Published in final edited form as:

AIDS. 2014 June 19; 28(10): 1421–1430. doi:10.1097/QAD.0000000000000292.

In-utero exposure to maternal HIV infection alters T-cell immune responses to vaccination in HIV-uninfected infants

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Abstract

Objective—In sub-Saharan Africa, HIV-exposed uninfected (HEU) infants have higher morbidity and mortality than HIV-unexposed infants. To evaluate whether immune dysfunction contributes to this vulnerability of HEU infants, we conducted a longitudinal, observational cohort study to assess T-cell immune responses to infant vaccines (*Mycobacterium bovis* BCG and acellular pertussis) and staphylococcal enterotoxin B (SEB). In total, 46 HEU and 46 HIV-unexposed infants were recruited from Khayelitsha, Cape Town.

Methods—Vaccine-specific T-cell proliferation (Ki67 expression) and intracellular expression of four cytokines [interferon- γ , interleukin (IL)-2, IL-13 and IL-17] were measured after whole blood stimulation with antigens at 6 and 14 weeks of age.

Results—HEU infants demonstrated elevated BCG-specific CD4⁺ and CD8⁺ T-cell proliferative responses at 14 weeks ($P = 0.041$ and 0.002 , respectively). These responses were significantly increased even after adjusting for birth weight, feeding mode and gestational age. Similar to BCG, increased CD4⁺ and CD8⁺ T-cell proliferation was evident in response to SEB stimulation ($P = 0.004$ and 0.002 , respectively), although pertussis-specific T cells proliferated comparably between the two groups. Within HEU infants, maternal CD4⁺ cell count and length of antenatal antiretroviral exposure had no effect on T-cell proliferation to BCG or SEB. HIV exposure

significantly diminished measurable cytokine polyfunctionality in response to BCG, *Bordetella pertussis* and SEB stimulation.

Conclusion—These data show for the first time, when adjusting for confounders, that exposure to HIV *in utero* is associated with significant alterations to CD4⁺ and CD8⁺T-cell immune responses in infants to vaccines and nonspecific antigens.

Keywords

cytokines; infants; maternal HIV; proliferation; T-cell immunity; vaccination

Introduction

An estimated 1.6 million infants are exposed to HIV during pregnancy and delivery annually; approximately 260 000 acquire HIV [1]. Due to the successful introduction of prophylactic interventions to prevent transmission of HIV, the mother-to-child transmission (MTCT) rates have been reduced to between 1 and 5% [2]. An infant born to an HIV-infected mother, but who remains uninfected after birth and during breastfeeding, is referred to as HIV-exposed uninfected (HEU) [3]. Exposure to HIV has clinical consequences, since HEU infants have increased morbidity and mortality compared to their unexposed counterparts [4–8]. The impact on clinical outcomes in HEU infants is independent of maternal HIV stage or socio-economic status [7]. The reasons for this are unclear, although one potential mechanism is through the in-utero exposure to HIV antigens – indeed some infants display immunological memory to HIV proteins [9–11]. HIV antigens might impact fetal immune maturation and responses to novel antigens in infants [12]. Alternatively, the increased immune activation and inflammation that are present during maternal HIV may have a long-term impact on the infant immune system that may affect the response to antigenic challenge after birth.

Neonatal innate, humoral, and cellular immunity differ to that of adults [13]. It is thought that neonates are poor type 1 T-helper (Th1) cytokine producers due to a lack of interleukin (IL)-12 production by dendritic cells [14–16]. However, Th1 cytokines, such as interferon-gamma (IFN- γ), IL-2, and tumor necrosis factors (TNFs), can be elicited in certain circumstances, such as with cytomegalovirus (CMV) infection or BCG vaccine [17], or in certain cytokine milieus [18–21]. Both HIV and pregnancy are characterized by alterations in Th1, Th2, and Th17 cytokines [22,23], and HIV infection may cause a Tc2-to-Tc1 bias [24] that may alter neonatal antigen responses in exposed, uninfected neonates [25].

Jones *et al.* [26] have demonstrated that HEU infants have lower specific antibody to routine infant vaccines at birth, but generate a robust response following vaccination. This indicates good humoral immunity, and raises questions regarding how intrauterine HIV exposure impacts cellular immune responses to mycobacterial and other common vaccine antigens. HEU infants provide an opportunity for studying not only the consequences of antigenic exposure and/or immune activation *in utero*, but also provide important insights for HIV vaccine development. To this end, we measured the ability of T cells to divide and express multiple cytokines to BCG, as well as *Bordetella pertussis*, in HEU compared to HIV-unexposed infants.

Methods

Study design

HIV-exposed uninfected and HIV-unexposed infants ($n = 46$ per group) were sequentially recruited postpartum from the Midwife Obstetric Unit in Khayelitsha site B, Western Cape Province, South Africa, between February 2010 and August 2012. All mothers were offered voluntary counseling and HIV testing at the time of antenatal care registration.

Eligibility criteria included birth weight greater than 2.4 kg, no complications during pregnancy or labor, no known household tuberculosis contacts, term gestation, and documented maternal HIV test results during this pregnancy. In addition, HEU infants tested HIV DNA PCR-negative at birth and 6 weeks. Khayelitsha has a high antenatal HIV prevalence of 30% [27], and dual therapy [zidovudine (ZDV) with single-dose nevirapine (NVP)] or HAART was offered to HIV-infected pregnant women, with NVP to the infants during this period [28].

BCG vaccine [Danish strain 1331; Statens Serum Institute (SSI)] was administered intradermally within 24 h after birth (standard of care) to HIV-unexposed infants and after HIV DNA PCR at 2–3 days of age for HEU to avoid BCG vaccine adverse events [29] (Supplemental Tables 1 and 2, <http://links.lww.com/QAD/A514>). Infants received all other routine vaccines according to the South African Expanded Program on Immunization (EPI) schedule including Oral Polio (OPV; Sanofi-Pasteur, Midrand, South Africa) at birth and 6 weeks, and Diphtheria-acellular Pertussis-Tetanus (DT_aP)-iPV/Hib (Sanofi-Pasteur) at 6, 10 and 14 weeks [30]. The study was approved by the Research Ethics Committees of the University of Cape Town and Stellenbosch University, and the University of Washington Institutional Review Board.

Sample collection

At birth, HEU infants had 500 µl blood collected into EDTA tubes for HIV DNA PCR. Between 1 and 3 ml was collected from both infant groups at 6 and 14 weeks of life into heparinized tubes.

Whole blood assay

Samples were placed in culture within 8 h of phlebotomy [31]. Whole blood was mixed (1:10) with warm Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, St. Louis, Missouri, USA) without additives, plated into a 24-well culture plate and incubated at 36°C with 5% CO₂ with the following antigens: 1×10^5 cfu/ml *Mycobacterium bovis* BCG (Danish strain 1331, SSI, Copenhagen, Denmark), 0.16 IU tetanus toxoid [TETAVAX, Aventis Pharma (Pty) Ltd, Mumbai, India] and 0.16 IU Pertussis antigens (Difco Bordetella Pertussis Antigen; BD Biosciences, San Jose, California, USA), and a negative control (medium alone).

After 24 h of incubation, 1 µg/ml staphylococcal enterotoxin B (SEB) was added to its required well and cells were incubated for a further 5 days. On the sixth day (day 5 for SEB), 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and 1.5 µg/ml

Ionomycin (Sigma-Aldrich) and 1.5 µg/ml Brefeldin A (Sigma-Aldrich) were added for the last 4 h of incubation. The cells were harvested in 20 mmol/l EDTA (Sigma-Aldrich). Red blood cells were lysed and white cells were stained with Pacific Blue Live/Dead stain [violet viability dye (VIVID); Invitrogen], fixed in BD FACS Lysing Solution (BD Biosciences) according to manufacturer's instructions and cryopreserved in a 10% DMSO-containing solution for storage at -80°C until analysis by flow cytometry [31].

Flow cytometry

Fixed, cryopreserved cells were thawed, permeabilized and stained for cellular markers: T-lymphocyte markers (CD3 and CD8), and intracellular cytokine markers (Th1/Tc1: IFN-γ and IL-2; Th2/Tc2: IL-13; Th17/Tc17: IL-17; and the proliferation marker: Ki67). Expression was measured by multiparameter flow cytometry (BD LSR Fortessa) using the following monoclonal antibodies: anti-CD3-APC-Cy7 clone UCHT1, anti-IFN-γ-Alexa Flour 700 clone B27 and anti-IL-17-PE-Cy7 clone BL168 (Biolegend, San Diego, California, USA); anti-CD8-PerCP-Cy5.5 clone SK1, anti-Ki67-FITC clone B56, anti-IL-2-APC clone 5344.111, anti-IL-13-PE clone JES10-5A2 (BD Biosciences, San Jose, California, USA); VIVID-Pacific Blue (Invitrogen, Brown Deer, Wisconsin, USA). Samples were acquired on a BD LSR Fortessa flow cytometer.

Data analysis

Postacquisition compensation and subsequent analysis was performed using FLOWJO V9.4.7 (Tree Star, Ashland, Oregon, USA). Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. After gating on live CD3⁺ cells, and CD8⁺ versus CD8⁻ cells (for CD4⁺ cells due to PMA-Ionomycin mediated CD4⁺ degradation in T cells) [32], CD8⁻ and CD8⁺ cells expressing Ki67⁺ were then quantified (Supplementary Fig. 1, <http://links.lww.com/QAD/A514>), and CD8⁻ considered as CD4⁺. Further, the following cytokine-expressing cells (IFN-γ, IL-2, IL-13 and IL-17) were gated within Ki67⁺ CD8⁻ and CD8⁺ cells (Supplementary Fig. 1, <http://links.lww.com/QAD/A514>). Fluorescence Minus One (FMOs) were used to define gates where expression was continuous. Boolean gating was used to quantify cells expressing a single or a combination of multiple cytokines. Data were analyzed with Pestle V1.7 software (VRC, NIH) for background subtraction of the negative control responses from that of specific antigens and Spice V5.22 software for CD8⁻ and CD8⁺ T cells breadth of cytokine responses.

Statistical analyses were performed using GraphPad Prism V5.0 (GraphPad Software Inc, La Jolla, California, USA) and Stata V11 (College Station, Texas, USA). The frequency of specific proliferating and cytokine-producing T cells between groups was compared using the Mann-Whitney *U*-test. Multiple comparison adjustment was performed using the Holm step-down procedure [33]. Multiple linear regression was used to estimate the association between HIV exposure status and log-transformed proliferative responses with adjustment for potential confounding variables identified *a priori*. A sample size of 46 per group allowed an 80% power to detect a 10% difference in the frequency of CD4⁺ki67⁺ cells, calculated using an independent cohort (two-tailed test at alpha = 0.05).

Results

Cohort characteristics

Evaluation of the clinical characteristics of HEU versus HIV-unexposed infants did not reveal any unexpected differences between the two groups (Supplemental Table 1, <http://links.lww.com/QAD/A514>). Median birth weight and the proportion of women who intended to exclusively breastfeed (EBF) infants at birth were lower in the HEU compared to HIV-unexposed infants [median 3.1; interquartile range (IQR) 2.8–3.4 kg versus 3.3; IQR 3.1–3.6 kg; $P = 0.009$ and 23.8 versus 100%; $P < 0.001$, respectively]. But by 6 weeks, both infant weight and the proportion of infants exclusively breastfed were similar between the two groups. In addition, the overall morbidity was higher in HEU versus HIV-unexposed infants, including the frequency of lower respiratory tract infection (Supplemental Table 1, <http://links.lww.com/QAD/A514>; 26 versus 13%; $P < 0.001$). All women except one were on antiretrovirals during pregnancy, 22 (48%) were on dual therapy, and 23 (50%) received HAART (Supplemental Table 1, <http://links.lww.com/QAD/A514>). Median CD4⁺ cell counts of mothers on dual therapy were significantly higher than those on HAART (396 versus 298 cells/ μ l; $P = 0.015$). All infants except one (98%) received postpartum NVP. Finally, the median age at receipt of BCG vaccine was significantly higher in HEU infants than that of the HIV-unexposed infants due to the turnaround time for HIV DNA PCR results (3 versus 0 days; $P < 0.001$).

Antigen-specific T-cell proliferative responses to BCG in HIV-exposed uninfected versus HIV-unexposed infants

Lymphoproliferation is an important measure of vaccine immunogenicity; therefore the frequency of Ki67⁺ CD4⁺ and CD8⁺ T cells to BCG stimulation was compared in HEU versus HIV-unexposed infants at 6 and 14 weeks of age. In response to BCG, there were no significant differences in the frequency of CD4⁺ or CD8⁺ cells expressing Ki67⁺ between the groups at 6 weeks of age (Fig. 1a). However, at 14 weeks, there was a higher frequency of both BCG-specific proliferating CD4⁺ (22 versus 9%; $P = 0.041$) and CD8⁺ (Fig. 1b; 16 versus 7%; $P = 0.002$) T cells in HEU infants. In a linear regression estimating the association between HIV exposure and proliferation, HIV exposure remained positively associated with CD4⁺ T-cell proliferation in response to BCG, at 14 weeks, when adjusting for potential confounders including birth weight, feeding mode at 6 weeks (feeding at birth was collinear and not included in the model), and gestational age, although not significantly so [adjusted coefficient 0.332, 95% confidence interval (CI) –0.057; 0.721].

Within the exposed infants, maternal CD4⁺ cell count was not predictive of CD4⁺ T-cell proliferative response to BCG at 6 or 14 weeks ($P = 0.172$ and 0.565 , respectively). Maternal CD4⁺ cell count had a weak negative correlation with CD8⁺ T-cell proliferation at 6 weeks ($P = 0.088$), but not at 14 weeks ($P = 0.571$). Median duration of exposure to antiretrovirals did not significantly affect the CD4⁺ or CD8⁺ proliferative response to BCG ($P = 0.731$ and 0.339 , respectively).

Polyfunctionality of BCG-induced T-cell cytokine expression in HIV-exposed uninfected versus HIV-unexposed infants

One of the important effector functions of proliferating T cells is their ability to produce cytokines, and generally, more polyfunctional responses are considered better (i.e. ability to produce more than one cytokine) [34–36]. However, when we assessed whether Ki67+ proliferating T cells could express mono or multiple cytokines in combination, the overall functionality of BCG-specific CD4⁺ and CD8⁺ T-cell responses was significantly less in the HEU infants when measured at 6 weeks (Fig. 2a and b; $P = 0.018$ and 0.013 , respectively). This lower HEU cytokine functionality was maintained through 14 weeks after vaccination for CD4⁺ (Fig. 2c; $P = 0.008$) and CD8⁺ (Fig. 2d; $P = 0.011$) T cells.

When evaluating the frequency of total T cells producing specific cytokines and combinations thereof following BCG stimulation, HEU infants exhibited a significantly lower frequency of single-expressing IFN- γ + and IL-13+ CD4⁺ T cells compared to HIV-unexposed infants at 6 weeks of age ($P = 0.027$ and $P = 0.011$, respectively), although the frequency of IL-13-producing cells was low for all infants (Fig. 2e). There were significantly higher frequencies of BCG-specific proliferating CD8⁺ T cells expressing no measurable cytokine in HEU compared with HIV-unexposed infants at 6 weeks (Fig. 2f) ($P < 0.001$).

The observations made at 6 weeks were more pronounced at 14 weeks, with higher frequencies of total CD4⁺ and CD8⁺ T cells expressing no cytokine in HEU than HIV-unexposed infants ($P = 0.022$ and < 0.001 , respectively), as were those of total CD8⁺ T cells expressing single cytokines: IL-2+ and IFN- γ + ($P < 0.001$ for both) (Fig. 2h). Conversely, the frequency of BCG-specific dual-expressing CD8⁺ IL-2+IL-17+ cells was lower in HEU than HIV-unexposed infants at 14 weeks ($P = 0.027$). There were no significant differences between HEU and HIV-unexposed infants in the frequency of total T cells making three cytokines in all the different combinations, or in making all four cytokines concurrently, although the frequencies were small (data not shown). Overall, HIV exposure markedly diminished the cytokine expressing T cells in response to BCG vaccination.

Antigen-specific T-cell proliferation and cytokine responses to *Bordetella pertussis* in HIV-exposed uninfected versus HIV-unexposed infants

To evaluate whether the effect of HIV exposure in response to BCG vaccination was applicable to other vaccines, we assessed T-cell proliferative and cytokine responses to pertussis vaccination at 14 weeks of age, 8 weeks after initial vaccination. Although there were no differences in T-cell proliferation after pertussis stimulation (Supplemental Fig. 2, <http://links.lww.com/QAD/A514>), HEU infants demonstrated significantly less overall functionality of proliferating CD4⁺ and CD8⁺ T-cell cytokine responses than HIV-unexposed infants ($P < 0.001$ and 0.013 , respectively) (Fig. 3a and b). Moreover, when evaluating the frequencies of total T cells producing cytokine, significantly higher CD4⁺ and CD8⁺ T cells expressing zero measurable cytokine were evident in response to pertussis stimulation in HEU when compared with HIV-unexposed infants ($P = 0.01$ and 0.021 , respectively). HEU had significantly fewer single-expressing IFN- γ +CD8⁺ T cells than HIV-

unexposed infants at 14 weeks of age (Fig. 3d; $P = 0.037$). HIV exposure therefore influences cytokine responses to vaccinations in addition to BCG.

T-cell proliferation and cytokine responses to staphylococcal enterotoxin B in HIV-exposed uninfected versus HIV-unexposed infants

We further characterized the T-cellular immunological responsiveness between HEU and HIV-unexposed infants by using the superantigen SEB. Similar to BCG, CD4⁺ and CD8⁺ T cells from HEU infants proliferated significantly more compared with HIV-unexposed infants when stimulated with SEB (43 versus 13%; $P = 0.004$ for CD4⁺ and 62 versus 20%; $P = 0.002$ for CD8⁺) at 14 weeks of age (Fig. 4). HEU infant T cells had lower overall cytokine functionality than HIV-unexposed infants (CD4⁺ $P = 0.002$; Fig. 5a, CD8⁺ $P < 0.001$; Fig. 5b). There was a remarkable lack of T-cell cytokine expression regardless of HIV exposure status in response to SEB.

In a linear regression estimating the association between HIV exposure and proliferation, HIV exposure was significantly associated with better CD4⁺ proliferative response to SEB at 14 weeks when adjusting for a priori confounders including birth weight, feeding mode at 6 weeks, and gestational age (coefficient 0.427, 95% CI 0.117; 0.737) (Supplemental Table 2, <http://links.lww.-com/QAD/A514>). In a subanalysis within the HIV-exposed infants, maternal CD4⁺ cell count was not significantly associated with CD4⁺ or CD8⁺ T-cell proliferative response to SEB at 14 weeks ($P = 0.873$ and 0.964 , respectively). Median duration of exposure to antiretrovirals was not predictive of the CD4⁺ or CD8⁺ proliferative response to SEB ($P = 0.199$ and 0.524 , respectively).

When evaluating the specific cytokines being produced following SEB stimulation at 14 weeks, the responses resembled that of BCG. The frequency of total CD4⁺ T cells in HEU infants making no measurable cytokine was significantly higher than HIV-unexposed infants ($P = 0.003$) (Fig. 5c). There were multiple differences in CD8⁺ T-cell responses to SEB (Fig. 5d): significantly higher frequency of total cells producing no measurable cytokine in HEU ($P < 0.001$), significantly lower single-IFN- γ ($P = 0.008$), double-IL-17+IL-13+ ($P = 0.039$) and triple IFN- γ +IL-17+IL-13+ ($P = 0.001$)-expressing CD8⁺ T cells.

Discussion

There is a limited understanding of the effects of in-utero HIV exposure on T-cell responses to currently available infant vaccines [25,26,34]. T-cell proliferation is an important measure of vaccine immunogenicity and T-cell function, and few studies have examined the effect of HIV exposure on this parameter in infants. In the current study, HIV exposure increased the proliferative response to BCG vaccination and to SEB until at least 14 weeks of age. However, the increased proliferation in the HEU infant T cells was associated with a reduced production of cytokine, with the majority of cells making no cytokine. Although it is possible that these cells were expressing cytokines not measured in our panel (such as TNF- α or IL-4), HEU infants clearly had impaired production of cytokines most frequently associated with vaccine immunogenicity. An alternative interpretation is that the actual number of cells expressing cytokine is similar in the HEU and HIV-unexposed infants, but that the proliferation has resulted in increased BCG-specific T cells in HEU

infants with a lower proportion that are polyfunctional. However, HEU infants still demonstrated impaired cytokine production when evaluating total T cells. In addition, there was no difference in proliferative response to pertussis, providing further evidence that the absolute numbers of T cells making cytokine in response to pertussis were deficient.

Polyfunctional T cells may be indicative of the quality of immune responses to vaccines and have been associated with better clinical prognosis during HIV and other infections [35–37]. Mansoor *et al.* [38] found no difference in polyfunctionality of CD4⁺ T-cell responses between HEU and HIV-unexposed infants in response to BCG given at birth when assessed at 12 weeks of age, although only Th1 cytokine responses were evaluated. In the present study, using a broader spectrum of cytokines (Th2/Tc2 and Th17/Tc17 in addition to Th1/Tc1), and a larger sample size, we had more power to detect differences between HEU and HIV-unexposed infants, and found reduced polyfunctionality of the vaccine antigen-stimulated CD4⁺ and CD8⁺ T cells within the HEU infants. Although the specific correlates of BCG-induced protection remain unknown, Th1 and Th17 responses are thought to be important [39,40]. In HEU infants, a significantly lower frequency of CD4⁺ T cells produced IFN- γ in response to BCG at 6 weeks of age. Likewise, HEU infants had significantly lower frequencies of polyfunctional CD8⁺ T cells making IL-17 in combination with IL-2 than HIV-unexposed infants.

Inclusion of the assessment of pertussis and SEB responses provided broader insights into the effect of HIV exposure on T-cell function. This is the first study comparing *B. pertussis*-specific T-cell responses in HEU versus HIV-unexposed infants. Similar to BCG, HEU cytokine responses to *B. pertussis* were significantly less polyfunctional than HIV-unexposed infants. A number of studies have determined that cellular immune response to pertussis is the best correlate of protection [41–43], and is vital for bacterial clearance [42–45]. Likewise, assessing T-cell responses to the superantigen SEB, which is used in many assays as a positive control, revealed important attributes of T-cell functionality in these infants. SEB stimulation results in signaling without the need for co-stimulatory molecules, and through multiple V β T-cell receptors (TCRS) [46,47]. When polyclonal stimulation with anti-CD3 and anti-CD28 was assessed in other studies, only HEU infants born to mothers with detectable plasma viral loads had increased proliferation compared to their unexposed counterparts [27]. Here, we observed increased T-cell proliferation to SEB in infants born to mothers on antiretroviral therapy.

Studies in this target population are prone to confounding by the inherent differences between HEU and HIV-unexposed infants, and previous studies have not always accounted for these biases [25,26,38,46–48]. In this study, the higher proliferative responses of HEU infants persisted after adjusting for confounders including breastfeeding, birth weight, and gestational age. Others have found that HEU infants had a reduced BCG-specific T-cell proliferation at 6 months, but these HIV-exposed infants were formula-fed [28]. It is feasible that the effects of maternal HIV exposure are only detectable in the first few months of life, and will be important to determine the duration of the effects of HIV exposure on cellular immunity through longer follow-up in a breastfed cohort.

Immunological differences observed in the present study between young HEU and HIV-unexposed infants may be due to in-utero perturbation of the TCR repertoire due to HIV antigen exposure [48,49], chronic immune activation and inflammation [25,34,50], and/or maternal antigens such as HIV and opportunistic pathogens [10,11]. Each of these may cause hyper-responsive T cells to proliferate to antigen, but possibly become exhausted with respect to cytokine response to both recall and polyclonal antigen stimulation [51]. It is possible that some of the immune differences observed here were due to exposure to ART *in utero*. Indeed, all but one of the infants in this study were exposed to ART that included ZDV. However, time of exposure to ART did not predict the proliferative response to BCG or SEB in this cohort. Also, ART has been observed to impair, not enhance, proliferation of T cells [52,53]. Whatever the mechanism, our findings demonstrate that although the T cells of HEU infants proliferate more readily to key antigens, the inability of these cells to express functional cytokine responses suggests that T-cell immunity on a per cell basis in these infants is impaired. These altered T-cell immunologic responses could contribute to the increased morbidity and mortality associated with HIV-exposure and have important implications for interventions to improve outcomes of this vulnerable group.

Acknowledgments

The study was supported by funding from the Elizabeth Glaser Pediatric AIDS Foundation Grant MV-00-9-900-01871-0-00, a Thrasher Foundation Early Career Award to HBJ (Grant number NR-0095), and a University of Washington Center for AIDS Research Developmental award (under P30AI027757). The authors are grateful to the Western Cape Department of Health for allowing use of their premises and the study staff, Nonkonzo Dziba and Eunice Mhlamba for assistance with recruitment and follow-up.

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Fig. 1. T-cell proliferative responses to BCG at 6 (a) and 14 (b) weeks: frequency of CD4⁺ (filled ovals and triangles on the left side of each graph) and CD8⁺ (open ovals and triangles on the right side of each graph) proliferating (Ki67+) T cells in whole blood of HIV-exposed uninfected infants (HEU: ovals) compared to HIV-unexposed infants (triangles)

(a) T-cell proliferative responses to BCG at 6 weeks; (b) T-cell proliferative responses to BCG at 14 weeks. Frequencies reported are the frequencies of proliferating cytokine producing T cells out of total CD4⁺ or CD8⁺ T cells. Bars indicate medians; $\alpha = 0.05$; statistical analysis was performed using Mann–Whitney *U* test.

Fig. 2. Cytokine production by BCG-specific T cells

(a–d) proportions of BCG-specific proliferating CD4⁺ and CD8⁺ T cells producing no cytokine, one, or a combination of cytokine(s) at 6 and 14 weeks of life in HIV-exposed uninfected (HEU) versus HIV-unexposed infants. (a) Comparison of the proportion of proliferating CD4⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s), based on expression of IL-2, IFN- γ , IL-13 and/or IL-17, at 6 weeks by HEU versus HIV-unexposed infants. (b) Comparison of the proportion of proliferating CD8⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) at 6 weeks by HEU versus HIV-unexposed infants. (c) Comparison of the proportion of proliferating CD4⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) at 14 weeks. (d) Comparison of the proportion of proliferating CD8⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) at 14 weeks by HEU versus HIV-unexposed infants. Statistical analysis was performed using Wilcoxon signed-rank test. (e–h) Frequency of total CD4⁺ (a

and c) and CD8⁺ (b and d) T cells expressing ki67 and no cytokine, each one, or each combination of IL-2, IFN- γ , IL-13 and/or IL-17, at 6 and 14 weeks of life in HEU versus HIV-unexposed infants. Each possible single cytokine or combinations thereof within the responding CD4⁺ and CD8⁺ T cells at 6 weeks (a and b) and 14 weeks (c and d). Frequency of HEU (blue) versus HIV-unexposed (red) T cells within the total CD4⁺ (left) and CD8⁺ (right) T cells that are proliferating and producing each specific cytokine or cytokine combination. Error bars and whiskers correspond to medians and interquartile ranges (IQRs), respectively. IFN, interferon; IL, interleukin; (*) HU significantly higher; (*) HEU significantly higher. * $P < 0.05$; ** $P < 0.05$ after adjustment for multiple comparisons; statistical analysis was performed using Wilcoxon signed-rank test and multiple comparison adjustment using the Holm step-down procedure.

Fig. 3. Cytokine production by *Bordetella pertussis*-specific T cells

(a, b) Proportions of *Bordetella pertussis*-specific proliferating CD4⁺ and CD8⁺ T cells producing no cytokine, one, or a combination of cytokine(s) at 14 weeks of life in HEU versus HIV-unexposed infants in response to *B. pertussis* antigens at 14 weeks of life. (a) Comparison of the proportion of cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) by proliferating CD4⁺ (left) and CD8⁺ (right) T cells in HEU versus HIV-unexposed infants in response to *B. pertussis* antigens at 14 weeks of life. (c, d) Frequency of total CD4⁺ (a) and CD8⁺ (b) T cells expressing ki67 and no cytokine, each one, or each combination of IL-2, IFN- γ , IL-13 and/or IL-17, at 6 and 14 weeks of life in HEU versus HIV-unexposed. Each possible single cytokine or combinations thereof of the cytokines IFN- γ , IL-2, IL-17, and IL-13 within the total CD4⁺ and CD8⁺ T cells. Frequency of HEU (blue) versus HIV-unexposed (red) T cells within the total CD4⁺ (left) and CD8⁺ (right) T cells expressing Ki67 and each specific cytokine or cytokine combination. Error bars and whiskers correspond to medians and interquartile ranges (IQRs), respectively. HEU, HIV-exposed uninfected; IFN, interferon; IL, interleukin. (*) HIV-unexposed significantly higher; (**) HEU significantly higher. * $P < 0.05$; ** $P < 0.05$ after adjustment for multiple comparisons; statistical analysis was performed using Wilcoxon signed-rank test and multiple comparison adjustment using the Holm step-down procedure.

Fig. 4. T-cell proliferative responses to SEB at 6 and 14 weeks

Frequency of $CD4^+$ (filled ovals and triangles on the left side of each graph) and $CD8^+$ (open ovals and triangles on the right side of each graph) proliferating T cells of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (triangles) presented on the right of each side of the plot. Frequencies reported are the frequencies of proliferating cytokine producing cells out of total $CD4^+$ or $CD8^+$ T cells. Bars indicate medians; $\alpha = 0.05$; statistical analysis was performed using Mann–Whitney *U*-test. SEB, staphylococcal enterotoxin B.

Fig. 5. Proportions of proliferating CD4⁺ and CD8⁺ T cells producing no cytokine, one, or a combination of cytokine(s) in response to SEB at 14 weeks of life in HIV-exposed uninfected (HEU) versus HIV-unexposed infants

(a) Comparison of the proportion of proliferating CD4⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s), based on expression of IL-2, IFN- γ , IL-13 and/or IL-17, at 14 weeks by HEU versus HIV-unexposed. (b) Comparison of the proportion of proliferating CD8⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) at 14 weeks by HEU versus HIV-unexposed infants. (c) Comparison of the proportion of proliferating CD4⁺ T cells expressing no cytokine (pink), any one (green), any combination of two (yellow), three (blue) or all four (red) intracellular cytokine(s) at 14 weeks. Statistical analysis was performed using Wilcoxon signed-rank test. (d) T cells that are SEB-specific and producing no cytokine, each one, or each combination of IL-2, IFN- γ , IL-13 and/or IL-17, at 14 weeks of life in HEU versus HIV-unexposed infants. Each possible single cytokine or combinations thereof within the responding CD4⁺ and CD8⁺ T cells at 14 weeks. Frequency of HEU (blue) versus HIV-unexposed (red) proliferating T cells within the total CD4⁺ (left) and CD8⁺ (right) T cells producing each specific cytokine or cytokine combination. Error bars and whiskers correspond to medians and interquartile ranges (IQRs), respectively. IFN, interferon; IL, interleukin; SEB, staphylococcal enterotoxin B. * $P < 0.05$; ** $P < 0.05$ after adjustment for multiple comparisons; statistical analysis was performed using Wilcoxon signed-rank test and multiple comparison adjustment using the Holm step-down procedure. * HIV-unexposed significantly higher, *HEU significantly higher.