The impact of HIV exposure and maternal *Mycobacterium tuberculosis* infection on infant immune responses to bacille Calmette-Guérin vaccination

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Objective: The objective of this study is to assess the effect of maternal HIV and *Mycobacterium tuberculosis (Mtb)* infection on cellular responses to bacille Calmette-Guérin (BCG) immunization.

Design: A mother–infant cohort study.

Methods: Samples were collected from mother–infant pairs at delivery. Infants were BCG-vaccinated at 6 weeks of age and a repeat blood sample was collected from infants at 16 weeks of age. BCG-specific T-cell proliferation and intracellular cytokine expression were measured by flow cytometry. Secreted cytokines and chemokines in cell culture supernatants were analysed using a Multiplex assay.

Results: One hundred and nine (47 HIV-exposed and 62 HIV-unexposed) mother–infants pairs were recruited after delivery and followed longitudinally. At birth, proportions of mycobacteria-specific proliferating T cells were not associated with either in-utero HIV exposure or maternal *Mtb* sensitization. However, in-utero HIV exposure affected infant-specific T-cell subsets [tumour necrosis factor-alpha (TNF-α) single positive proliferating CD4$^+$ T cells and interferon-gamma (IFN-γ), TNF-α dual-positive CD4$^+$ T cells]. Levels of TNF-α protein in cell culture supernatants were also significantly higher in HIV-exposed infants born to *Mtb*-sensitized mothers. In the presence of maternal *Mtb* sensitization, frequencies of maternal and newborn BCG-specific proliferating CD4$^+$ T cells were positively correlated. Following BCG vaccination, there was no demonstrable effect of HIV exposure or maternal *Mtb* infection on infant BCG-specific T-cell proliferative responses or concentrations of secreted cytokines and chemokines.

Conclusion: Effects of maternal HIV and *Mtb* infection on infant immune profiles at birth are transient only, and HIV-exposed, noninfected infants have the same potential to respond to and be protected by BCG vaccination as HIV-unexposed infants.

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Introduction

Many studies have reported that HIV-exposed infants have increased rates of morbidity and mortality compared with infants not exposed to HIV *in utero* [1−10] including high rates of tuberculosis (TB) early in life, even if they do not acquire HIV infection themselves [11]. A number of factors might contribute to this observation, not least social considerations, including immunological differences, some of which have been shown to persist into childhood. However, their direct impact on clinical outcomes is not understood [12−22].

The developing immune system can be primed *in utero* by sensitization to maternal infections [23−26]. Published data show that a proportion of infants display cytokine responses to mycobacterial antigens already at birth, prior to apparent exposure to *Mycobacterium tuberculosis (Mtb)*, bacille Calmette-Guérin (BCG) vaccination or environmental mycobacteria [27−29].

We hypothesized that in-utero exposure of infants to HIV and/or mycobacterial antigens might impact their immune responses to BCG vaccination, as considerable heterogeneity has been observed. We conducted a comprehensive analysis of immune responses in paired maternal and infant blood samples at delivery and following infant BCG vaccination with the live attenuated strain of *Mycobacterium bovis*.

Materials and methods

Study setting

The study was conducted between March 2009 and June 2011 in Khayelitsha, Western Cape, South Africa. The TB incidence in Khayelitsha was 1389 per 100 000 and the HIV prevalence amongst pregnant women registering for antenatal care was 33.1% in 2010 [30]. The comprehensive HIV prevention of mother-to-child transmission (PMTCT) programme at the study site has been described previously [31].

Recruitment of participants and study measures

The study was approved by the Universities of Cape Town (382/2008) and Stellenbosch (N08/10/278) and the National Research Ethics Service, England (07/H0720/178) and local health authorities. Following antenatal provision of study-specific information, eligible postpartum mothers and their infants were recruited and a blood sample collected from mothers and infants within 24 h of delivery. Women with active TB were excluded from the study (see figure, Supplemental Digital Content 1, http://links.lww.com/QAD/A614), and full eligibility criteria have been previously reported [31]. All HIV-exposed infants were tested at 4 weeks to exclude HIV infection using standardized HIV PCR (Amplicor HIV-a DNA kit, Version 1.5; Roche, Branchburg, New Jersey, USA) BCG vaccine (unadjuvanted Danish strain 1331; Statens Serum Institut, Copenhagen, Denmark) was administered at 6 weeks of age to HIV-uninfected infants, as per WHO recommendations [32]. A second blood sample was collected from infants at 16 weeks of age to assess immune responses to BCG vaccination.

Data were analysed for four groups of mothers and infants, based on maternal antenatal HIV results with confirmatory testing at delivery (Abbott Determine HIV-1/2; Abbott, Tokyo, Japan) and QuantiFERON-TB Gold In-Tube (QFN: Qiagen, Hilden, Germany) results as a measure of *Mtb* sensitization.

Laboratory assays

Six-day whole blood Ki67 lymphoproliferation assay
Whole blood (125 µl, diluted 1:10 with warm RPMI 1640) was incubated with 5×10^5 CFU/ml BCG vaccine, or 1 µg/ml Staphylococcal enterotoxin B or media alone as positive and negative controls, respectively, in 24-well plates at 37°C, 5% CO_2, 80% humidity for 6 days, following a method adapted from Soares *et al.* [33]. BCG was used to stimulate mycobacterial recall responses in newborns and mothers and to assess the response to BCG vaccination in 16-week-old infants. After 24 h, 150 µl of supernatant was collected and stored at -80°C for cytokine analysis. On day 6, 2.5 µg/ml Brefeldin A, 26 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 2.6 µg/ml Ionomycin (Sigma-Aldrich, St Louis, Missouri, USA) were added for the last 4 h of culture. Cells were harvested with EDTA, red cells lysed and white cells stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Eugene, Oregon, USA). Fixed white cells were stored at -80°C in 10% DMSO.

Thawed white cells were permeabilized and stained with optimized concentrations of fluorescent-conjugated mAbs (BD Biosciences, San Jose, California, USA); anti-CD8 PerCP-Cy5.5 (RPA-T8), anti-IFN-γ Alexa

Fluor 700 (B27), anti-IL-2 FITC (MQ1-17H12), anti-TNF-α PE-Cy7 (MAb11), anti-Ki67 PE (B56), anti-IL-17A Alexa Fluor 647 (N49-853) and anti-CD3 Qdot 605 (UCHT1; Invitrogen). Ki67 is a nuclear protein expressed during active phases of the cell cycle, but absent in resting cells, making it a useful marker of proliferation [34].

PMA and ionomycin were used to assess the capacity of antigen-specific T cells to produce cytokine by restimulating cells for the last 4 h of culture. However, PMA and ionomycin strongly downregulate the surface expression of $CD4^+$ on T cells; therefore, the $CD3^+$ $CD8^-$ T-cell subset was used in place of the $CD4^+$ T-cell subset. $CD8^-$ T cells are subsequently considered equivalent to $CD4^+$ T cells, consistent with previous publications [33,35].

Multiparameter flow cytometry and controls

A BD LSRFortessa Flow Cytometer (model 649225B7; five lasers, 19 detectors) using FACSDiva software (BD Biosciences) was used to acquire the entire sample. Optimal photomultiplier tube voltages were established for this study. Single-stained antimouse or antirat beads immunoglobulin, κ, beads were used to calculate compensation in FACSDiva. Dead cells were excluded using viability stain.

Multiparameter flow cytometry data analysis

Data were analysed using FlowJo v 9.4.11 (TreeStar, Ashland, Oregon, USA) by a single operator using a predetermined gating strategy and template (see figure, Supplemental Digital Content 2, http://links.lww.com/QAD/A614). Boolean gating was used to determine combinations of antigen-specific cytokine-producing cells. Background subtraction was performed using Pestle v 1.7 (VRC, NIH) and display of multiple combination of cells was performed using SPICE v 5.22 [36]. Samples were excluded from analysis if there was no distinct population of live cells or contained less than 1000 live $CD3^+$ T cells.

Multiplex analysis

Customized plates (MILLIPLEX MAP; Millipore, Billerica, Massachusetts, USA) were used to quantify 20 cytokines and chemokines in supernatants collected after 24 h of culture (Supplemental Digital Content 3, http://links.lww.com/QAD/A614) following manufacturer's instructions. Beads were analysed on a Bio-Plex array reader (Bio-Rad, Hercules, California, USA). The standard curve for all analytes ranged from 3.2 to 10 000 pg/ml. Unstimulated sample values were subtracted from stimulated sample values. Values less than the lower limit of detection of the assay were assigned a value of 1.6 pg/ml and values greater than the upper limit were assigned a value of 10 000 pg/ml.

Statistical analysis

Statistical analysis was performed using SPSS (version 20), GraphPad Prism (version 5.0a, 2008) and Statistica (v10). A two-way analysis of variance (ANOVA) or Kruskal–Wallis test was used to test the interaction of maternal HIV and QFN status with frequencies of specific T cells. Bonferroni adjustment was applied to correct for multiple comparisons; the corrected *P* value is reported. A mixed ANOVA was used to analyse paired infant responses. Multiplex data was analysed using a three-way repeated measures ANOVA with mixed models or a generalized estimating equations (GEEs) model, depending on the distribution of data. Spearman's rank order correlation was used to assess the association of maternal and infant immune responses.

Results

Participant characteristics

One hundred and nine mother–infant pairs were enrolled, of which 47 women (43%) were HIV-infected and 62 (57%) HIV-uninfected. Of this cohort, 95 infants (87%) were followed up to 16 weeks of age (Supplemental Digital Content 1, http://links.lww.com/QAD/A614). Of these, 39 infants (41%) were HIV-exposed and 56 (59%) were HIV-unexposed. One infant (1%) was found to be HIV-infected at 4 weeks of age and was referred for rapid initiation of antiretroviral treatment (mother–infant pair subsequently excluded from analysis). There was no significant difference in gestation and birth weight between HIV-exposed and unexposed infants (Supplemental Digital Content 4, http://links.lww.com/QAD/A614).

The mean $CD4^+$ cell count among HIV-infected women was 474 cells/μl (SD 252); the median viral load was 730 copies/ml [interquartile range (IQR) 357–3925]. Antenatal zidovudine or HAART was received by 41 (89%) women; 30 (65%) women received intrapartum nevirapine or HAART.

Data were analysed in four groups, according to maternal HIV and *Mtb* sensitization status: HIV−QFN− ($n = 27$), HIV−QFN+ ($n = 35$), HIV+QFN− ($n = 25$) and HIV+QFN+ ($n = 20$). One HIV-infected mother had an indeterminate QFN result; maternal and infant data were used when considering the effect of HIV status alone, but not when analysing all four groups.

In-utero HIV exposure affected T-cell subsets in newborn infants; however, overall mycobacteria-specific proliferative T-cell responses were unaffected by HIV-exposure or maternal *Mycobacterium tuberculosis* sensitization

There were no differences in the frequency of BCG-specific $Ki67^+CD4^+$ or $CD8^+$ T cells between the four groups of infants at birth ($P = 0.25$ and $P = 0.73$), or in the total expression of intracellular cytokines in these cells between the four groups of infants (see table, Supplemental Digital Content 5, http://links.lww.com/QAD/A614). However, some differences were observed amongst T-cell subsets. At birth, HIV-exposed, uninfected infants

displayed increased frequencies of BCG-specific TNF-α single-positive Ki67$^+$CD4$^+$ T cells (median = 0.34%, IQR 0.17−1.89), compared with infants born to HIV-uninfected mothers (median = 0.12%, IQR 0.00−0.49), $P = 0.04$. Exposed infants also showed an increased frequency of BCG-specific Ki67$^+$CD4$^+$ IFN-γ$^+$TNF-α$^+$ T cells compared with unexposed infants (median 0.2%, IQR 0.04−0.99 vs. 0.00%, IQR 0.00−0.08), $P = 0.007$. Frequencies of TNF-α single-positive CD8$^+$ T cells (0.17, IQR 0.00−0.99) were increased in HIV-exposed infants compared with undetectable frequencies in unexposed infants, $P = 0.007$. IL-2 single-positive CD8$^+$ T cells were increased in HIV-exposed compared with unexposed infants (0.25%, IQR 0.09−0.96 vs. 0.11%, IQR 0.00−0.89, $P = 0.007$).

Consistent with the results from flow cytometric data, in-utero HIV exposure and maternal Mtb sensitization were also associated with significant differences in BCG-specific secreted cytokines and chemokines. At birth, HIV-exposed, uninfected infants had significantly higher concentrations of TNF-α in response to BCG antigens than unexposed infants ($P = 0.03$). When comparing the four groups of infants, maternal HIV infection only had

an effect on infant TNF-α and granulocyte macrophage colony-stimulating factor responses in the presence of maternal Mtb sensitization (Fig. 1). A higher IFN-γ concentration to BCG antigens was also seen in HIV-unexposed infants born to Mtb-sensitized mothers than HIV-exposed infants born to Mtb-unsensitized mothers (Fig. 1). Maternal Mtb sensitization was associated with higher sCD40L responses to BCG antigens in HIV-unexposed, but not in HIV-exposed infants (Fig. 1).

Positive association between some mycobacteria-specific responses in mothers and their infants at birth

We used identical immunological assays to determine association between maternal HIV infection and Mtb sensitization with maternal responses to BCG antigens at delivery. Amongst HIV-infected women, Mtb-sensitized participants had higher frequencies of BCG-specific CD4$^+$ and CD8$^+$ Ki67$^+$ T cells than Mtb-unsensitized women (Fig. 2). Mtb-sensitized, HIV-infected women also had significantly higher frequencies of BCG-specific proliferating CD4$^+$ T cells expressing IFN-γ, TNF-α and IL-2 than Mtb-unsensitized, HIV-infected women
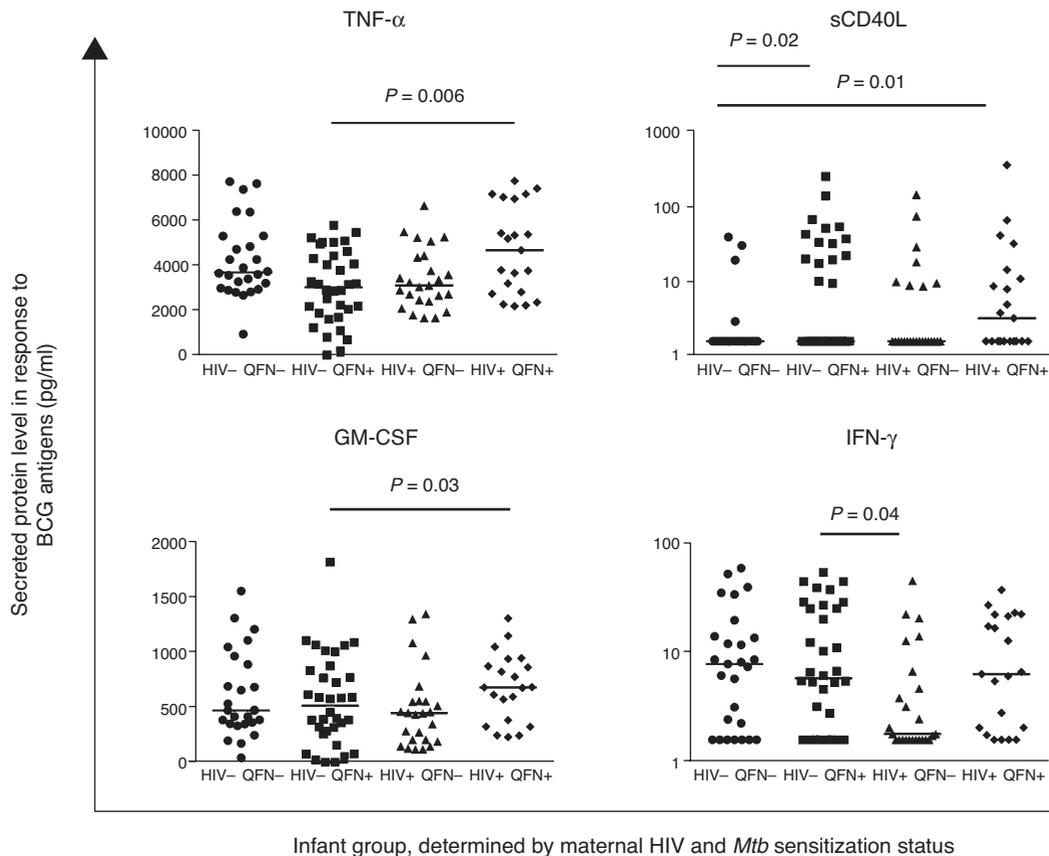
Fig. 1. Concentration of tumour necrosis factor-alpha, GM-CSF, sCD40L and interferon-gamma *in vitro* in response to bacille Calmette-Guérin antigens in newborn infant samples. Horizontal lines represent median values. Only comparisons with $P < 0.05$ are given. IFN-γ and sCD40L data are presented using a log scale.
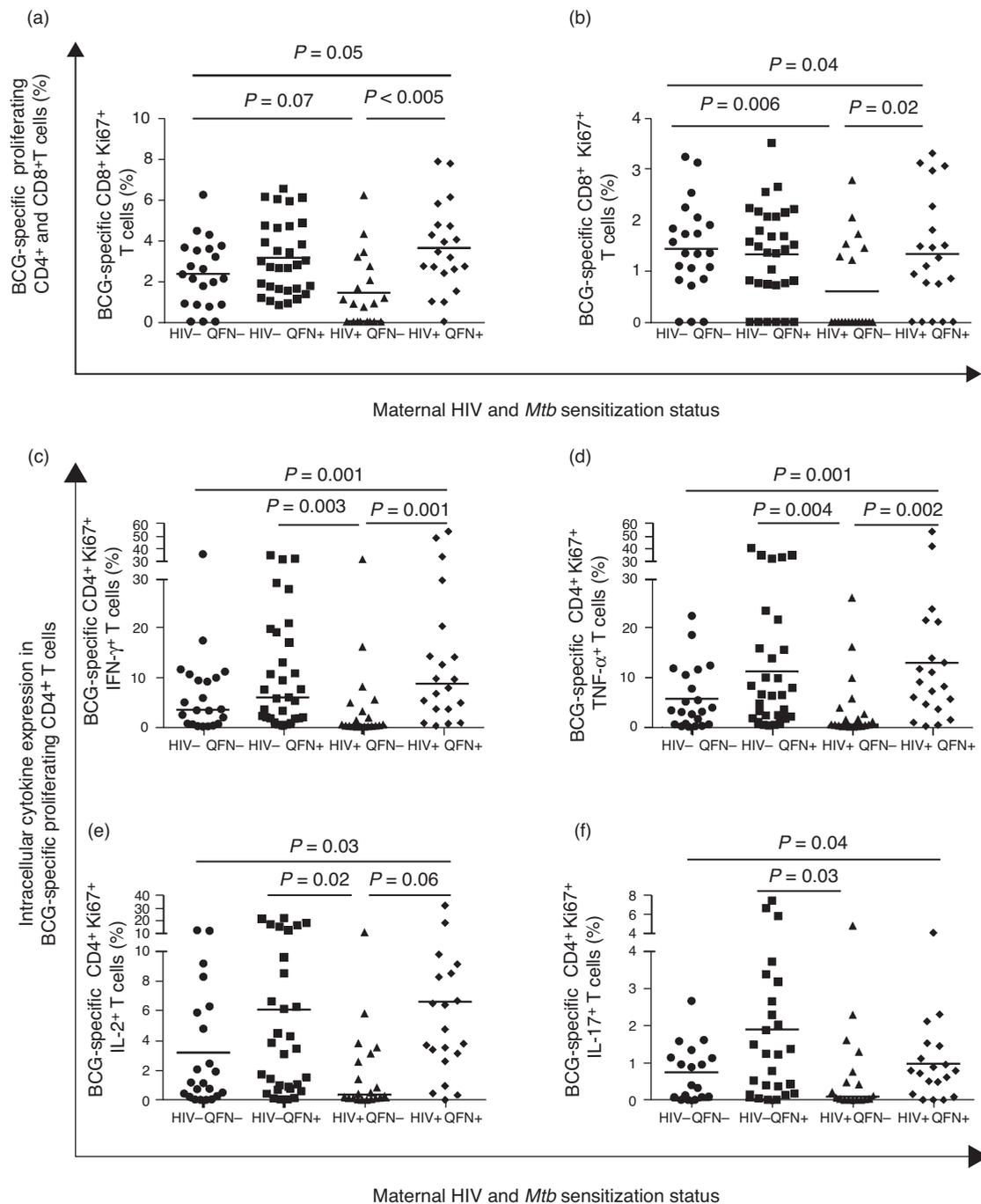
Fig. 2. **Maternal T-cell responses to BCG antigens.** CD4$^+$ (a) and CD8$^+$ (b) T cell proliferation in response to bacille Calmette-Guérin stimulation and frequencies of BCG-specific CD4$^+$ Ki67$^+$ T cells expressing IFN-γ (c), TNF-α (d), IL-2 (e) or IL-17 (f) in women at delivery. Groups of mothers: HIV−QFN− ($n = 22$, $n = 18$ for IL-17); HIV−QFN+ ($n = 31$, $n = 25$ for IL-17); HIV+QFN− ($n = 20$, $n = 18$ for IL-17) and HIV+ QFN+ ($n = 20$, $n = 19$ for IL-17). Square root transformed data are presented in (a) and (b); horizontal lines represent mean response. Only comparisons with adjusted $P < 0.05$ are given.

(Fig. 2). In agreement with these findings, the concentration of IFN-γ in BCG-stimulated cell culture supernatants was also significantly higher in *Mtb*-sensitized than *Mtb*-unsensitized women (Fig. 3). Interestingly, such differences were not observed amongst HIV-uninfected women. In addition, the frequency of CD8$^+$ T cells expressing TNF-α

($P = 0.006$), IL-2 ($P = 0.03$) and IL-17 ($P = 0.01$) differed significantly between the four maternal groups, Supplemental Digital Content 6 (http://links.lww.com/QAD/A614).

We then correlated the maternal responses with paired infant responses. In the presence of maternal *Mtb*
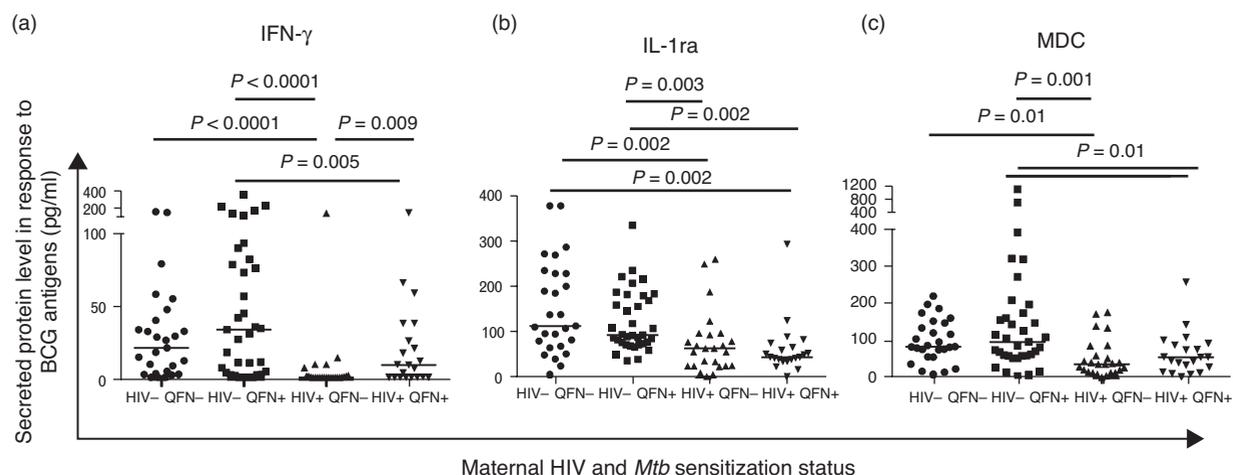
Fig. 3. Interferon-gamma, interleukin-1ra and MDC responses to bacille Calmette-Guérin antigens in cell culture supernatants in mothers at delivery. Horizontal lines represent median values. Only comparisons with $P < 0.05$ are given.

sensitization, frequencies of maternal and newborn infant BCG-specific proliferating CD4$^+$ T cells were positively correlated, $r_s = 0.43$, $P = 0.03$; however, no association was observed in the absence of maternal Mtb sensitization, $r_s = 0.02$, $P = 0.92$. This association did not persist following infant BCG vaccination. Moreover, at birth, the frequency of BCG-specific Ki67$^+$CD4$^+$ and CD8$^+$ IL-2$^+$ T cells was associated with the frequency of the same cells in mothers (Table 1). At 16 weeks, this correlation persisted in the CD8$^+$, but not the CD4$^+$ compartment, and association was strongest in HIV-infected, Mtb-sensitized mothers and their infants, $r_s = 0.65$, $P = 0.01$. Maternal and newborn infant CD4$^+$Ki67$^+$ TNF-α^+ T-cell frequencies were also positively correlated; this association was strongest in the context of maternal Mtb sensitization, but it did not persist at 16 weeks of age. An

association of maternal and infant TNF-α concentration was also observed at birth and persisted in 16-week-old infants born to HIV-uninfected, Mtb-sensitized mothers, $r_s = 0.43$, $P = 0.01$ (Supplemental Digital Content 7, http://links.lww.com/QAD/A614). Further strong associations between concentrations of maternal and newborn infant cytokines (IL-1β, IL-1Ra, IL-6 and IL-10) were observed (Supplemental Digital Content 7, http://links.lww.com/QAD/A614).

Responses to bacille Calmette-Guérin vaccination in infants at 16 weeks of age were robust and independent of maternal HIV or *Mycobacterium tuberculosis* status

In order to determine the potential impact of infant responses at birth on the subsequent immunogenicity of

Table 1. Association between maternal and infant bacille Calmette-Guérin specific CD4$^+$ and CD8$^+$ Ki67$^+$ cytokine$^+$ T-cell frequencies at birth.

| Group		CD4$^+$Ki67$^+$				CD8$^+$Ki67$^+$			
		IFN-γ^+	IL-2$^+$	TNF-α^+	IL-17$^+$	IFN-γ^+	IL-2$^+$	TNF-α^+	IL-17$^+$
Overall cohort ($n=49$)	r_s	0.27	**0.49**	**0.39**	0.23	0.14	**0.45**	0.22	−0.17
	P	0.07	**<0.0005**	**0.006**	0.15	0.36	**0.001**	0.13	0.29
HIV-negative ($n=32$)	r_s	0.29	**0.53**	**0.43**	0.28	0.37	**0.50**	0.12	−0.03
	P	0.11	**0.002**	**0.02**	0.16	0.04	**0.004**	0.66	0.88
HIV-positive ($n=17$)	r_s	0.25	0.37	0.30	0.06	−0.18	0.29	0.30	−0.42
	P	0.33	0.14	0.24	0.85	0.49	0.26	0.09	0.16
QFN− ($n=22$)	r_s	−0.05	**0.45**	0.02	−0.14	−0.24	**0.44**	−0.24	−0.32
	P	0.84	**0.03**	0.93	0.60	0.28	**0.04**	0.28	0.23
QFN+ ($n=26$)	r_s	0.32	**0.49**	**0.52**	**0.48**	0.36	**0.50**	**0.47**	−0.10
	P	0.11	**0.01**	**0.007**	**0.02**	0.07	**0.01**	**0.02**	0.65
HIV−QFN− ($n=14$)	r_s	−0.05	**0.56**	0.20	−0.08	0.02	**0.58**	−0.12	−0.22
	P	0.88	**0.04**	0.49	0.82	0.94	**0.03**	0.70	0.54
HIV−QFN+ ($n=18$)	r_s	0.44	**0.49**	**0.56**	0.52	0.59	0.44	**0.55**	0.09
	P	0.07	**0.04**	**0.02**	0.31	**0.01**	0.06	**0.02**	0.74
HIV+ QFN− ($n=8$)	r_s	−0.10	0.17	−0.12	−0.22	−0.40	0.00	−0.51	−0.48
	P	0.81	0.70	0.78	0.68	0.33	1.00	0.20	0.33
HIV+ QFN+ ($n=8$)	r_s	−0.23	−0.17	−0.10	0.25	0.19	0.63	0.04	−0.70
	P	0.59	0.69	0.82	0.59	0.65	0.09	0.93	0.08

Data were square root transformed and analysed using Spearman's rank order correlation, $P < 0.05$ were considered significant and are highlighted in bold. IFN-γ, interferon-gamma; IL, interleukin; TNF-α, tumour necrosis factor-alpha.

delayed BCG vaccination at 6 weeks of age, identical assays were repeated in infants at 16 weeks of age.

BCG vaccination induced strong immune responses in all infants: there was a significant increase in the frequency of $CD4^+Ki67^+$ T cells in infants following vaccination (mean 36.84%, SD 3.50) compared with prevaccination (mean 3.35%, SD 1.5), $P < 0.0005$. $CD8^+Ki67^+$ T cells also significantly increased (mean prevaccination 2.19%, SD 1.18, mean postvaccination 10.18% SD 1.80), $P < 0.0005$. Similarly, post-BCG vaccination, there was a highly significant

increase in BCG-specific $CD4^+$ and $CD8^+ Ki67^+ IFN-\gamma^+$, $TNF-\alpha^+$, $IL-2^+$ and $IL-17^+$ T cells, $P < 0.0005$ ($P = 0.003$ for $CD8^+ IL-17^+$ T cells). The expansion of these cells was similar for all infants, with no statistically significant differences between groups of infants (Supplemental Digital Content 8, http://links.lww.com/QAD/A614).

At 16 weeks of age, all infants displayed similar frequencies of BCG-specific $CD4^+$ and $CD8^+$ proliferating T cells, irrespective of maternal HIV and *Mtb* infection status (Fig. 4). Furthermore, there was no effect
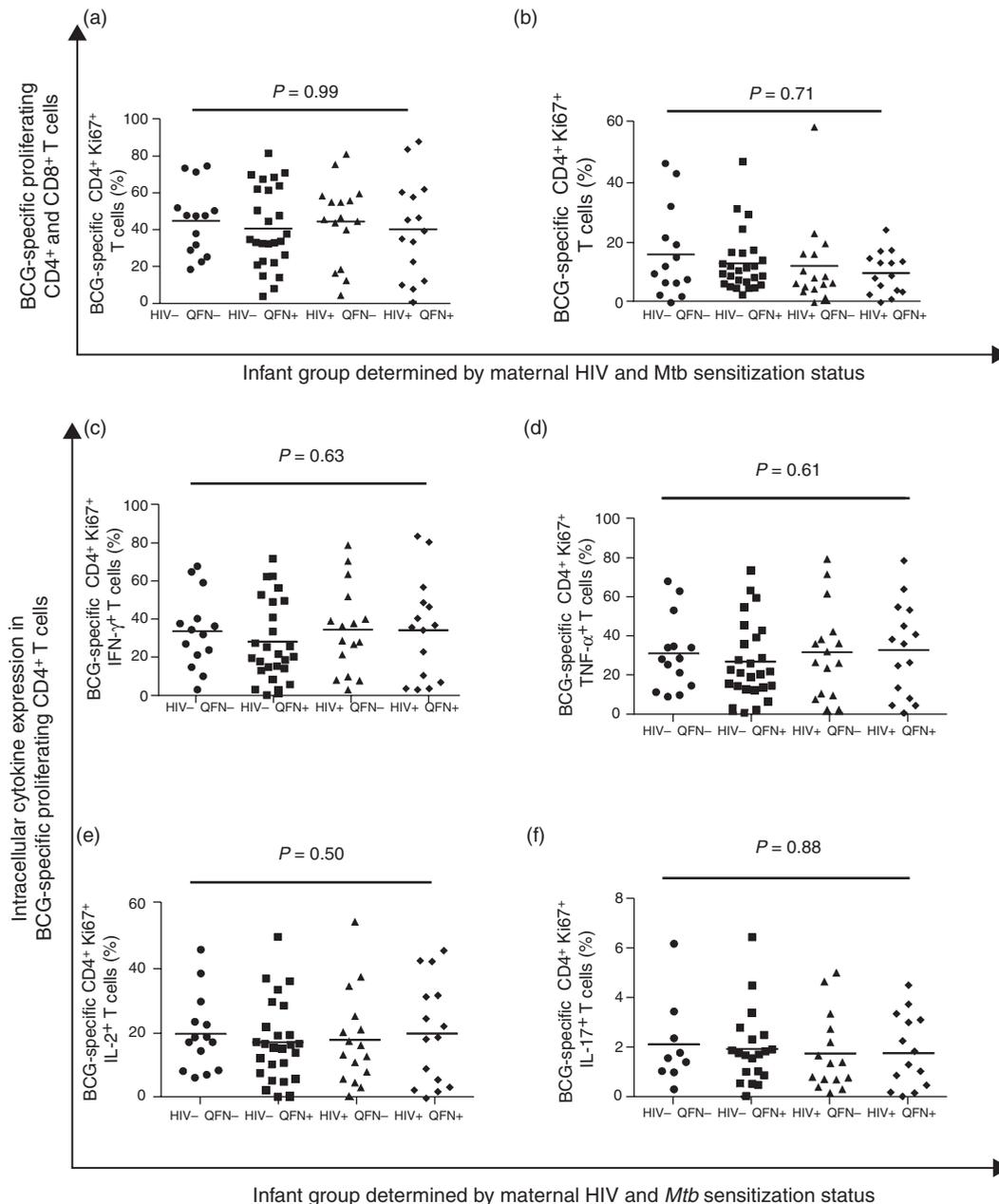
Fig. 4. **Frequency of bacille Calmette-Guérin specific Ki67$^+$ CD4$^+$ and CD8$^+$ T cells following infant BCG vaccination.** BCG-specific proliferating CD4$^+$ T cells (a), and CD8$^+$ T cells (b). Frequencies of BCG-specific CD4$^+$ Ki67$^+$ T cells expressing IFN-γ^+(c), TNF-α (d), IL-2 (e) or IL-17 (f). Horizontal lines represent median values. Only comparisons with $P < 0.05$ are given.

of maternal HIV infection or *Mtb* sensitization on the frequency of BCG-specific proliferating CD4$^+$ T cells expressing intracellular IFN-γ, TNF-α, IL-2 or IL-17, Fig. 4, or CD8$^+$ T cells expressing intracellular IFN-γ ($P = 0.77$), TNF-α ($P = 0.83$), IL-2 ($P = 0.37$) or IL-17 ($P = 0.20$), see figure, Supplemental Digital Content 9, http://links.lww.com/QAD/A614.

We examined the quality of the CD4$^+$ proliferative response to BCG antigens by examining distinct functional populations. The distribution of these cells were similar amongst infants, irrespective of maternal HIV ($P = 0.26$) or *Mtb* sensitization status ($P = 0.76$), Supplemental Digital Content 10, http://links.lww.com/QAD/A614. BCG-specific IL-2$^+$ CD8$^+$ T cells contributed a very small proportion of the overall response; however, the frequency was significantly different between the four groups of infants, $P = 0.005$. HIV-unexposed infants had higher frequencies of these cells (median = 0.18%, IQR 0.09−0.73) than HIV-exposed infants, (median = 0.04%, IQR 0.00−0.19, $P = 0.002$). Infants born to *Mtb*-sensitized mothers (median = 0.18%, IQR 0.03−0.70) also had an increased frequency of these cells compared with infants born to unsensitized mothers (median = 0.05%, IQR 0.00−0.16), $P = 0.02$.

Following BCG vaccination at 6 weeks of age, there were no differences in the cytokines or chemokines detectable in cell culture supernatants amongst any group of infants, and there was no significant effect of maternal HIV or *Mtb* sensitization alone or in combination (data not shown).

Discussion

Our study is the first to investigate the effects of both maternal HIV infection and *Mtb* sensitization on BCG-induced immune responses in mother−infant pairs. Despite the differences in T cell responses between HIV-infected and HIV-uninfected mothers and some differences in their infants at birth, immune responses following BCG vaccination were strikingly similar amongst all infants by 16 weeks of age. This implies that HIV-exposed and unexposed infants have the same potential to respond to BCG immunization at 6 weeks of age, irrespective of maternal *Mtb* sensitization, and are as likely to benefit from vaccination. Given the high risk of *Mtb* exposure early in life in HIV-exposed infants, this is an important finding [11,37].

There is compelling evidence that foetal HIV-exposure can 'prime' the developing immune system resulting in a more activated and mature immunophenotype [13−16,19,38−40]. It has been postulated that HIV-exposed, uninfected infants may therefore be more responsive to unrelated antigens [27]. A previously unexplored hypothesis is that sensitization to mycobacteria *in utero* might 'prime' the immune system such that infants have recall responses to mycobacterial antigens after birth. In mice, it has been shown that *Mtb* can prime the foetal immune system, but this had not previously been explored in humans [41].

In our cohort, we did not observe any effect of HIV exposure or maternal *Mtb* sensitization on T-cell proliferation or total intracellular expression of cytokine in response to BCG antigens in newborn infants. However, there were some differences in frequencies of subsets of proliferating T cells expressing TNF-α IFN-γ, IL-2 in various combinations. These results were corroborated by results of secreted cytokines. It seems therefore that exposure to HIV *in utero* may prime specific aspects of the response to mycobacterial antigens at birth and that maternal Mtb sensitization can modulate this to some extent. The underlying mechanisms for this are not clear but could include HIV or *Mtb* antigen, and maternal cells or antigen-loaded microvesicles might traverse the placental barrier; alternatively, maternal cytokines or chemokines may cross the placenta and influence the immunological milieu of the developing foetus. In support of this, we observed correlations between maternal and infant cellular and cytokine responses to mycobacterial antigens at birth, a few of which persisted to 16 weeks of age.

In addition to infant responses, we measured paired maternal mycobacterial responses in order to explore possible reasons for the differences in infant immune responses at birth. We showed that HIV-infected mothers demonstrate significant differences in responses to BCG antigens dependent on prior *Mtb* sensitization; this effect was not observed amongst HIV-uninfected women, which was somewhat surprising. A potential explanation is that memory responses to BCG are severely impaired in HIV-infected women, but boosting occurs though cross-priming of BCG memory cells by *Mtb* [42]. It has been established that central memory T-cell responses to childhood vaccines are lost early in the course of adult HIV infection and remain severely impaired [43,44]. In contrast, vaccine recall responses are detectable for many years in HIV-uninfected individuals [44,45]. We speculate that in HIV-uninfected women, the pool of memory cells induced by childhood BCG vaccination is maintained and cross-priming by *Mtb* does not increase this population of BCG memory cells further. Experiments analysing the differential responses between BCG and *Mtb*-specific antigens would be required to investigate this hypothesis.

Participating HIV-infected women were more likely to reside in an informal housing structure than HIV-uninfected women. This alludes to additional socio-economic disadvantage in this group and suggests that there are likely to be unmeasured differences between the groups of women, for example in diet, maternal

microbiome and microbial exposure that might act as potential confounders. Most relevant is *Mtb* exposure; we used the QFN test to determine Mtb sensitization and repeated clinical screening of mothers and infants to exclude TB disease. However, given the factors discussed, the intensity of exposure might be different in the different groups.

An important strength of our study was the measurement of infant responses to mycobacterial antigen pre and post-BCG vaccination in order to ascertain whether differentially affected immune responses at birth affect vaccine immunogenicity. Our data show that BCG vaccination is associated with a substantial increase in BCG-specific T-cell proliferation and intracellular cytokine expression, independent of HIV exposure and maternal *Mtb* sensitization. There are few studies that have examined antigen-specific responses pre and post-BCG vaccination in infants exposed to HIV *in utero* and none that have addressed whether maternal Mtb infection can additionally alter this response. In a different study setting, using different methodology, Van Rie *et al.* [27] found that the pattern of change in secreted IFN-γ response to BCG vaccination was altered in some, but not all, HIV-exposed, uninfected infants, in contrast to our own results.

Following BCG vaccination, we found no difference in the frequency of proliferating T cells, the expression of intracellular cytokines or the concentration of secreted cytokines between infants. However, HIV-exposed, uninfected infants and infants born to *Mtb*-sensitized mothers had significantly higher frequencies of BCG-specific IL-2 single-positive CD8$^+$ T cells than HIV-unexposed infants. Although this small subset of CD8$^+$ T cells may not have any relevance for the protection afforded by the BCG vaccine, these cells may reflect infant antigenic CD8$^+$ 'memory'. Interestingly, an association between maternal and infant CD8$^+$ IL-2$^+$ T cells was still observed at 16 weeks postdelivery, particularly amongst infants born to *Mtb*-sensitized mothers.

Mansoor *et al.* [46] also reported comparable BCG immunogenicity in HIV-exposed and unexposed infants. In contrast to the short-term stimulation assay used in their laboratory, we utilized a 6-day proliferation assay to assess central memory responses, which are thought to be critical for long-term vaccine-induced protection [47]. Our data are also consistent with a study from Uganda, which showed no difference in secreted cytokines in response to mycobacterial antigens amongst BCG-vaccinated infants, irrespective of maternal HIV status [48]. Another study from Malawi found a tendency towards reduced proliferative capacity in response to purified protein derivative, but similar responses to BCG antigens in HIV-exposed, uninfected infants compared with HIV-unexposed infants [14]. Mazzola *et al.* [49], however, reported a higher proportion of proliferating CD4$^+$ T cells and lower proportion of γδ$^+$ cells in

HIV-exposed infants than unexposed infants. Unlike our study, none of these studies also assessed the impact of maternal *Mtb* sensitization on infant BCG responses.

We have previously reported that concentrations of specific antibody are influenced by in-utero HIV-exposure, but that antibody responses to non-BCG vaccines were unaffected [31]. Our findings of cellular immune responses to BCG vaccination reported here mirror these observations.

A limitation of our study was enrolment of modest numbers of mother–infant pairs at a single centre. Enrolment was consecutive and representative of the women delivering at the community health facility, but the cohort may not be representative of other areas of the resource-poor world. We were unable to correlate BCG-induced responses with protection against TB, which would require very large cohorts, making the detailed immunological studies challenging.

In summary, despite the effect of maternal HIV and *Mtb* sensitization on maternal mycobacteria-specific responses and infant responses at birth, our data suggest that HIV-exposed infants have the same potential to respond to BCG immunization administered at 6 weeks of age as HIV-unexposed infants and are therefore as likely to benefit from this vaccine.

Acknowledgements

Conflicts of interest

The authors declare no conflicts of interest.

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