

Neonatal innate cytokine responses to BCG controlling T-cell development vary between populations

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Background: The protective effect of *Mycobacterium bovis* BCG vaccination against infection and atopy varies between populations.

Objective: To identify differences in neonatal responses to BCG between diverse populations and study longitudinal associations with memory T-cell responses.

Methods: Cord blood mononuclear cells were collected from Papua New Guinean (PNG) and Western Australian (WA) newborns. Toll-like receptor (TLR)-2, TLR4, and TLR9 mRNA expression and *in vitro* BCG-stimulated ($\pm$ IFN- γ priming) innate cytokine responses were compared. When PNG infants were 3 months old, PBMCs were stimulated *in vitro* with *Mycobacterium*-purified protein derivative (PPD) to determine memory T-cell responses.

Results: BCG-induced IL-10 and IFN- γ responses were significantly higher in cord blood mononuclear cells of PNG newborns, and TLR2 and TLR9 expression was significantly higher and TLR4 expression lower compared with WA newborns. High neonatal IL-10 and low IFN- γ responses to BCG were found to promote the development of PPD-memory

T_H2 responses in infancy, whereas neonatal BCG-TNF α responses inhibited the development of PPD-IL 10 responses. When primed with IFN- γ , BCG-induced TNF- α , IL-12p70, and in particular IFN- γ responses were enhanced to a significantly higher extent in WA than in PNG newborns. In response to IFN- γ priming and BCG stimulation, natural killer cells of WA newborns produced IFN- γ , whereas natural killer cells of PNG newborns contributed only indirectly to this response.

Conclusion: Neonatal BCG-related innate immune responses control the differentiation of T_H memory responses and vary between populations. This may explain differences in the effects of BCG vaccination between populations. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: BCG, neonatal, allergy, atopy, innate immune system, TLR, IL-10, IFN- γ , T_H1, T_H2, Papua New Guinea

Mycobacterium bovis BCG is given to millions of neonates in low-income countries as a vaccine against *Mycobacterium tuberculosis*. Some studies suggest that BCG can also protect against the development of atopic disease, but findings have been inconsistent.¹⁻⁵ Interestingly, from these studies a pattern is emerging that the allergeo-protective effects of BCG are more evident in children from low-income countries and less so in children from high-income countries. Together with evidence that the efficacy of BCG to protect against *M tuberculosis* infections also varies between populations,⁶⁻⁸ this suggests that the effects of BCG on the developing immune system are influenced by genetic and/or environmental factors.

Toll-like receptors (TLRs) are key receptors for the recognition of pathogens and activate cells of the innate immune system, thereby also orchestrating downstream adaptive immune responses. BCG is known to activate a number of these pathways, including TLR2, TLR4, and putatively TLR9.^{9,10} TLR expression and function can be modulated by genetic variation¹¹ and environmental factors including maternal smoking and microbial exposure.¹²⁻¹⁴ We therefore postulate that differences in TLR-mediated innate immune responses are responsible for the variation in BCG responses reported between children in high-income and low-income countries.

The role of the innate immune system in BCG-induced protection against atopy can be explained by different mechanisms. The first is based on the production of IL-12. Although the neonatal immune system has a reduced capacity to stimulate IL-12 responses that drive maturation of T_H1 responses,¹⁵ these deficiencies can be overcome by potent microbial immunostimulants such as BCG vaccination.^{16,17} The consequently early development of T_H1

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Abbreviations used

CBMC:	Cord blood mononuclear cell
IFN γ R:	IFN- γ receptor
LPS:	Lipopolysaccharide
LTA:	Lipoteichoic acid
GM:	Geometric mean
NK:	Natural killer
PNG:	Papua New Guinea
PPD:	<i>Mycobacterium</i> -purified protein derivative
SEGM:	SE of the geometric mean
TLR:	Toll-like receptor
WA:	Western Australia

responsiveness has been proposed to counteract atopy-associated T_H2 responses.^{18,19} A second explanation involves the production of IL-10 is under conditions of persistent immune challenge that has been inversely associated with the development of atopy.^{20,21} It has been suggested that BCG preferentially stimulates dendritic cells to produce IL-10, and not IL-12, which primes naive T cells to develop into IL-10-producing T cells without a strong T_H1 or T_H2 bias.^{22,23} We therefore postulated that the ability of the neonatal innate immune system to produce IL-10, IL-12, and other key factors that influence T_H-cell differentiation determines the longer-term outcomes of neonatal BCG vaccination.

In this study, we aimed to identify major differences in BCG-related neonatal innate immune function in children born in a high-income (Western Australia; WA) versus low-income country (Papua New Guinea; PNG) and verify the influence of neonatal innate immune responses on the subsequent development of memory T-cell responses. In addition, because *in vivo* the neonatal innate immune system may experience a certain level of IFN- γ priming that could be relevant for responses in this age group,²⁴ we compared the responsiveness of PNG and WA cord blood mononuclear cells (CBMCs) to BCG after *in vitro* IFN- γ priming.

METHODS**Study populations**

Pregnant women were recruited in PNG through Goroka Hospital (n = 50) and in WA through private/state antenatal clinics in the metropolitan area of Perth (n = 50). Goroka is highly endemic for respiratory pathogens, but because of its location at high altitude (1500-1900 m above sea level), the prevalence of malaria infections is low. Inclusion criteria for enrolment of newborns were an uncomplicated pregnancy, a birth weight of at least 2000 g, no acute neonatal infection, and no severe congenital abnormality. Although antenatal HIV testing is standard in WA, it is recommended but not routine in PNG. Children of mothers who were known to be HIV-positive were excluded. Differences in obstetrics and newborn characteristics are further described in Table I. PNG (and not WA) newborns received BCG vaccination within the first 3 days of life, and a venous blood sample to determine memory T-cell responses was collected when they were 3 months old.

A control group of 15 healthy adult volunteers was recruited from WA (6 men, 9 women; mean age, 31 years).

Written informed consent was obtained from parents and volunteers. Ethical approval was obtained from the PNG Medical Research Advisory Council and the Ethics Committee of the Princess Margaret Hospital for Children, Perth, WA.

Cell isolations

Umbilical cord blood samples (2 $\times$ 20 mL) were collected in sterile tubes containing an equal volume of RPMI 1640 (Invitrogen-Life Technologies,

Melbourne, Australia) and preservative-free heparin (20 U/mL). Venous blood samples (2 mL) were collected from the PNG infants in 100 U preservative-free heparin. CBMCs and PBMCs were isolated by Ficoll-Hypaque (Alexis-Shield, Oslo, Norway) gradient centrifugation and cryopreserved in 50% heat-inactivated FCS (JRH Biosciences, Lenexa, Ks) and 7.5% dimethyl sulfoxide. Identical protocols and consumables were used in both settings. Cells from PNG study children were transported to Perth for further experiments.

***In vitro* CBMC and PBMC cultures**

Cord blood mononuclear cells and adult PBMCs were cultured in a concentration of 1×10^6 cells/mL with 1640 RPMI/10% non-heat inactivated FCS in 96-well plates with medium alone (control), BCG (5×10^4 colony forming units/mL; Aventis Pasteur, Toronto, Canada) in the absence or presence of recombinant human IFN- γ (0.5 μ g/mL; BD Biosciences Pharmingen, San Diego, Calif), Staphylococcus aureus lipoteichoic acid (LTA) (Invivogen, San Diego, Calif) (2 μ g/mL) or Escherichia coli lipopolysaccharide (LPS) (Alexis biochemicals, San Diego, Calif) (5 μ g/mL). mRNA expression was measured in cells cultured for 6 hours (LTA and LPS) or 24 hours (BCG), and cytokine protein levels were measured in supernatants after 24 hours (LTA and LPS) or 48 hours (BCG) of culture. PNG infant PBMCs were cultured in a concentration of 1×10^6 cells/mL with 1640 RPMI/5% heat-inactivated human AB serum (Invitrogen-Life Technologies) in 96-well plates with medium alone (control) and *Mycobacterium*-purified protein derivative (PPD, CSL Limited, Parkville, Australia; 10 μ g/mL) for 96 hours.

Cytokine protein detection

IFN- γ , TNF- α , IL-12p70, IL-6, IL-10, IL-5, and IL-13 levels were measured in supernatants by in-house time-resolved fluorometry, as described elsewhere.²⁵ Samples with concentrations below the detection limit were given the value corresponding to half the lowest concentration that could be detected in this set of samples.

Quantitative RT-PCR

Total RNA was isolated from cells by using the RNAqueous 96-well kit (Ambion, Austin, Tex). Reverse transcription was performed by using the Omniscript kit (Qiagen, Valencia, Calif) with oligo-dT (Promega, Madison, Wis) and Superscript (GeneWorks, Hindmarsh, Australia). Intron-spanning primers were obtained from <http://pga.mgh.harvard.edu/primerbank> and designed in-house by using Primer Express Software (Applied Biosystems, Carlsbad, Calif). Reverse-transcribed RNA samples were diluted 1:5 and quantitated by real-time PCR using QuantiTect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems). Copy numbers were determined by 10-fold serial dilutions of plasmid standards and normalized to the reference gene ubiquitin-conjugating enzyme E2D 2 (Geneworks).

Flow cytometry

Brefeldin A (5 μ g/mL; Sigma-Aldrich, Steinheim, Germany) was added for the last 16 hours of culture. Cells were diluted in fluorescence-activated cell sorting buffer (1×10^6 cells/mL), incubated on ice with 20 μ L anti-CD3, CD56, CD4, or CD8 (diluted between 1:2 and 1:200), washed, and fixed overnight with formaldehyde (Univar, Seven Hills, Australia, 0.4% in PBS) at 4°C. Cells were washed and briefly incubated in perm-wash (0.1% saponin [Sigma-Aldrich], 0.5% BSA in PBS) before adding anti-IFN- γ -fluorescein isothiocyanate (BD Pharmingen) and run through a 4-color FACSCalibur (BD Biosciences). Results were analyzed by using FlowJo software (Tree Star Inc, Stanford, Calif).

Statistical methods

Because cytokine concentrations were not normally distributed, data were log-transformed, and nonparametric tests were used to test for significant differences between groups (Mann-Whitney), within groups (Wilcoxon

TABLE I. Obstetrics and newborn characteristics

	PNG	WA	<i>P</i> value
Mean age of mother (y)	26.4 ± 5.4	32.9 ± 4.6	<.001
Gravida			
1 (%)	38	43	
2 (%)	23	19	
3 or more (%)	39	38	.898
Delivery by cesarean (%)	0	39	<.001
Premature bleeding (%)	0	2	.467
Antibiotic use in pregnancy (%)	0	10	.037
Fetal distress (%)	8	2	.367
Stained liquor amnii (%)	2	0	.533
Male newborn (%)	56	46	.359
Mean gestational age (wk)	39.5 ± 1.6	39.5 ± 1.3	.829
Mean birth weight (kg)	3.3 ± 0.6	3.5 ± 0.4	.214
Mean birth length (cm)	50 ± 3.2	51 ± 2.3	.591
Mean head circumference (cm)	33 ± 1.6	35 ± 1.2	<.001
Antibiotic use, newborn (%)	0	5	.210
Oxygen use, newborn (%)	4	5	.859

Continuous variables are expressed as means ± SDs.

signed-ranks test), or for correlations (Spearman ρ correlation). Pearson χ^2 and Fisher exact tests (when cells contained numbers equal or lower than 5) were performed to test for differences in prevalence. Multivariate linear regression analysis was used to study associations between receptor expressions and neonatal innate cytokine responses, and associations between neonatal innate BCG responses and T_H -cell cytokine responses at 3 months of age. Differences were considered to be statistically significant when *P* values were less than .05. All analyses were performed by using the statistical package SPSS 15.0 (SPSS Inc., Chicago, Ill).

RESULTS

Population characteristics

Obstetric and newborn characteristics are described in Table I. Significant differences between PNG and WA newborns included the age of the mothers, prescription of antibiotics to mothers during pregnancy, birth by cesarean section, and neonatal head circumferences. Thirty-one percent of the WA study mothers were atopic as determined by a positive skin prick test to common allergens, whereas the atopic status of the PNG mothers was unknown. Furthermore, PNG but not WA mothers were vaccinated against tetanus in the third trimester of pregnancy according to standard clinical procedures in PNG (60% received 1 dose, 24% 2 doses, 10% unknown).

Differences in BCG-related neonatal innate immune function

Differences between adult PBMCs and PNG and WA CBMCs are summarized in this article's Table E1 in the Online Repository at www.jacionline.org. In line with reports for LPS responses in adults and newborns,^{24,26} IFN- γ and TNF- α responses to BCG were significantly lower in WA and PNG CBMCs than adult PBMCs, whereas IL-6 responses were similar.

As shown in Fig 1, A, PNG CBMCs produced significantly more IL-10 (*P* = .002) and IFN- γ (*P* = .017) in response to BCG than WA CBMCs. In contrast, stimulation with the classic TLR ligands LTA and LPS that bind to a single TLR (TLR2 and TLR4, respectively), whereas BCG signals through both (and others) simultaneously, induced significantly higher

IL-6, IL-10, and TNF- α responses in WA than PNG CBMCs (Table E1).

The expression of TLR2 (*P* = .001) and TLR9 (*P* = .005) mRNA was significantly higher and that of TLR4 (*P* < .001) significantly lower in resting CBMCs of PNG than WA newborns (Fig 1, B).

Within the WA study population, differences were found in relation to the mode of delivery and maternal atopy. BCG-IFN- γ responses were significantly higher when children were born by cesarean section (geometric mean [GM] ± SE of the GM [SEGM], 55.5 ± 27.3 pg/mL) compared with natural delivery (GM ± SEGM, 11.3 ± 4.6 pg/mL; *P* = .047); consequently, the significant difference in BCG-IFN- γ responses between PNG and WA newborns was further increased when adjusting for the mode of delivery (data not shown). In addition, CBMCs of children born by cesarean section expressed significantly higher levels of TLR2 (GM ± SEGM, 59.0 ± 11.7 × 10⁻³ normalized copies) and TLR9 (GM ± SEGM, 11.5 ± 1.8 × 10⁻³ normalized copies), but significantly lower levels of TLR4 (GM ± SEGM, 6.5 ± 1.7 × 10⁻³ normalized copies) than CBMCs of children born by natural delivery (TLR2: GM ± SEGM, 51.2 ± 11.9 × 10⁻³ normalized copies, *P* = .003; TLR9: GM ± SEGM, 9.6 ± 1.6 × 10⁻³ normalized copies, *P* = .024; TLR4: GM ± SEGM, 8.5 ± 1.7 × 10⁻³ normalized copies, *P* = .056). In relation to maternal atopy, CBMCs of WA newborns whose mothers were atopic produced significantly less BCG-TNF- α (GM ± SEGM, 3.6 ± 1.1 pg/mL) and expressed significantly more TLR2 (GM ± SEGM, 81.2 ± 12.9 × 10⁻³ normalized copies) than children born to nonatopic mothers (BCG-TNF- α , GM ± SEGM, 13.3 ± 3.5 pg/mL, *P* = .026; TLR2, GM ± SEGM, 45.6 ± 9.0 × 10⁻³ normalized copies, *P* = .045).

There were no differences in relation to other population characteristics such as number of gravida, maternal age, sex, gestational age, or tetanus vaccination (data not shown).

Next, multivariate linear regression analysis was used to determine associations between neonatal cytokine responses to BCG (dependent variables) and TLR2, TLR4, and TLR9 expression (independent variables, studied simultaneously), while coadjusting for the mode of delivery and the study population. Significant positive associations were found between TLR2 expression and BCG-induced IL-6, IL-10, and TNF- α responses, whereas significant inverse associations were found between TLR4 expression and BCG-induced IL-10, TNF- α , and IFN- γ responses (Table II).

BCG responses under optimal conditions of IFN- γ priming

Exogenous IFN- γ significantly enhanced BCG-induced production of IL-12p70, IFN- γ , and TNF- α within both study groups, but the magnitude of this effect was much stronger for WA than PNG newborns (see this article's Table E2 in the Online Repository at www.jacionline.org). This was most marked for BCG-IFN- γ production itself, which was enhanced 21-fold (median) in PNG newborns but 390-fold (median) in WA newborns (*P* < .001). Although IFN- γ priming significantly increased IL-6 and IL-10 production in WA CBMCs, this was not the case for PNG cells in which responses remained similar or tended to be down-regulated. Under IFN- γ priming conditions, BCG-induced IFN- γ (*P* < .001), IL-12p70 (*P* < .001), TNF- α (*P* < .001), and IL-6

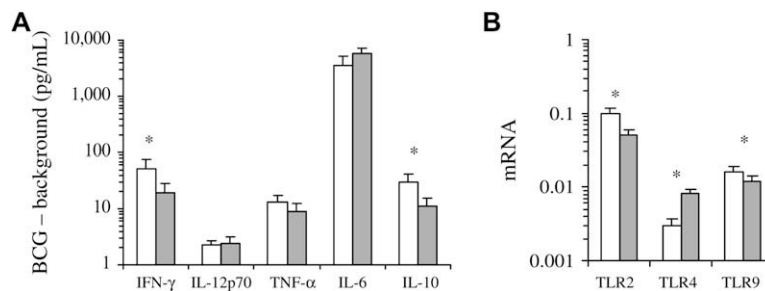


FIG 1. Innate cytokine responses to BCG and TLR expression in CBMCs. CBMCs of PNG newborns (white bars, $n = 47$) and WA newborns (gray bars, $n = 47$; **A**) were stimulated *in vitro* with BCG, and cytokine responses were measured in supernatants (presented as BCG-induced responses minus background levels), and (**B**) TLR2, TLR4, and TLR9 mRNA expression was measured in resting cells. Bars represent GMs and 95% CIs. *Significant differences between PNG and WA newborns are indicated at the .05 level.

TABLE II. Multivariate linear regression analysis for TLR expression predicting BCG-induced cytokine expression in CBMCs of WA and PNG newborns

	BCG-IFN- γ		BCG-IL-12p70		BCG-TNF- α		BCG-IL-6		BCG-IL-10	
	$\beta \pm SE$	<i>P</i>	$\beta \pm SE$	<i>P</i>	$\beta \pm SE$	<i>P</i>	$\beta \pm SE$	<i>P</i>	$\beta \pm SE$	<i>P</i>
TLR2	0.03 $\pm$ 0.20	.874	-0.03 $\pm$ 1.80	.985	0.64 $\pm$ 0.19	.002	0.52 $\pm$ 0.23	.030	0.81 $\pm$ 0.18	<.001
TLR4	-0.40 $\pm$ 0.18	.033	1.20 $\pm$ 1.81	.509	-0.56 $\pm$ 0.18	.003	-0.21 $\pm$ 0.21	.323	-0.51 $\pm$ 0.16	.004
TLR9	-0.07 $\pm$ 0.14	.619	-0.75 $\pm$ 1.46	.612	0.17 $\pm$ 0.14	.218	0.03 $\pm$ 0.16	.833	-0.14 $\pm$ 0.13	.276

Multivariate linear regression analysis studying associations between TLR2, TLR4, and TLR9 mRNA expression in resting CBMCs (independent variables) and *in vitro* CBMC cytokine responses to BCG (respective dependent variables), coadjusting for the study population and mode of delivery. Both the dependent and independent continuous variables are studied as z -scores (for cytokine responses of log-transformed BCG minus background concentrations).

responses ($P < .001$) were significantly higher in WA than PNG CBMCs (Fig 2).

Resting PNG CBMCs expressed significantly more IFN- γ receptor (IFN- γ R)-2 mRNA than WA CBMCs ($P < .001$), and a similar trend was seen for IFN- γ R1 ($P = .062$; Fig 2, A). Significant inverse associations were found between IFN- γ R1 and IFN- γ R2 expression and the exogenous IFN- γ -induced change in BCG-IL-12p70 production (IFN- γ R1, ρ , -0.438 , $P < .001$; IFN- γ R2, ρ , -0.530 , $P < .001$), and between IFN- γ R2 expression and the exogenous IFN- γ -induced change in BCG-induced TNF- α production (ρ , -0.224 ; $P < .037$), IL-6 (ρ , -0.311 ; $P = .003$) and IL-10 (ρ , -0.258 ; $P = .016$).

Population characteristics such as mode of delivery, atopic status, and tetanus vaccination had no effect on responsiveness to IFN- γ priming and expression of IFN- γ R1 and IFN- γ R2 (data not shown).

Role of natural killer cells in IFN- γ -primed BCG-IFN- γ responses

The percentage of CD3⁺CD56⁺ natural killer (NK) cells was similar in PNG (mean $\pm$ SEM, $14.8 \pm 5.2\%$) and WA CBMCs (mean $\pm$ SEM, $9.3 \pm 3.3\%$; $P = .307$). In response to BCG and IFN- γ stimulation, intracellular IFN- γ production by neonatal NK-cells was found to be essentially restricted to the WA population (Fig 3). BCG and IFN- γ stimulation did not induce the production of IFN- γ in neonatal CD4⁺ or CD8⁺ T cells (data not shown).

When CBMC were predepleted of NK cells before IFN- γ and BCG stimulation, IFN- γ levels were significantly reduced on average 0.75-fold in WA CBMCs and 0.85-fold in the more modest responding PNG CBMCs ($P = .018$; data not shown).

Longitudinal effect of neonatal innate BCG responses on T_H-cell responses

In response to *in vitro* PPD stimulation, PBMCs obtained from PNG study infants at 3 months of age ($n = 44$) produced average (GM $\pm$ SEM) responses of 638.4 ± 168.2 pg/mL for PPD-IFN- γ , 23.8 ± 5.0 pg/mL for PPD-IL-5, 66.1 ± 8.1 pg/mL for PPD-IL-13, and 6.5 ± 1.1 pg/mL for PPD-IL-10.

Longitudinal associations between neonatal innate cytokine responses to BCG (independent variables, studied simultaneously) and memory T_H-cell responses to PPD at 3 months of age (dependent variables) were studied by using multivariate linear regression analysis. Neonatal BCG-IL-10 responses promoted PPD-IL-5 responses at 3 months of age, whereas neonatal BCG-IFN- γ responses inhibited both IL-5 and IL-13 responses to PPD in 3-month-olds (Table III). Neonatal BCG-TNF- α responses inhibited PPD-specific IL-10 responses. When IFN- γ -primed neonatal BCG responses were studied, only the significant association between neonatal BCG-TNF- α responses and PPD-IL-10 responses at 3 months of age remained.

Neonatal BCG responses were not associated with a PHA-specific T-cell response at 3 months of age, except for IFN- γ -primed BCG-TNF- α responses that inhibited PHA-IL-10 and promoted PHA-IL-5 and PHA-IL-13 responses (data not shown).

DISCUSSION

This study has identified important differences in innate immune responsiveness between PNG and WA newborns. The first is that BCG-induced IFN- γ and IL-10 responses that predict T_H-cell differentiation are significantly elevated in PNG newborns. The second is that IFN- γ responsiveness is constrained

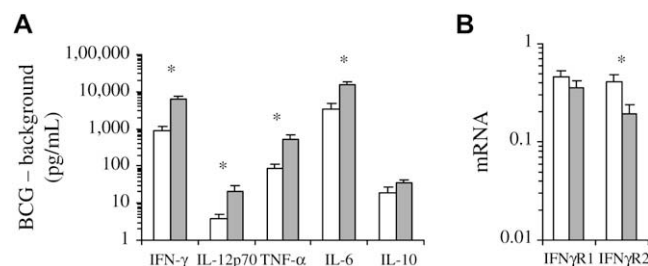


FIG 2. Effect of exogenous IFN- γ priming on BCG-induced neonatal innate cytokine responses, and IFN- γ R expression in CBMCs. **(A)** CBMCs of PNG newborns (white bars, $n = 47$) and WA newborns (gray bars, $n = 47$) were primed with IFN- γ and stimulated *in vitro* with BCG, and cytokine responses were measured (presented are BCG-induced responses minus background levels), and **(B)** IFN- γ R1 and IFN- γ R2 mRNA expression was measured in resting cells. Bars represent GMs and 95% CIs. *Significant differences between PNG and WA newborns are indicated at the .05 level.

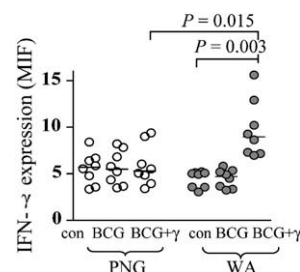


FIG 3. NK cell IFN- γ production. Intracellular expression of IFN- γ (median intensity fluorescence, MIF) in NK cells (CD3⁺ CD56⁺ cells) was measured in CBMCs of PNG (white circles, $n = 8$) and WA newborns (gray circles, $n = 8$) that were cultured with medium alone (control; con), BCG, or BCG plus exogenous IFN- γ .

TABLE III. Multivariate linear regression analysis for neonatal BCG response longitudinally predicting PPD-specific T_H-cell responses at 3 months of age in the PNG study population

Neonatal BCG response	T _H -cell response at 3 months of age							
	PPD-IFN- γ		PPD-IL-5		PPD-IL-13		PPD-IL-10	
	$\beta \pm SE$	<i>P</i> value	$\beta \pm SE$	<i>P</i> value	$\beta \pm SE$	<i>P</i> value	$\beta \pm SE$	<i>P</i> value
IFN- γ	-0.21 ± 0.17	.226	-0.29 ± 0.16	.076	-0.31 ± 0.17	.078	0.10 ± 0.17	.548
IL-12p70	0.02 ± 0.16	.907	-0.16 ± 0.15	.321	-0.27 ± 0.16	.106	-0.12 ± 0.16	.465
TNF- α	-0.20 ± 0.19	.291	-0.01 ± 0.18	.951	-0.05 ± 0.19	.808	-0.44 ± 0.19	.026
IL-6	0.29 ± 0.21	.179	-0.33 ± 0.20	.114	0.12 ± 0.21	.593	0.15 ± 0.21	.479
IL-10	-0.17 ± 0.20	.404	0.40 ± 0.20	.047	-0.04 ± 0.21	.865	-0.02 ± 0.21	.994

Multivariate linear regression analysis studying associations between *in vitro* CBMC responses to BCG (independent variables) and *in vitro* PBMC responses to PPD at 3 months of age (respective dependent variables; $n = 44$). Both the dependent and independent continuous variables are studied as z -scores of log-transformed delta concentrations (BCG/PPD response minus background level).

in PNG newborns, with IFN- γ primed NK cells of WA but not PNG newborns able to produce IFN- γ in response to BCG.

There is increasing evidence that TLR pathways play an important role in the development of atopic disease. For example, we have recently shown that infant allergic disease is associated with presymptomatic differences in TLR function at birth.²⁷ TLR expression and function can be modulated by genetic variation¹¹ and environmental factors,^{8,28-30} including *in utero* exposures that are more common in low-income compared with high-income countries, such as infections with malaria parasites,¹⁴ high exposure to animals,¹² and cigarette smoke.¹³ Consistent with the low (but rising) prevalence of atopic disease in low-income countries, infants born under adverse living conditions may therefore be relatively protected against atopic disease by a genetic and/or an environmental control of their neonatal TLR-mediated immune responses that are vigorously activated after BCG vaccination at birth.

Two different mechanisms can explain the presumed allergo-protective effect of BCG. The first is the activation of proinflammatory innate immune responses that stimulate the early development of T_H1 and counteract T_H2 responses.¹⁶⁻¹⁹ This mechanism would fit our observation that neonatal BCG-IFN- γ responses inversely predict PPD-specific IL-5 and IL-13 responses at 3 months of age. The second mechanism involves the production of IL-10. Besides indications that BCG-IL-10 drives the development of IL-10 producing immune regulatory T cells,^{22,23} in mice TLR-mediated IL-10 responses have been shown to impair dendritic cell function and T_H1 responses in the neonatal period.^{31,32} This typical neonatal effect of IL-10

might offer an explanation for observations that any capacity of BCG to prevent atopic disease seems to be limited to children vaccinated at a very young age.^{33,34} Despite opposing effects, both mechanisms can contribute to the differentiation of postnatal T_H1 and T_H2 responses, depending on individual production patterns.

The role of T_H cells in atopic disease varies with the maturation status of the immune system that, besides age, depends on environmental and genetic factors. For example, established atopic disease is associated with enhanced T_H2 and likely T_H1 responses, whereas earlier in life, the T_H1 compartment is suppressed in children who go on to develop atopy.^{35,36} Our observations that *in vitro* cord BCG responses longitudinally predict PPD-specific IL-5, IL-13, and IL-10 at 3 months of age therefore above all verify the relevance of the neonatal innate immune response to BCG for the development of T_H-cell responses, without proclaiming a role of T_H1 versus T_H2 responses in atopy development. This would require a more detailed study of the ontogeny of T_H-cell responses, including complementary data on the atopic status of the children at different ages. Moreover, postnatal BCG-specific T_H-cell responses are likely to be further influenced by environmental factors.³⁷ Unfortunately, we did not know the atopic status of the PNG study infants, nor were we in the position to carry out a comparable longitudinal study in the WA population because in most industrialized countries, low-risk children are no longer vaccinated with BCG.

The production of IFN- γ is suboptimal in newborns, but nevertheless can be produced by neonatal cytotoxic T cells and NK cells in response to strong microbial stimulants in quantities

that may be physiologically sufficient to prime innate immune cells *in vivo*.^{38,39} After *in vitro* IFN- γ priming, proinflammatory cytokine responses (IFN- γ , TNF- α , and IL-12p70) to BCG were enhanced in both WA and PNG CBMCs, but to a markedly higher extent in cells of WA than PNG newborns. Furthermore, NK cells of WA but not PNG newborns produced IFN- γ and therefore contribute directly to the elevated BCG-IFN- γ response after IFN- γ priming. On the contrary, NK cells of PNG newborns did not produce IFN- γ , but likely contribute to the overall IFN- γ response by producing other cytokines that stimulate IFN- γ production in other cell types. Further studies will be needed to clarify the mechanisms underlying this difference in IFN- γ responsiveness between neonatal populations, which could include differences in IFN- γ receptor expression and signaling,^{40,41} TLR function,⁴² or restricted production of proinflammatory cytokines such as IL-12, IL-15, and TNF- α .⁴⁰ The observation that IFN- γ -primed neonatal BCG responses did not predict the development of memory T_H-cell responses in the PNG study cohort may question the relevance of this *in vitro* response. However, we cannot exclude the importance of this response in other neonatal populations such as in WA, where neonatal innate cytokine responses were highly enhanced by exogenous IFN- γ . Although we found that neither PNG nor WA neonatal T cells produced IFN- γ in response to BCG ($\pm$ IFN- γ), we cannot rule out that the precursor frequency of prenatally primed memory T cells was too low to be detectable.

Finally, it is important to note that BCG is primarily administered to protect children against *Mycobacterium* infections and not to prevent atopy. Although microbial clearance requires the activation of T_H1 responses, there are indications that T_H1-driving innate immune pathways can be detrimental if unregulated. For example, in neonatal mice, the administration of IL-12 has been shown to increase risks for growth retardation and death, whereas concurrent generation of innate IL-10 responses protect against excessive or uncontrolled inflammatory responses to microbial exposure.^{32,43} A suppression of T_H1-mediated innate immune responses and reduced IFN- γ responsiveness may therefore be beneficial in the early postnatal period. In other words, the activation of anti-inflammatory IL-10 responses and redundant production of proinflammatory responses in the early postnatal period may provide a trade-off between microbial protection and systemic inflammation, in particular in environments where newborns experience high levels of microbial exposure.

In summary, we have shown that BCG-related neonatal innate immune responses influence the development of T_H responses in early infancy and differ between children born in an industrialized versus a nonindustrialized country. This may affect resistance to *M. tuberculosis* infections and the risk for atopic disease in later life.

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Clinical implications: BCG vaccination may prevent allergies in children in low-income countries but not in high-income countries as a result of differences in neonatal innate immune function.

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TABLE E1. TLR-mediated cytokine responses in WA and PNG newborns, and WA adult controls

	PNG newborn (n = 47)	WA newborn (n = 47)	WA adult (n = 15)
IFN- γ			
LTA	2 (2-19)	2 (2-2)*	6 (2-14)‡
LPS	2 (2-15)	2 (2-23)	26 (5-80)†
BCG	99 (9-215)	18 (2-113)*	410 (205-3,420)†‡
BCG + IFN- γ			
IL-12p70			
LTA	2 (2-2)	2 (2-2)	2 (2-2)
LPS	2 (2-2)	2 (2-2)	2 (2-2)
BCG	2 (2-3)	2 (2-2)	2 (2-2)
BCG + IFN- γ	2 (2-9)	23 (9-65)*	12 (2-50)†
TNF- α			
LTA	2 (2-2)	4 (2-25)	45 (16-153)†‡
LPS	2 (2-32)	33 (4-126)*	152 (47-305)†‡
BCG	13 (2-50)	7 (2-49)	62 (43-134)†‡
BCG + IFN- γ	113 (26-320)	756 (273-1,390)*	776 (500-1,083)†
IL-6			
LTA	5,807 (48-13,217)	22,615 (15,262-28,087)*	16,229 (10,900-23,222)†‡
LPS	3,971 (2-13,783)	29,100 (16,013-39,526)*	17,010 (11,403-21,372)†‡
BCG	9,303 (1,489-18,684)	5,224 (3,091-12,109)	6,285 (2,677-12,037)
BCG + IFN- γ	7,993 (971-16,728)	15,571 (8,519-23,423)*	7,729 (4,251-12,965)‡
IL-10			
LTA	92 (6-212)	206 (136-313)*	462 (276-666)†‡
LPS	65 (4-163)	302 (144-491)*	414 (273-514)†
BCG	47 (13-99)	15 (2-40)*	69 (30-88)‡
BCG + IFN- γ	39 (2-79)	44 (17-66)	60 (38-72)

CBMCs from PNG and WA newborns and PBMCs from WA healthy adult controls were stimulated *in vitro* with TLR2 ligand *Staphylococcus aureus* lipoteichoic acid (LTA; 2 μ g/mL), TLR4 ligand *Escherichia coli* lipopolysaccharide (LPS; 5 μ g/mL), or BCG (5×10^4 CFU/mL) $\pm$ recombinant human IFN- γ (0.5 μ g/mL). TLR-mediated cytokine responses were measured in supernatants by time-resolved fluorometry, and cytokine background concentrations in unstimulated cell cultures were subtracted. Presented are the median and interquartile concentrations (pg/mL) per group, with significant differences (Mann-Whitney, $P < .05$) between *PNG and WA cords, †PNG cords and WA adults, ‡WA cords and WA adults.

TABLE E2. IFN- γ priming-induced fold change in BCG responses

	PNG newborns	WA newborns	<i>P</i> value
IFN- γ	21 (4-60)	390 (76-1396)	<.001
IL-12p70	1.0 (1.0-1.9)	5.8 (1.3-8.6)	<.001
TNF- α	26 (2-13)	55 (19-234)	<.001
IL-6	1.1 (0.8-1.5)	2.7 (1.6-5.3)	<.001
IL-10	1.0 (0.7-1.1)	2.3 (1.6-5.3)	<.001

Median (and interquartile ranges) of ratio between BCG-induced cytokine responses in the absence of presence of exogenous IFN- γ .