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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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Received: 15 August 2014; revised: 30 October 2014; accepted: 31 October 2014.

DOI:10.1097/QAD.0000000000000536

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AIDS 2015, **29**:155–165

Keywords: bacille Calmette-Guérin, HIV infection, HIV-exposed, immunogenicity, *Mycobacterium tuberculosis* infection, uninfected infants, vaccination

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

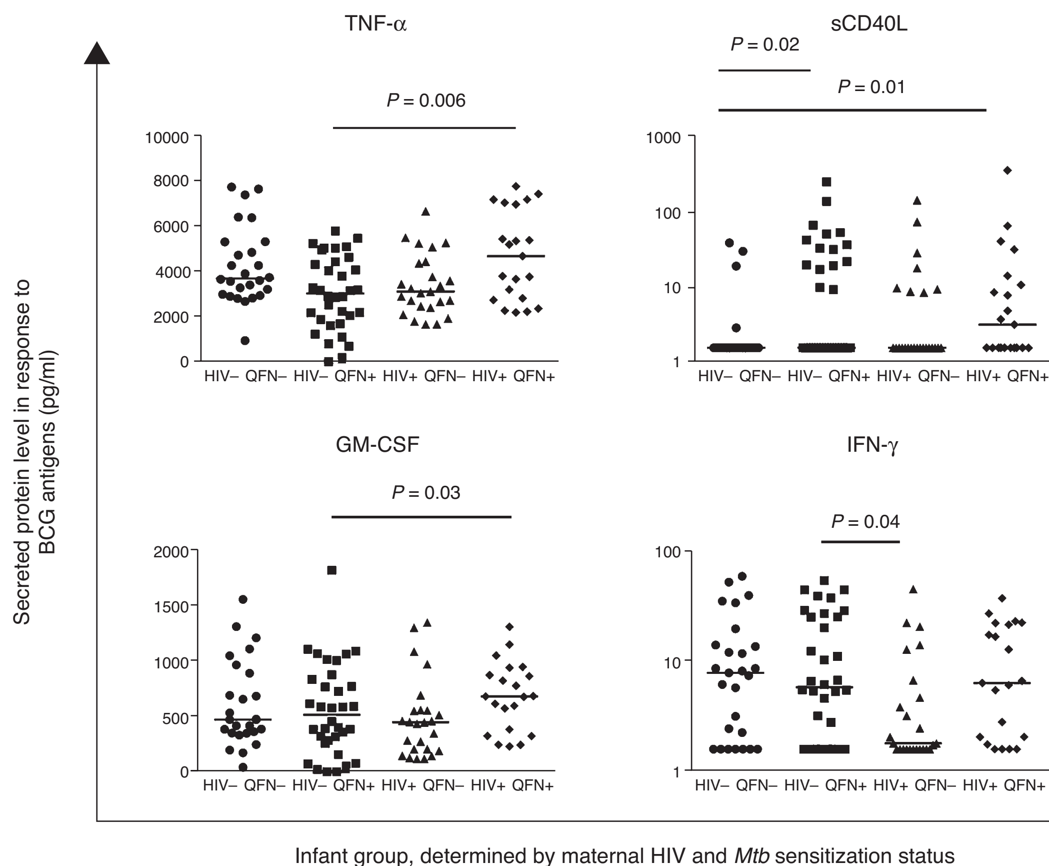

Fig. 1. Concentration of tumour necrosis factor-alpha, GM-CSF, sCD40L and interferon-gamma *in vitro* in response to bacille Calmette-Guérin antigens in newborn infant samples. Horizontal lines represent median values. Only comparisons with $P < 0.05$ are given. IFN-γ and sCD40L data are presented using a log scale.