45 (2)	.78
Weight, kg	2.21 (1.68–2.45)	2.17 (1.60–2.45)	.10	2.23 (1.74–2.44)	2.15 (1.50–2.42)	.05	2.18 (1.65–2.45)	2.20 (1.67–2.46)	.68
MUAC, mm	82 (8)	81 (8)	.14	82 (8)	80 (8)	.26	83 (8)	82 (8)	.34
Part B. At bleeding	n (%)	n (%)	*P* Value	n (%)	n (%)	*P* Value	n (%)	n (%)	*P* Value
OPV in campaign	52 (20%)	44 (21%)	.73	24 (21%)	15 (16%)	.37	28 (19%)	29 (25%)	.23
Not breastfed	2 (1%)	2 (1%)	.82	2 (2%)	0 (0%)	.20	0 (0%)	2 (2%)	.11
Disease symptoms	149 (57%)	128 (62%)	.30	63 (56%)	61 (66%)	.12	86 (58%)	67 (58%)	.98
Received medication	48 (18%)	34 (16%)	.58	19 (17%)	18 (20%)	.61	29 (20%)	16 (14%)	.23
	50 (10–90) centile	50 (10–90) centile	*P* Value	50 (10–90) centile	50 (10–90) centile	*P* Value	50 (10–90) centile	50 (10–90) centile	*P* Value
Weight, kg	3.15 (2.30–3.60)	2.95 (2.20–3.70)	.03	3.25 (2.40–3.65)	2.90 (2.10–3.75)	.002	3.05 (2.00–3.55)	3.00 (2.30–3.70)	.86
Age, days	30 (26–39)	31 (26–38)	.40	30 (26–38)	32 (26–41)	.53	30 (26–39)	31 (26–37)	.54
	mean (SD)	mean (SD)	*P* Value	mean (SD)	mean (SD)	*P* Value	mean (SD)	mean (SD)	*P* Value
Height, cm	50 (3)	50 (3)	.39	50 (3)	49 (3)	.06	50 (3)	50 (3)	.58
MUAC, mm	99 (12)	97 (13)	.09	99 (11)	94 (14)	.01	98 (12)	99 (12)	.83

For categorical observations, statistical test for difference between randomization groups is by χ^2 test, alternatively Fisher exact test for outcome sizes ≤5. Normally distributed numerical values are tested with *t*-test, presented with mean (standard deviation). Non-normally distributed numerical values are tested with Kruskal–Wallis, presented as median (10%–90%-tiles).

Abbreviations: BCG, bacillus Calmette–Guerin; HIV, human immunodeficiency virus; MUAC, mid-upper arm circumference; OPV, oral polio vaccine; SD, standard deviation.

OPV was distributed to infants in regular campaigns outside the routine vaccination program. Symptoms include an axillary temperature >37.5°C or maternally reported cold, coughing, fever, vomiting, diarrhea on day of bleed. Medication of the infant was primarily analgesics, antibiotics or medicine to relieve stomach pain.

[a] Reference population is mothers with a valid HIV test.

supernatants were diluted 1:4 with culture medium due to the relatively high levels of cytokines in these cultures; all other supern atants were analyzed undiluted.

Differential Count

A whole-blood differential count was performed on the EDTA-treated blood by ABX Pentra60 (Horiba, France). Samples with an improper separation and gating of the detected cell subsets as assessed by visual inspection of the scatter plot produced by the ABX Pentra60 were repeated up to 3 times; poor quality analyses were ultimately excluded.

A blood film was microscopically inspected for malaria parasitemia; none was found.

The sample size was not based on power calculations as no comparable study with similar outcome and aim had been carried out before. However, based on experiences from previous investigations of heterologous immunity effects of vaccines on cytokine production stratified by sex [10] we aimed for a sample size of 400 infants, 200 in each randomization group.

Statistical Analysis

Analyses were performed using STATA 12 (StataCorp LP, College Station, Texas).

Cytokine Responses

The valid range of the Luminex assay was defined by the range of the standard concentrations used for the standard curve. After censoring the standard concentrations with an observed concentration falling outside a set limit of 80%–120% of the expected (theoretic) concentration, the remaining highest and lowest standard concentrations defined the upper and lower limit, respectively. Observations outside this range were considered nondetectable and hence treated as missing in the statistical analysis.

Log-transformed cytokine outcomes comparing BCG vaccinated vs BCG unvaccinated infants were analyzed by Tobit multiple regression to account for observations outside the range of the standard curve of the assay [11]. Geometric means (GM) and geometric mean ratios (GMR) were computed by retransforming the coefficients using the exponential function.

For a limited number of cytokine measurements, >50% of samples had a value outside the assay range, prevailingly above the upper limit with very few or none below the lower limit. These outcomes were analyzed by Poisson regression, providing prevalence ratios (PR) describing the risk of having a value above the upper limit of the assay. GMRs and PRs are reported with 95% confidence interval (CI). All estimates including interaction analyses were adjusted for weight at randomization.

Collective Cytokine Responses

To test the effect of BCG on overall nonspecific cytokine responsiveness irrespective of stimulation, a collective test for each cytokine was performed including all conditions, except PPD, adjusting for the main effect of stimulation. This, however, was only performed if the test of heterogeneity for the BCG effect across the different stimulations was nonsignificant in the particular analysis, indicating that the stimulations could be merged.

Effect on Cytokine Response Ratios

The ratios of IFN-γ to IL-5 and TNF-α to IL-10 responses were analyzed as crude markers of the balance of Th1 vs Th2 cytokine responses and the pro- vs anti-inflammatory cytokine responses, respectively; we report geometric mean ratio-ratios (GMRR).

Differential Counts

Log-transformed differential counts were analyzed by linear regression using bootstrap to obtain confidence intervals [12], adjusted for weight at randomization.

Sensitivity Analysis

BCG-vaccinated infants in the control group were censored (19%; Figure 1) as the objective was to investigate the true immunological effect of early BCG vaccination. The censored infants were among the heaviest. Hence, in order to test the robustness of our model for the cytokine outcomes including the potential implication of the censoring, we also excluded the 19% heaviest infants in the BCG group in a sensitivity analysis. To assess whether differences in cell counts could explain observed differences in cytokine production, we furthermore analyzed the effect of BCG on cytokines adjusted for monocyte or lymphocyte counts.

Effect Modification

As previous observational studies have indicated that sex [13, 14] and season (rainy: June to November; dry: December to May) of vaccination [15, 16] may modify the effect of BCG, we also stratified the analysis by these factors.

Ethical Considerations

The BCG RCT and the immunological substudy (clinicaltrials.gov: NCT00625482) were approved by the National Committee on Health Ethics of the Ministry of Health in Guinea-Bissau, and a consultative approval was obtained from the Danish National Committee on Biomedical Research Ethics.

RESULTS

A total of 625 infants from the BCG trial were visited and assessed for enrollment in the immunology study (Figure 1). We obtained a blood sample from 525 infants. At bleeding, 48 infants (19%) in the control group had received BCG, and 4 infants had received Penta vaccine. After exclusion of these 52 infants, 473 infants remained. Of these, we obtained a valid measurement of in vitro cytokine production (n = 467) and/or a valid differential count (n = 394) from 468 infants; more females (n = 263) than males (n = 205) were enrolled, since more females are LBW.

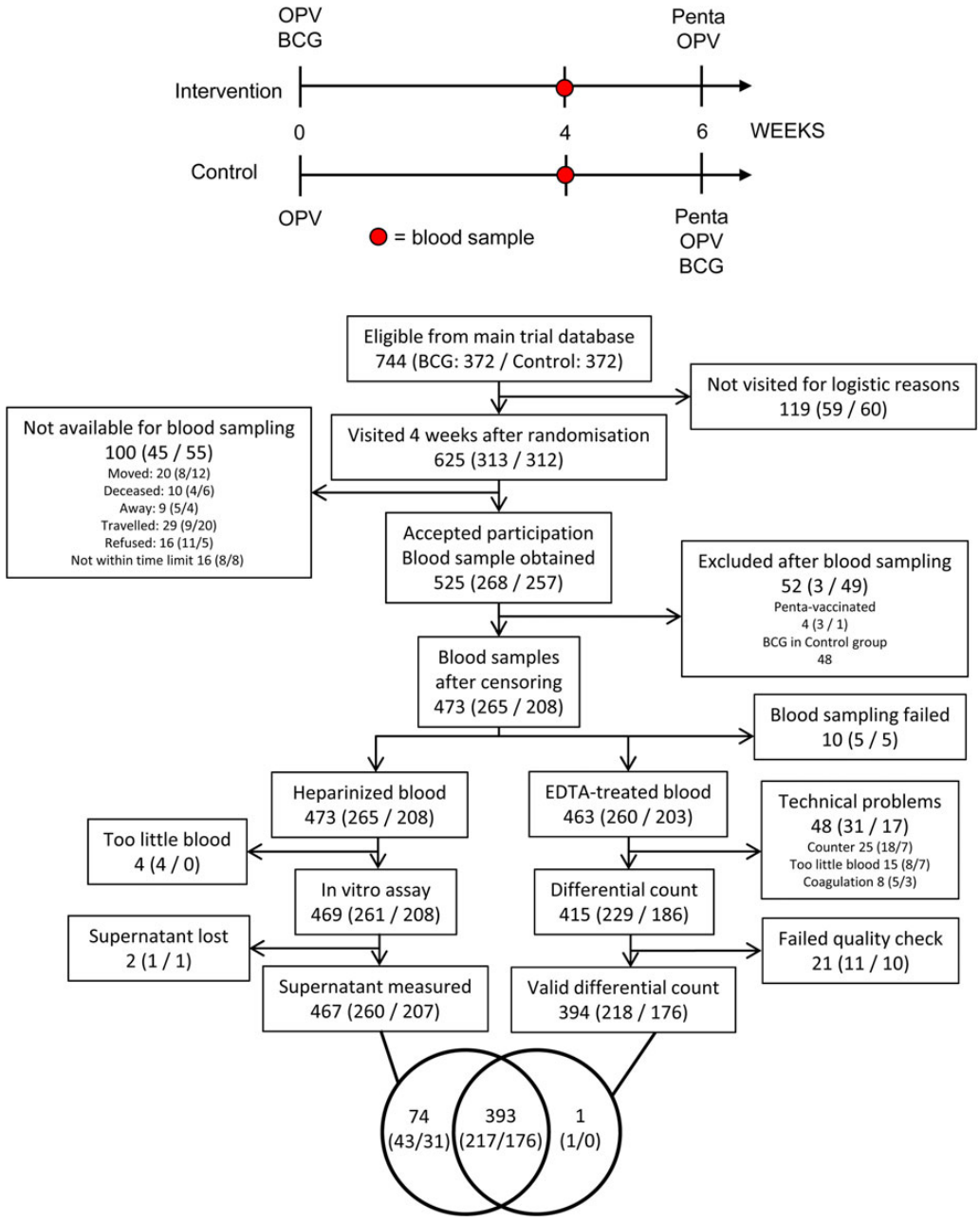

Figure 1. Flow chart of the study participants and the outline of study. The infants in the present study were recruited from the participants of the randomized trial of early BCG in low-birth-weight infants during the period between 18 April 2011 and 12 January 2012. Infants were visited and bled 4 weeks (±7 days) after randomization. The figures presented are the number of participants (Early BCG / control). Abbreviations: BCG, bacillus Calmette–Guerin; EDTA, ethylenediaminetetraacetic; OPV, oral polio vaccine.

Comparability of Groups

The chance of being BCG vaccinated in the control group increased with enrollment weight and other anthropometric measures, but the difference was only significant in males (data not shown). Hence, excluding the BCG vaccinated infants in the control group meant that, among males, the BCG group was generally larger and less frequently premature at enrollment than the no-BCG group, whereas there was no difference among females (Table 1). No other background characteristics differed between the 2 randomization groups.

We compared the 468 infants in the immunological study with the main trial participants during the same period. The infants enrolled in the immunological study tended to be of a slightly higher socioeconomic status, although this was only significant for roof type ($P = .04$; data not shown). This most likely reflects that infants living in the Bissau city and nearest suburbs

Table 2. Effect of BCG Vaccination on In Vitro cytokine Production, Overall and Stratified by Sex

			All (n = 467)		Male (n = 204)	Female (n = 263)	
		Obs in Range	GMR (95% CI)	*P* Value	GMR (95% CI)	GMR (95% CI)	*P* Value*
Medium	IL-1β	85%	1.33 (.97–1.83)	.08	1.10 (.68–1.77)	**1.55 (1.02–2.37**)	.29
	IL-6	87%	1.27 (.90–1.80)	.17	1.10 (.66–1.85)	1.43 (.91–2.26)	.46
	TNF-α	96%	**1.30 (1.05–1.60)**	**.01**	1.16 (.84–1.59)	**1.42 (1.08–1.88)**	.34
	IL-5	60%	0.98 (.77–1.25)	.86	0.90 (.62–1.30)	1.06 (.76–1.48)	.51
	IL-10	76%	1.13 (.85–1.52)	.40	1.04 (.67–1.62)	1.21 (.82–1.79)	.62
	IL-17	62%	1.00 (.73–1.38)	.98	0.81 (.50–1.30)	1.20 (.78–1.83)	.22
	IFN-γ	82%	**1.40 (1.04–1.88)**	**.03**	1.12 (.72–1.76)	**1.66 (1.12–2.46)**	.20
PMA	IL-1β	91%	1.20 (.91–1.57)	.19	1.15 (.76–1.73)	1.25 (.87–1.79)	.76
	IL-6	79%	**1.32 (1.02–1.69)**	**.03**	1.32 (.90–1.92)	1.32 (.95–1.84)	.98
	TNF-α	61%	1.24 (.95–1.63)	.11	1.23 (.81–1.86)	1.26 (.88–1.79)	.93
	IL-5	89%	0.91 (.70–1.19)	.51	0.98 (.66–1.46)	0.87 (.62–1.23)	.66
	IL-10	98%	1.05 (.84–1.30)	.69	1.11 (.80–1.55)	1.00 (.75–1.34)	.65
	IL-17[a]	46%	1.18 (.99–1.41)	.06	1.10 (.86–1.41)	1.26 (.98–1.61)	.45
	IFN-γ[a]	20%	**1.13 (1.03–1.25)**	**.01**	1.09 (.95–1.25)	**1.17 (1.02–1.34)**	.50
PPD	IL-1β	94%	**1.47 (1.16–1.85)**	**.001**	1.29 (.90–1.83)	**1.63 (1.19–2.21)**	.33
	IL-6	57%	**1.40 (1.18–1.66)**	**<.001**	1.27 (.98–1.64)	**1.51 (1.20–1.89)**	.33
	TNF-α	96%	**1.62 (1.39–1.89)**	**<.001**	**1.62 (1.29–2.04)**	**1.63 (1.33–1.99)**	.98
	IL-5	94%	**2.36 (1.98–2.81)**	**<.001**	**2.10 (1.61–2.73)**	**2.58 (2.05–3.26)**	.25
	IL-10	98%	**1.21 (1.02–1.43)**	**.03**	1.08 (.83–1.39)	**1.32 (1.05–1.65)**	.24
	IL-17	98%	**2.81 (2.32–3.40)**	**<.001**	**2.35 (1.76–3.13)**	**3.23 (2.51–4.16)**	.10
	IFN-γ	69%	**13.96 (9.98–19.52)**	**<.001**	**9.27 (5.63–15.27)**	**18.89 (12.17–29.33)**	**.03**
Pam	IL-1β	95%	**1.36 (1.13–1.63)**	**.001**	1.07 (.81–1.41)	**1.63 (1.28–2.08)**	**.02**
	IL-6[a]	22%	**1.13 (1.02–1.25)**	**.02**	1.16 (.99–1.36)	1.10 (.96–1.26)	.62
	TNF-α	85%	**1.29 (1.12–1.48)**	**<.001**	**1.28 (1.04–1.58)**	**1.30 (1.08–1.56)**	.93
	IL-5	98%	0.99 (.84–1.16)	.86	0.95 (.75–1.22)	1.02 (.82–1.26)	.71
	IL-10	89%	1.11 (.96–1.30)	.16	1.09 (.87–1.37)	1.13 (.93–1.39)	.80
	IL-17	98%	1.03 (.89–1.18)	.74	0.96 (.77–1.19)	1.08 (.89–1.31)	.41
	IFN-γ	84%	**1.39 (1.04–1.86)**	**.03**	1.09 (.70–1.69)	**1.68 (1.14–2.46)**	.15
LPS	IL-1β[a]	42%	1.14 (.97–1.34)	.10	1.08 (.84–1.39)	1.19 (.96–1.46)	.58
	IL-6[a]	14%	1.01 (.94–1.09)	.72	1.02 (.90–1.16)	1.01 (.91–1.11)	.81
	TNF-α[a]	34%	1.01 (.88–1.15)	.88	1.02 (.84–1.25)	1.00 (.84–1.19)	.86
	IL-5	97%	0.99 (.86–1.13)	.86	0.95 (.77–1.17)	1.02 (.85–1.23)	.63
	IL-10	56%	1.02 (.84–1.24)	.84	1.06 (.79–1.43)	0.99 (.76–1.28)	.71
	IL-17	98%	0.96 (.82–1.11)	.56	0.88 (.70–1.10)	1.03 (.84–1.25)	.30
	IFN-γ	57%	1.21 (.86–1.71)	.27	1.17 (.69–1.98)	1.25 (.79–1.96)	.86
CL075	IL-1β	85%	1.11 (.88–1.38)	.38	1.15 (.83–1.61)	1.06 (.79–1.43)	.72
	IL-6	61%	1.05 (.86–1.27)	.65	1.04 (.78–1.40)	1.04 (.80–1.35)	.99
	TNF-α	71%	1.08 (.86–1.36)	.49	1.16 (.83–1.63)	1.01 (.75–1.38)	.56
	IL-5	83%	0.89 (.76–1.05)	.18	0.95 (.74–1.21)	0.85 (.68–1.06)	.53
	IL-10	99%	0.85 (.71–1.01)	.07	1.01 (.78–1.33)	**0.73 (.57–.93)**	.07
	IL-17	93%	0.90 (.73–1.12)	.34	0.83 (.60–1.14)	0.97 (.73–1.30)	.46
	IFN-γ[a]	34%	1.03 (.88–1.21)	.68	1.12 (.88–1.42)	0.97 (.79–1.19)	.38

Estimates presented as geometric mean ratios (GMR) comparing BCG-vaccinated to non-vaccinated infants, adjusted for weight at randomization. Estimates with a significance of $P < .05$ are in bold. The proportion of observations within the detection range of the cytokine assay is indicated (Obs in range).

Abbreviations: BCG, bacillus Calmette–Guerin; CI, confidence interval; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PPD, purified protein derivative; TNF, tumor necrosis factor.

[a] Due to >50% of measurements being outside the assay working range the estimate is obtained by Poisson regression presenting the relative risk of having a measurement above the upper detection limit of the assay.

* Test of interaction between BCG and sex, adjusted for weight at enrolment.

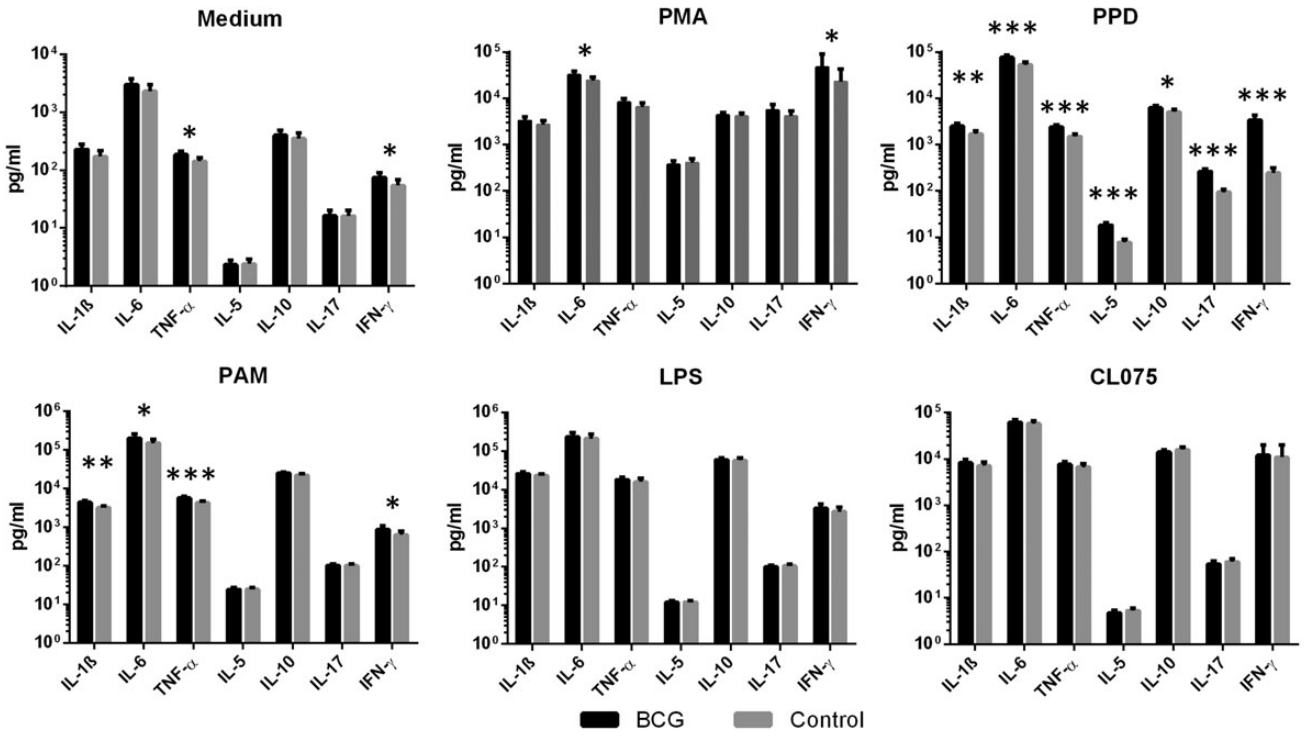

Figure 2. Geometric means (GM) of in vitro cytokine concentrations for BCG-vaccinated and non-vaccinated infants, estimated by use of Tobit regression. Note the log-scale of the axis. Observations outside the assay range are included as missing in the model producing the GM estimates. Responses of IL-17 and IFN-γ to PMA/ionomycin; IL-6 to Pam3CSK4; IL-1β, IL-6 and TNF-α to LPS; and IFN-γ to CL075 have >50% of observations outside assay range; hence the estimated GM for these are subject to some uncertainty. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: BCG, bacillus Calmette–Guerin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor.

were prioritized for logistic reasons. Furthermore, a larger proportion of infants in the immunology study were enrolled during the rainy season ($P < .01$). This skewed seasonal distribution reflects that for logistical reasons fewer infants were enrolled in the immunological study during the first and last few weeks of the study, which fell in the dry season.

BCG Effect on In Vitro Cytokine Production

For all cytokines, BCG vaccination was associated with a significantly higher response to PPD (Table 2 and Figure 2). Particularly the IFN-γ response was strongly increased (geometric mean ratio [GMR]: 13.96 [95% CI: 9.98–19.52]).

For most of the non-PPD stimulation, BCG was associated with increased production of several cytokines. The differences reached statistical significance for the production of IL-1β (GMR: 1.36 [95% CI, 1.13–1.63]), IL-6 (prevalence ratio (PR): 1.13 [95% CI, 1.02–1.25), TNF-α (GMR: 1.29 [95% CI, 1.12–1.48), and IFN-γ (GMR: 1.39 [95% CI, 1.04–1.86) to the TLR2/1 agonist Pam3CSK4, and for IL-6 (GMR: 1.32 [95% CI, 1.02–1.69) and IFN-γ (PR: 1.13 [95% CI, 1.03–1.25) to the positive control PMA/ionomycin. Responses of TNF-α (GMR: 1.30 [95% CI, 1.05–1.60]) and IFN-γ (GMR: 1.40 [95% CI, 1.04–1.88) to the culture medium control were also significantly higher in the BCG-vaccinated group.

BCG had no significant effect on heterologous IL-5 or IL-10 responses. BCG was not associated with a reduced response for any of the cytokine outcomes (Table 2).

When analyzing the responses to all non-specific stimuli collectively, BCG was found to increase responses of IL-1β (GMR: 1.25 [95% CI, 1.04–1.49), IL-6 (GMR: 1.26 [95% CI, 1.04–1.53), TNF-α (GMR: 1.21 [95% CI, 1.05–1.39), and IFN-γ (GMR: 1.31 [95% CI, 1.02–1.69]) (Figure 3). IL-10 responses could not be analyzed collectively in the present analysis, as the estimates of the main effect of BCG for the different stimulations were too heterogeneous.

Sensitivity Analysis

To test the robustness of our model, we performed a sensitivity analysis censoring the 19% heaviest-at-enrolment infants in the intervention group. This censoring did not change the conclusion regarding the BCG effects on cytokine responses described above (Supplementary Table 1). Moreover, including adjustment for monocyte or lymphocyte counts, respectively, did not produce estimates substantially different from those presented in Table 2 (data not shown).

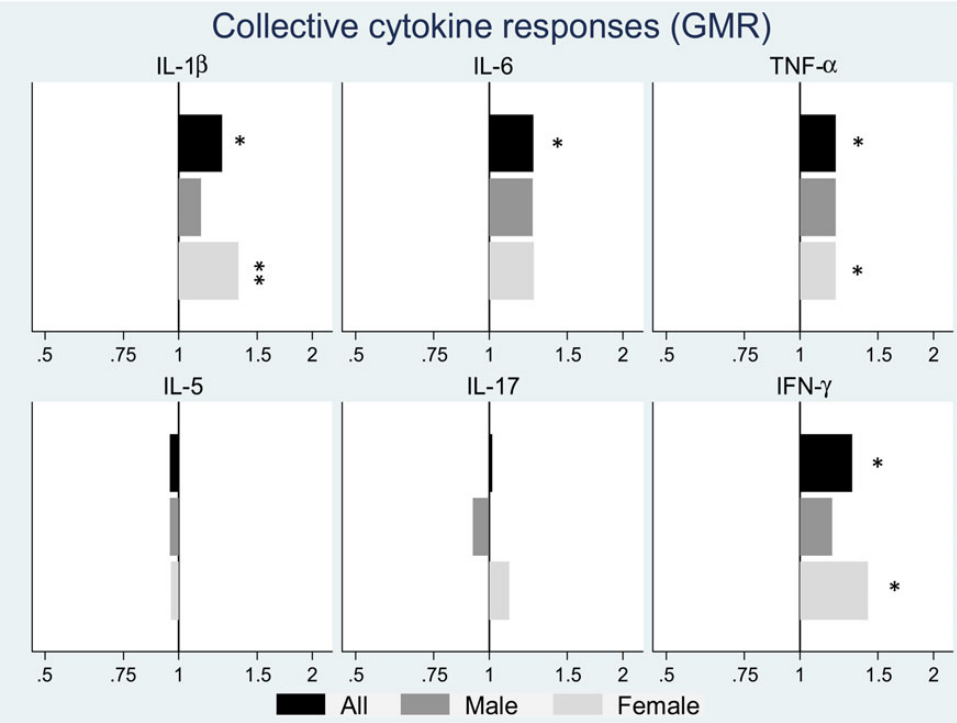

Figure 3. Geometric mean ratio (GMR) of in vitro cytokine production, comparing BCG-vaccinated to nonvaccinated overall and stratified by sex. The cytokine concentrations are analyzed collectively for all innate stimuli (medium alone, PMA/ionomycin, Pam3CSK4, LPS, CL075, excluding PPD). Estimates are adjusted for weight at randomization. For IL-10 a collective estimate could not be obtained due to rejected test of homogeneity of estimates of the BCG effect across the stimulations. A GMR >1 may be interpreted as an increasing effect of BCG on the outcome. $^{*}P < .05$; $^{**}P < .01$. Abbreviations: BCG, bacillus Calmette–Guerin; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PPD, purified protein derivative; TNF, tumor necrosis factor.

BCG Effect on Cytokine Ratios

The effect of BCG was larger in respect to TNF-α than IL-10, as BCG was associated with a significantly higher ratio of TNF-α to IL-10 responses to PPD (geometric mean ratio-ratio (GMRR): 1.34 [95% CI, 1.17–1.54]), Pam3CSK4 (GMRR: 1.16 [95% CI, 1.02–1.31]), and CL075 (GMRR: 1.30 [95% CI, 1.06–1.59]) (Figure 4).

BCG was also associated with a higher ratio of IFN-γ to IL-5 responses in the culture medium alone (GMRR: 1.41 [95% CI, 1.05–1.90]), PMA/ionomycin (GMRR: 1.70 [95% CI, 1.22–2.38]), PPD (GMRR: 4.76 [95% CI, 3.63–6.23]), and Pam3CSK4 (GMRR: 1.35 [95% CI, 1.03–1.77]) (Figure 4).

Effect Modification by Sex

There may have been a tendency toward a stronger specific, as well as nonspecific effect of BCG in females, although an effect modification by sex was only significant for the IL-1β response to Pam3CSK4 ($P = .03$ for interaction) and IFN-γ responses to PPD ($P = .03$ for interaction; Table 2).

There was no significant sex-difference in the effect of BCG on collective nonspecific responses (Figure 3) or the ratios of IFN-γ to IL-5 or TNF-α to IL-10 (data not shown).

Effect Modification by Season

Overall, the association between BCG and cytokine responses tended to be more pronounced in the dry season, although the effect modification by season was only significant for some of the outcomes: a significant effect modification by season on the effect of BCG was found for IL-1β (test for interaction with season $P = .01$), TNF-α ($P = .005$), IL-5 ($P = .01$), IL-17 ($P = .04$), and IFN-γ ($P = .001$) responses to PPD, and IL-17 responses to Pam3CSK4 ($P = .03$) and to the TLR4-agonist LPS ($P = .05$). A seasonal effect modification was also found for the basal secretion of IL-1β ($P = .04$), IL-6 ($P = .03$), TNF-α ($P = .01$), IL-10 ($P = .04$), and IFN-γ ($P = .02$) in the culture medium control (Supplementary Table 2). Analyzed collectively, there was a significant interaction between season and BCG for TNF-α ($P = .04$) and IL-17 ($P = .02$) responses (Figure 5).

BCG Effect on Differential Counts

Overall, BCG was not significantly associated with changes in the differential cell counts, although a borderline significant increasing effect was seen for monocytes (GMR: 1.09 [95% CI, 1.00–1.18]). In females, an effect of BCG was found for total leukocytes (GMR: 1.07 [95% CI, 1.01–1.12]), monocytes

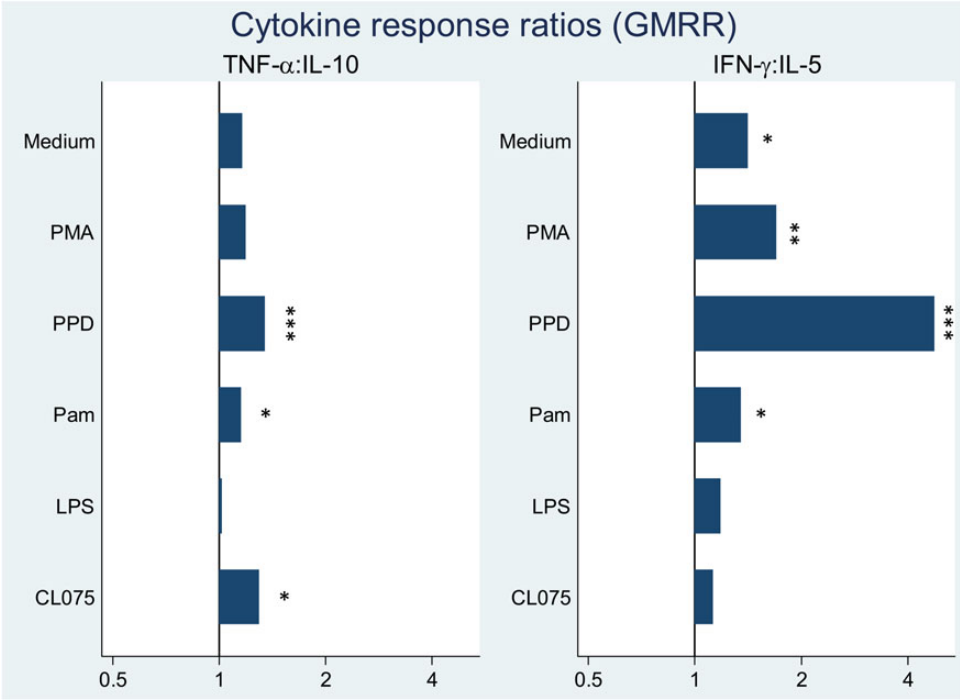

Figure 4. Geometric mean ratio-ratio (GMRR) of TNF-α vs IL-10 and IFN-γ vs IL-5 in vitro cytokine production, comparing BCG-vaccinated to nonvaccinated. Estimates are adjusted for weight at randomization. A GMRR >1 may be interpreted as an increasing effect of BCG on the ratio. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: BCG, bacillus Calmette–Guerin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PPD, purified protein derivative; TNF, tumor necrosis factor.

(GMR: 1.10 [95% CI, 1.00–1.20]) and basophils (GMR: 1.21 [95% CI, 1.04–1.40]), whereas there was no effect in males (Supplementary Table 3).

DISCUSSION

BCG vaccination has been shown to induce beneficial protective effects on neonatal mortality through protection against other infections than tuberculosis, but the mechanisms mediating this protection are not known. In the present study, BCG vaccination of West African LBW neonates increased the in vitro cytokine responses to specific as well as nonspecific stimuli, including baseline (unstimulated) cytokine production. BCG increased the responses of Th1-polarizing cytokine (IFN-γ), increased more pro-inflammatory than anti-inflammatory responses (TNF-α : IL-10 ratio), and induced a higher production of cytokines typically derived from monocytes (IL-1β, IL-6, TNF-α). There may have been a tendency of BCG having stronger effects on cytokine responses when immunized in the dry season. The effects were more frequently seen for females than males, although the effect modification by sex was rarely significant.

Strengths and Limitations of the Study

In contrast to several previous studies on the effect of BCG, in this randomized study we were able to study a BCG-naive control group. However, for ethical reasons we could not control the timing of the vaccinations after enrollment; 19% of the infants in the control group received BCG before bleeding and hence were excluded from the analysis. These infants were generally larger at randomization, and their exclusion could have partially biased the results. However, adjustment for enrollment weight did not change the estimates of the BCG effect. In addition, the sensitivity analysis censoring the 19% heaviest infants in the BCG arm did not change the estimates of the BCG effect. Hence, the exclusion of these infants in the control group did not seem to bias our results.

The whole blood assay did not enable a resolution of the cellular sources of the cytokine production; hence the distinction between monocyte-derived innate (Il-1β, IL-6, TNF-α) from T-cell derived adaptive (IL-5, IL-10, IL-17, IFN-γ) responses or a Th1- vs Th2-polarization is merely an assumption and should be interpreted as such. The responses to LPS were relatively high with many observations above the assay detection range. This may have reduced the sensitivity of the analysis to detect effects for this particular stimulation. It should be borne in mind that multiple testing was not adjusted for in the analyses, and the results should therefore be interpreted with caution. However, the effects of BCG vaccination toward a pro-inflammatory cytokine profile was seen for many of the stimuli and consistently so for the cytokines typically derived from monocytes, strongly suggesting

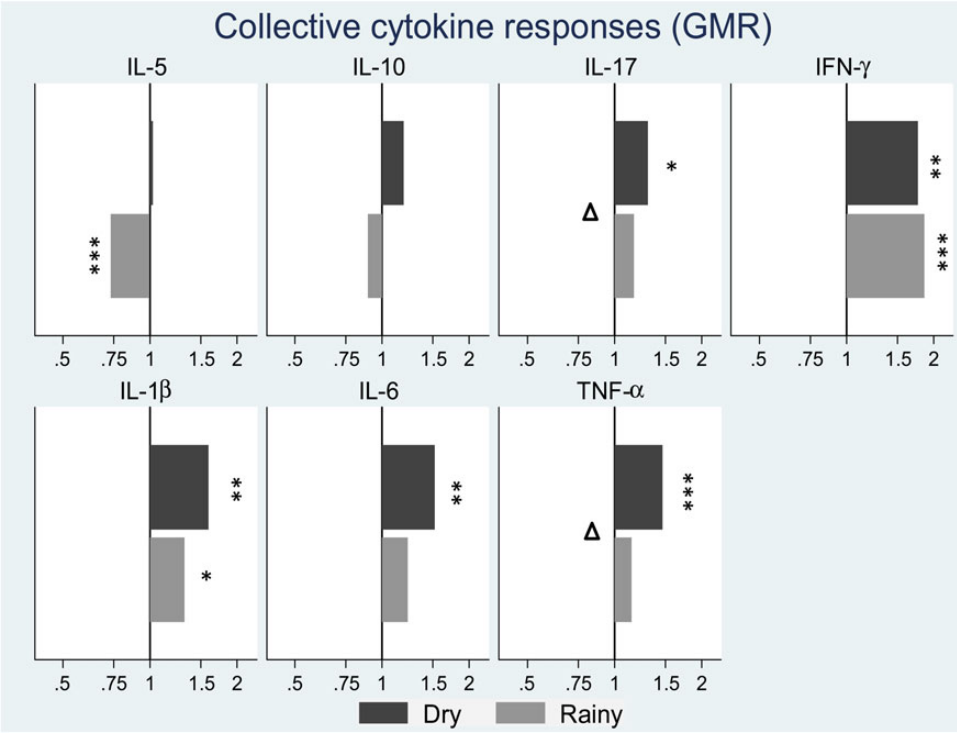

Figure 5. Geometric mean ratio (GMR) of in vitro cytokine production, comparing BCG-vaccinated to nonvaccinated stratified by season at randomization. The cytokine concentrations are analyzed collectively for all innate stimuli (medium alone, PMA/ionomycin, Pam3CSK4, LPS, CL075, excluding PPD). Estimates are adjusted for weight at randomization. A GMR >1 may be interpreted as an increasing effect of BCG on the outcome. A significant interaction ($P < .05$) between BCG and season is indicated with a triangle. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: BCG, bacillus Calmette–Guerin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PPD, purified protein derivative; TNF, tumor necrosis factor.

that the effects observed are a true biological phenomenon. The randomization was nonblinded to the mothers and the field team members. However, the technicians processing the samples at all stages of the laboratory work were blinded to the randomization.

Previous Studies and Potential Mechanisms

Studies on effects of BCG on heterologous cytokine responses in infants are few, heterogeneous in design, and frequently fail to include a comparable control group; some studies found little or no effect [17, 18], whereas anti-inflammatory [19], nonbiased [20, 21], or pro-inflammatory effects [22] have been suggested. A mixed Th1 and Th2 cellular response to other vaccine antigens was found in Gambian infants [23]. On a molecular level, BCG vaccination has recently been found to induce epigenetic reprogramming of monocytes in adults by an increased H3K4-tri-methylation in the promoter regions of TLR4, IL-6, and TNF-α. This change in the cellular epigenetic program, also termed "trained innate immunity" [24], was found to be imperative for the increased inflammatory responsiveness of monocytes to heterologous stimulation by a broad array of pathogens. Both epigenetic changes and increased heterologous responses could be detected 3 months after BCG vaccination [25] and even 1 year after BCG vaccination, albeit at lower levels [26]. The present study suggests that these effects are also found in infants; BCG vaccination seemed to induce both a Th1-polarizing (IFN-γ) innate response and an increased pro-inflammatory cytokine response (IL-1β, IL-6, and TNF-α) in Guinean 4-week-old infants, including an increased baseline cytokine production.

The observation that more outcomes were found significantly affected by BCG in females than males could be associated with a difference in power due to the relatively higher number of enrolled females in the study. This, however, may not be the only factor. The possible sex-differential effects of BCG on mortality may be age-dependent. The previous smaller RCT found that males had a particular benefit from BCG within the first weeks of life [7], corroborated by preliminary data from the main trial. The present immunological evaluation 4 weeks after randomization may therefore have missed the strongest effects of BCG for males and may explain why the effects tended to be stronger in females.

Importantly, the present study aligns well with key observations on mortality and morbidity from the previous epidemiological studies and RCTs of BCG. First, the rapid survival

benefit after vaccination indicates that the innate immunity is enhanced by BCG [7], and the present study demonstrates a potentiating effect by BCG on innate cytokine responses. Second, BCG particularly reduces sepsis mortality [8] and the enhanced pro-inflammatory cytokine responses to innate stimulation may facilitate an appropriate immunological response to bacterial insult. Third, the strongest protective effect of BCG against all-cause mortality may take place within the first few weeks after administration in males, but is delayed in females [7], and the present study suggests that 1 month after BCG vaccination, the immunological effects of BCG are stronger in females than males.

In conclusion, BCG vaccination of LBW infants has significant effects on the heterologous cytokine responsiveness primarily by means of increased Th1-related and inflammatory innate immune responses ("trained innate immunity"). Neonatal BCG may thus contribute to the maturation of the infant immune system, and this may account for beneficial effects of BCG against heterologous infectious challenge.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. The Bandim Health Project is a department at the Statens Serum Institut (SSI). None of the investigators is on the payroll of SSI. SSI is the producer of the BCG vaccine used in the present study. However, SSI was not involved in the design of the study, data collection, data analysis, or the writing of the article. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Benn CS, Netea MG, Selin LK, Aaby P. A small jab—a big effect: Non-specific immunomodulation by vaccines. Trends Immunol **2013**; 34:431–9.
2. Shann F. Nonspecific effects of vaccines and the reduction of mortality in children. Clin Ther **2013**; 35:109–14.
3. Roth A, Jensen H, Garly M-L, et al. Low birth weight infants and Calmette-Guerin bacillus vaccination at birth: Community study from Guinea-Bissau. Pediatr Infect Dise J **2004**; 23:544–50.
4. Kristensen I, Aaby P, Jensen H. Routine vaccinations and child survival: Follow-up study in Guinea-Bissau, West Africa. BMJ **2000**; 321:1435–8.
5. Roth A, Gustafson P, Nhaga A, et al. BCG vaccination scar associated with better childhood survival in Guinea-Bissau. Int J Epidemiol **2005**; 34:540–7.
6. Garly ML, Martins CL, Bale C, et al. BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: A non-specific beneficial effect of BCG? Vaccine **2003**; 21:2782–90.
7. Biering-Sorensen S, Aaby P, Napirna BM, et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. Pediatr Infect Dis J **2012**; 31:306–8.
8. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis **2011**; 204:245–52.
9. Skogstrand K, Thorsen P, Norgaard-Pedersen B, et al. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem **2005**; 51:1854–66.
10. Jorgensen MJ, Fisker AB, Sartono E, et al. The effect of at-birth vitamin A supplementation on differential leucocyte counts and in vitro cytokine production: An immunological study nested within a randomised trial in Guinea-Bissau. Br J Nutr **2013**; 109:467–77.
11. Andersen A. Statistical Analysis of Population-Based Immunological Studies: Faculty of Health and Medical Sciences. Copenhagen: University of Copenhagen, **2012**.
12. Mooney CZ, Duval RD. Bootstrapping: A nonparametric approach to statistical inference. Newbury Park, CA: Sage, **1993**.
13. Stensballe LG, Nante E, Jensen IP, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: A beneficial effect of BCG vaccination for girls community based case-control study. Vaccine **2005**; 23:1251–7.
14. Valentiner-Branth P, Perch M, Nielsen J, et al. Community cohort study of *Cryptosporidium parvum* infections: Sex-differential incidences associated with BCG and diphtheria-tetanus-pertussis vaccinations. Vaccine **2007**; 25:2733–41.
15. Roth A, Sodemann M, Jensen H, et al. Vaccination technique, PPD reaction and BCG scarring in a cohort of children born in Guinea-Bissau 2000–2002. Vaccine **2005**; 23:3991–8.
16. Miles DJ, van der Sande M, Crozier S, et al. Effects of antenatal and postnatal environments on CD4 T-cell responses to *Mycobacterium bovis* BCG in healthy infants in the Gambia. Clin Vaccine Immunol **2008**; 15:995–1002.
17. Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guerin vaccination. J Immunol **1999**; 163:2249–55.
18. Djuardi Y, Sartono E, Wibowo H, Supali T, Yazdanbakhsh M. A longitudinal study of BCG vaccination in early childhood: The development of innate and adaptive immune responses. PLoS One **2010**; 5: e14066.
19. Jason J, Archibald LK, Nwanyanwu OC, et al. Clinical and immune impact of *Mycobacterium bovis* BCG vaccination scarring. Infect Immun **2002**; 70:6188–95.
20. Akkoc T, Aydogan M, Yildiz A, et al. Neonatal BCG vaccination induces IL-10 production by CD4+ CD25+ T cells. Pediatr Allergy Immunol **2010**; 21:1059–63.

21. Libraty DH, Zhang L, Woda M, et al. Neonatal BCG vaccination is associated with enhanced T-helper 1 immune responses to heterologous infant vaccines. Trials Vaccinol **2014**; 3:1–5.
22. Lalor Maeve K, Ben-Smith A, Gorak-Stolinska P, et al. Population differences in immune responses to bacille Calmette-Guérin vaccination in infancy. J Infect Dis **2009**; 199:795–800.
23. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. J Immunol **2002**; 168: 919–25.
24. Netea MG. Training innate immunity: The changing concept of immunological memory in innate host defence. Eur J Clin Invest **2013**; 43:881–4.
25. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A **2012**; 109:17537–42.
26. Kleinnijenhuis J, Quintin J, Preijers F, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun **2014**; 6:152–8.