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Altered innate immune development in HIV-exposed uninfected infants

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Abstract

BACKGROUND—Early in life HIV-exposed uninfected (HEU) infants are at an increased risk of morbidity and mortality from infectious disease compared to HIV-unexposed (UE) infants. To improve our understanding of the mechanisms underlying their increased risk, we contrasted innate immune development between HEU and UE infants in a developing world setting, where early-life infectious disease risk is exceptionally high.

METHODS—A prospective longitudinal cohort of HEU and UE newborns was established and the most detailed characterization to date of HEU infant immune development was performed. Single-cell cytokine production was analyzed by flow cytometry after stimulation of whole blood with pathogen associated molecular patterns (PAMP).

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Conflict of Interest Declaration:

No author has a commercial or other association that might pose a conflict of interest. There is no conflict of interest to declare.

Statement Regarding Previously Presented Data:

HIV-exposed infant data has not been previously presented, however, data from the HIV-unexposed infant group used for comparison has recently been published and is referenced accordingly in this manuscript: Reikie BA, Adams RC, Ruck CE, Ho K, Leligowicz A, et al. (2012) Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life. *PLoS One* 7: e44763.

RESULTS—Monocyte, classical dendritic cell and plasmacytoid dendritic cell composition was similar between HEU and UE infants throughout the first year of life. However, HEU mononuclear cells mounted an enhanced pro-inflammatory response to PAMP stimulation, both in quantity of cytokine produced per-cell and in proportion of responder cells. Significant differences in cytokine production were detected on the single cell level in a PAMP-specific pattern, but only at 2 and 6 weeks of age; all differences normalized by 12 months of age.

CONCLUSIONS—This time course of innate immune deviation early in life corresponds to the clinical window of vulnerability to infections in HEU infants and may be at least partially responsible for their increased morbidity and mortality from infectious disease.

Keywords

HIV exposed uninfected; HIV uninfected; HIV in sub-Saharan Africa; immune development; innate immunity; early life immunity

INTRODUCTION

More than 2 million babies are born to HIV-infected mothers every year¹. With improved implementation of prevention of mother to child transmission programs, an increasing majority of these infants are HIV uninfected². However, an increased risk of morbidity and mortality has been observed in HEU infants compared to HIV-unexposed (UE) infants³⁻⁹. The etiology of the increased infection risk in HEU infants is unknown.

Risk of severe infections is greatest early in life, especially for acute respiratory infections, which are the leading cause of mortality in HIV-exposed infants^{10,11}. Specifically, the risk for severe lower respiratory tract infection (LRTI) is greatest during life's earliest months^{7,12}. We previously reported a similar incidence of infectious episodes between UE and HEU infants but noted the frequency of severe infectious events was substantially higher in HEU infants⁹ (using the Division of AIDS Table for Grading Severity of Adult and Pediatric Adverse Events¹³). The relative risk for severe infectious events in HEU compared to UE infants was 3.5 in the first 6 months of life; the majority being LRTIs. This strongly suggests an altered host response to pathogens in HEU vs. UE infants.

Innate immunity orchestrates the initial response to pathogens while shaping future adaptive responses¹⁴, therefore differences in early life innate immunity between HEC and UE infants may be associated with increased risk of infectious morbidity¹⁵.

It is essential to understand how common exposures impact the trajectory of innate immune development in resource poor regions such as sub-Saharan Africa, where the risk of infant morbidity and mortality is the greatest¹⁶. This region carries the heaviest burden of HIV. For example, in South Africa the prevalence of HIV infection in pregnant women visiting antenatal clinics is 30%¹⁷.

In this study, we provide the most comprehensive functional analysis of antigen presenting cells (APCs) in HEU, and the most detailed description of HEU innate immune development to date. Cytokine secretion was characterized in South African infants over the first year of life to determine if differences between HEU and UE innate immune development exist.

METHODS

Ethics Statement

The Health Research Ethics Committee of Stellenbosch University and the Institutional Review Board of the University of British Columbia approved the study (Protocol H09-02064 and H11-01947, respectively). Informed consent was obtained from next of kin, care givers or guardians on behalf of infant participants.

Prospective Birth Cohort Study Design

A prospective, longitudinal cohort study commenced in 2009 in Cape Town, South Africa, to evaluate immune function early in life. Infants born from mothers infected or uninfected with HIV were enrolled at birth at Tygerberg Academic Hospital. Exclusion criteria included: (1) Parent or legal guardian was unable to read and/or comprehend the consent process, (2) diagnosis of a significant chronic medical condition, or (3) any maternal febrile illness within the last 24 hours. Infants were confirmed HIV-negative by HIV-PCR. Infants were seen by health professionals and blood was collected at 2 and 6 weeks, 6 and 12 months of age. Maternal and infant data were collected as previously described⁹.

Blood sample processing

3–5ml of peripheral blood was drawn into sodium-heparin tubes and then immediately processed as described previously^{18,19}. Samples were diluted 1:1 with Roswell Park Memorial Institute-1640 media and added to prefabricated stimulation plates containing PAMPs at concentrations that elicit optimal cytokine expression¹⁸. Six TLR and NOD agonists were used, which represent canonical bacterial vs. viral stimuli, e.g. bacteria (CpG, PAM), Gram negative bacteria (LPS), Gram positive bacteria (peptidoglycan), single stranded RNA viruses (R848), or double stranded RNA viruses (pI:C). CpGA (CpG_{Coley}) stimulates signaling through TLR9, PAM3CSK4 (PAM_{EMC microcollections}) signals via TLR2/1, 0111:B4 LPS (LPS_{In vivo Gen}) through TLR4, Peptidoglycan (PGN_{In vivo Gen}) via TLR2 and NOD1/2, TLR7/8 is stimulated by R848_{In vivo Gen}, and pI:C_{Amersham} through TLR3²⁰. Stimulation plates contained the vesicle transport inhibitor Brefeldin A (added at T=0h for PGN, PAM, LPS and R848, or at T=3h for pI:C and CpG as described¹⁸) and were incubated for 6h at 37°C in 5% CO₂. Cell pellets were stored frozen in FACS Lysing Solution_{BD Biosciences} as previously described¹⁸.

Staining, acquisition and flow cytometric analysis

A detailed description of antibodies (source, clone, and dilution), machine set up, and data acquisition compliant with MiFlowCyt reporting standards^{21,22} is provided in supplementary methods. Samples were prepared for flow cytometric analysis as previously described¹⁸. Stained and fixed cells were analyzed on a FACS Aria flow cytometer_{BD Biosciences} set up using a biological standard, according to published guidelines²². B-cells, monocytes, classical dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) were differentiated, and intracellular TNF- α , IL-6, IL-12 and IFN- α were detected. 300,000 events were acquired per sample for analysis with FlowJo software_{Tree Star} and gates were set based on the fluorescence-minus-one principle²³. Negative controls from

unstimulated samples that produced cytokine above cutoffs were negligible and were subtracted from stimulated samples²³.

Statistical analysis

This study was exploratory with the intent to generate, and not to test a particular hypothesis. The data were strongly interdependent, although the degree of correlation is not quantified between e.g. different cytokines produced in response to particular PAMPs. Therefore, controlling specifically for familywise error rate due to multiple comparisons was inappropriate²⁴. The null-hypothesis was that no differing trends in longitudinal data would be identified between HEU vs. UE innate immune development. The p-values from Mann-Whitney test comparisons between proportion of responder cells and cytokine produced per-cell in HEU vs. UE APC are reported (Supplementary Table 1/ Table 3 and Supplementary Table 2 / Table 4, respectively); p-values < 0.05 were considered significant for the purpose of trend identification. To assess polyfunctionality, the percentage of cells in a given cytokine-combination category was calculated (e.g. there are 7 possible cytokine-combination categories for 3 cytokines in which at least one cytokine is positive). Each of the 7 cytokine-combination categories was compared using Mann-Whitney test. Total response (shown as total bar height, Supplementary Figure 3) for each cell type was compared by Student's t-test. Bonferroni correction for multiple comparisons was applied to compare infant housing characteristics between HEU and UE, and $p < 0.01$ was considered significant.

RESULTS

Racial background and housing attributes differed between HIV-exposed uninfected and HIV-unexposed infants

No significant difference was identified between HEU and UE mothers' age, education, smoking or alcohol consumption during pregnancy. The mean antenatal CD4 count of HIV positive mothers was 337 (range 131–673). A significant difference was identified in racial composition between HEU and UE groups. Eighty-one percent of HEU were of African origin, whereas 71% of UE had mixed racial backgrounds ($p=0.001$). HEU infants did not breastfeed, however failure to thrive was not identified by anthropometrics throughout the study period. The primary caregiver was the mother for 73% and 80% of HEU and UE infants respectively. The majority (58%) of HEU infants lived in informal housing, compared to 15% of UE infants ($p=0.007$), with the same proportion of HEU infants lacking access to running water relative to UE infants ($p=0.007$). More people occupied UE infant households (mean 5.8) versus HEU households (mean 3.9) ($p=0.01$), and UE infants also shared a room with more occupants (mean 3.4) relative to HEU infants who shared with an average of 2.3 ($p=0.008$) (Table 1).

Monocyte, cDC and pDC cell composition was similar, and B-cell composition differed between HIV-exposed uninfected and HIV-unexposed infants

Monocytes, cDC, pDC and B-cells were identified by flow cytometry as illustrated in Figure 1a. The relative proportion of each cell type was compared between HEU and UE groups (Figure 1b) and between subjects of African and Mixed racial backgrounds (Figure 1c).

Differences in proportion of B-cells were observed between HEU and UE groups at 2 weeks (UE>HEU) and between African and Mixed groups at 2 weeks and 6 months (Mixed>African). Differences were also observed between African and Mixed racial groups in cDC proportions at 6 weeks (African>Mixed) and pDC proportions at 6 months (Mixed>African). No other differences were identified in HEU versus UE APC subsets.

Enhanced pro-inflammatory response of monocytes and conventional dendritic cells in HIV-exposed uninfected vs. HIV-unexposed infants

Whole blood was stimulated with PAMPs prior to single cell analysis of cytokine production (IL-6, IL-12, TNF- α and IFN- α) in monocytes, cDCs, pDCs, and B-cells. B-cells did not produce a detectable amount of cytokine in response to PAMP stimulation. The overall trend of evolving reactivity of HEU and UE infant monocytes, cDC and pDCs was similar throughout the first year of life (Figure S1), however when compared at individual time points several differences were observed between groups (Figure 2). HEU monocyte and cDC responses are illustrated in Figure 2a, and these cell types produced significantly more IL-6 and TNF- α in response to stimulation with the bacterial PAMPs, PAM and LPS. HEU cDCs also produced more IL-6 and IL-12 in response to PGN and the ssRNA-like ligand R848 (IL-6 only). All ten significantly different responses detected at 2 weeks represented higher responses in HEU neonates compared to their UE counterparts. Blood samples collected at 6 weeks of age similarly demonstrated higher monocyte and cDC responses in HEU as compared to UE, but differences were restricted to bacterial LPS (TLR4) stimulation only. On the other hand, at 6 weeks UE monocytes produced more IL-12 in response to pI:C (dsRNA). Only a single difference was detected between HEU and UE pDC, with HEU pDC producing more IL-6 in response to R848, although the median response was similar between groups (Figure 2b). The only difference detected past 6 weeks was for UE monocytes producing more TNF- α in response to R848 stimulation at 6 months. By 12 months of age any difference in the reactivity between HEU and UE mononuclear cells had completely disappeared (Supplementary Table 1).

HIV-exposed uninfected infant mononuclear cells produced more cytokine on a per-cell basis than their HIV-unexposed counterparts

Measuring the proportion of cells responding to stimulation does not account for the strength of response per cell. We therefore quantified the difference in mean fluorescence intensity (MFI) between HEU and UE infants for monocyte and cDC (Figure 3a), and pDC (Figure 3b). The overall trend of evolving cytokine production per cell was similar between groups throughout the first year of life (Figure S2), however, multiple differences were detected when MFI was compared between the two groups (Supplementary Table 2). At 2 weeks of life HEU monocytes produced more IL-12 per-cell for all PAMPs tested except for the viral stimuli R848. Similarly, HEU cDC produced more IL-12 per-cell for the bacterial PAMPs (PGN, LPS, PAM). HEU monocytes and cDC produced more IL-6 in response to TLR2/1 stimulation. HEU cDC displayed higher TNF- α production in response to stimulation of TLR4 and TLR7/8. By 6 weeks of age HEU and UE monocyte cytokine production was similar, with no significant difference detected except the UE monocyte TLR3 response, which was the only instance where UE APCs mounted a higher per-cell response compared

to the HEU group. HEU pDC produced more TNF- α and IFN- α per-cell in response to stimulation of TLR7/8 and TLR9, but only within the first 6 months of life.

Early life variability detected in polyfunctional mononuclear cell responses of HIV-exposed uninfected and HIV-unexposed infants

Total proportion of monocytes (Figure S3a), cDC (Figure S3b), and pDC (Figure S3c) that responded to PAMP stimulation was compared between HEU and UE infants (total bar height). Overall, a greater proportion of HEU monocytes and cDC responded to bacterial ligand stimulation (PAM, LPS) up to 6 weeks of age. Conversely, UE monocytes responded more strongly to viral ligand stimulation (pI:C and R848) at 6 months. No significant differences in total responders were detected at 12 months of age.

Mononuclear cell responses were subsequently differentiated into mono- and polyfunctional subtypes. For monocytes and cDC, 7 permutations were theoretically possible for cytokine expression of TNF- α and/or IL-6 and/or IL-12 (Figure S3a and Figure S3b, respectively). For pDC as well there were 7 possible permutations for TNF- α and/or IL-6 and/or IFN- α (Figure S3c). Comparisons between HEU and UE were performed for each subtype's percent responder cells. Overall twenty-seven differences were identified, sixteen at 2 weeks, nine at 6 weeks, none at 6 months, and only two at 12 months of age (Figure 4a). Of the differences identified between HEU and UE, 13 were found in response to LPS, 4 in response to PAM, 3 for PGN, 2 for CpG, 2 for pI:C and 3 for R848 (Figure 4b). At 2 weeks of age, when most differences between HEU and UE were observed, the majority of differences were detected in cytokine production in cDCs. For all statistical differences the HEU subset responses were higher than UE responses.

Comparisons of antigen presenting cell responses between groups defined by race were dissimilar to comparisons between groups defined by maternal HIV infection

Due to differences in racial composition between HEU and UE groups, additional analysis was performed to compare groups defined by race (African vs. Mixed descent). Contrasting the increased proportion of HEU vs. UE APCs responding primarily to bacterial stimuli at 2 weeks of age (Supplementary Table 1), less than a third of the differences were observed in African vs. Mixed race infants (Supplementary Table 3). No pattern was evident that would indicate a greater responsiveness of any cell type to a particular stimuli or at a particular time point. Differences in per-cell cytokine production were identified between infants of African and Mixed race. Infants of mixed race exhibited a pattern of stronger per-cell responses to PRR stimulation at 6 weeks of age (Supplementary Table 4). Specifically, Mixed race cDC produced more TNF- α , IL-6 and IL-12 in response to PGN. More TNF- α and/or IL-6 was produced by Mixed race cDC after LPS and PAM stimulation at 6 weeks. African monocytes produced more TNF- α and IL-12 in response to R848 at 2 weeks and 6 months, respectively. R848 elicited more IL-12 from Mixed race cDC at 6 weeks, and IFN- α from African pDC at 2 weeks.

DISCUSSION

HEU infants are at an increased risk of life-threatening infections³⁻⁹ and differences in innate immunity in early life potentially contribute to their vulnerabilities¹⁵. This study examined early life HEU innate immune development, and contrasted it to UE infants using multiparameter flow cytometry to measure cytokine production in monocytes, cDC and pDC. Overall, HEU innate antigen presenting cells responded more strongly than UE to stimulation with PAMPs in both the proportion of responder cells and quantity of cytokine produced per cell. The majority of differences occurred at the earliest time points and in response to bacterial PAMPs.

Previous comparisons of early life innate immunity in HEU relative to UE demonstrate altered secretion of immune mediating cytokines, increased soluble indicators of inflammation^{25,26} and cell surface receptor expression suggestive of APC activation in HEU²⁷. Many of these observations have been in cord blood and these changes are no longer detected later in life. Functional comparison of natural killer (NK) cell activity at one month of age also demonstrates an increase of an intermediate NK phenotype for activation and perforin expression in HEU vs. UE, which 'normalizes' by one year²⁸. These findings are in line with our observations at the cellular level. Our single-cell focus now provides the sensitivity to detect differences of smaller magnitude (often less than 15% for any single parameter). Analysis focused at the single-cell level also offers the necessary resolution to observe trends when comparing HEU and UE innate immune development, which resulted in the most striking observations. These data delineate the time (<6 weeks) and stimulus (bacterial PAMPs) restricted nature of differences in innate immune ontogeny between HEU and UE infants. Time-restricted differences in innate immune responses to PAMPs may be pertinent to corresponding periods of increased susceptibility of HEU infants to infectious diseases.

Additional resolution was provided by detecting APCs ability to produce more than one cytokine following stimulation (termed 'polyfunctionality'), which is a functional parameter previously correlated with clinical outcomes²⁹. We subdivided innate responses into polyfunctional subgroups and observed heightened responses in HEU, which were almost exclusively induced by bacterial PAMPs in cDC and monocytes. In contrast, no consistent difference in polyfunctionality between HEU vs. UE was observed following stimulation with viral PAMPs, or in pDCs, which are instrumental in viral responses. These data suggest differences in polyfunctionality may be pathogen- and correspondingly also APC subtype-specific. However, our data do not allow for inference of causality for eg. bacterial infections; they solely indicate that differences in innate response to these PAMPs exist in a time frame closely associated with increased risk for severe infection.

The etiological factors driving the observed early life differences in innate immune ontogeny between HEU and UE are likely multifactorial^{6,30}. Future analysis will be needed to evaluate how genetic and environmental differences between HEU and UE infants impact innate immune ontogeny. Just as direct comparisons would provide a better understanding of why there is variability between observations of innate immune development in resource rich settings³¹⁻³³ versus resource poor settings³⁴⁻³⁷. For example, lacking access to running

water correlates positively with expression of IL-10 in childhood³⁸. The majority (58%) of HEU infants in our study lacked access to running water, whereas only 15% of UE infants did. However, HEU APCs responded more strongly to PAMPs, suggesting that elevated IL-10 production associated with this basic measure of sanitation was unlikely a dominant factor influencing difference in innate immune development. Antiretroviral therapy (ART) exposure *in utero* and in the perinatal period may also impact immune development. In adults, ART is associated with anemia, neutropenia, relative lymphopenia and down regulation of select pattern recognition receptor genes³⁹⁻⁴². However, there are currently no data measuring the effect of ART exposure on functional innate immune ontogeny early in life.

Varied racial background was identified between groups (81% vs 29% African in HEU vs UE respectively) of our cohort. Different ethnic groups (with varied genetic backgrounds by extrapolation) can exhibit varied innate immune responses to, and protection from, infectious challenge^{43,44}. More specifically, TLR polymorphisms are associated with heterogeneity of innate responses⁴⁵. In order to test the relative impact of race on our observations we redefined comparison groups by racial background (African vs. Mixed). Given the increased African composition in the HEU group, we expected to find a similar pro-inflammatory pattern in African vs. Mixed responses as was seen in HEU vs. UE. This was not observed. When proportion of responder APCs (to PAMP stimulation) was compared between African and Mixed groups, only 6 differences were detected, which was within the expected level of error using a p-value of <0.05. When the amount of cytokine produced per-cell was compared, a pattern emerged that may suggest pro-inflammatory cDC in Mixed race vs. African infants at 6 weeks. This pattern was weaker and distinct in its timing compared to changes in HEU vs. UE (and does not support a pro-inflammatory pattern in African vs. Mixed). Therefore, while genetic variability is a probable contributor, differences in racial composition between groups was likely not the cardinal factor determining the observed variability in innate immune responses between HEU and UE infants.

Differences in breastfeeding practices may have contributed to the differences in innate immune development between HEU and UE. Breast milk contains compounds that modulate PRR-mediated immune responses, including immunoglobulins, antimicrobial proteins/peptides, nucleotides and oligosaccharides^{46,47}. Breast milk can also alter TLR responses to PRR specific agonists⁴⁸. Clinical evidence indicates that the time period of increased morbidity from diarrheal infections in HEU infants coincides with the average time of weaning⁴⁹. The median duration of exclusive breastfeeding in UE infants of this cohort was 12 weeks, while HEU infants were not breastfed as recruitment occurred prior to the shift towards recommending breastfeeding for infants born to HIV positive mothers⁹. However, it is noteworthy that breastfeeding provides greatest protection from diarrheal disease as opposed to respiratory tract infections⁵⁰, which was the leading cause of severe illness and hospitalization of HEU in this study, and is the leading cause of infectious morbidity and mortality in HEU infants^{7,12}. Lack of breastfeeding is thus likely to only partially explain increased morbidity and altered immune status in HEU vs. UE infants.

Our goal had not been to delineate precise etiological cause-effect relationships, but to first determine if differences in innate immunity between HEU and UE existed at all, and if so, for how long they persisted. Our data indicate that innate immune response to PRR stimulation differed between HEU and UE infants. Specifically, innate ontogeny initially followed a different trajectory in HEU compared to UE infants, but this difference became less apparent as the infant matured. No clear etiology for these differences can at this stage be assigned, as several factors may have contributed to our observation of significant differences in innate immune response between HEU and UE in the earliest postnatal period. Follow-up studies can now focus on examining the relative contribution of potential etiological factors (ranging from HIV-exposure to variables associated with host genetics) to differences in innate immune ontogeny. Our data support the notion that early life represents a window of significant vulnerability for altered immune development^{14,51}. Elucidating the mechanisms that drive these changes in innate immune development may move us closer to identifying targeted strategies to decrease infectious morbidity and mortality in HEU infants. This has potentially broad implications for the rapidly growing population of HIV-exposed uninfected infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. WHO. Towards universal access: scaling up priority HIV/AIDS interventions in the health sector: progress report. 2009.
2. UNAIDS. How to get to zero: Faster. Smarter. Better. UNAIDS World AIDS Day Report. 2011
3. Thea DM, St Louis ME, Atido U, et al. A prospective study of diarrhea and HIV-1 infection among 429 Zairian infants. *N Engl J Med*. Dec 2; 1993 329(23):1696–1702. [PubMed: 8232458]
4. Brahmabhatt H, Kigozi G, Wabwire-Mangen F, et al. Mortality in HIV-infected and uninfected children of HIV-infected and uninfected mothers in rural Uganda. *Journal of acquired immune deficiency syndromes*. Apr 1; 2006 41(4):504–508. [PubMed: 16652060]
5. Marinda E, Humphrey JH, Iliff PJ, et al. Child mortality according to maternal and infant HIV status in Zimbabwe. *The Pediatric infectious disease journal*. Jun; 2007 26(6):519–526. [PubMed: 17529870]

6. Filteau S. The HIV-exposed, uninfected African child. *Tropical medicine & international health : TM & IH*. Mar; 2009 14(3):276–287. [PubMed: 19171011]
7. Koyanagi A, Humphrey JH, Ntozini R, et al. Morbidity among human immunodeficiency virus-exposed but uninfected, human immunodeficiency virus-infected, and human immunodeficiency virus-unexposed infants in Zimbabwe before availability of highly active antiretroviral therapy. *The Pediatric infectious disease journal*. Jan; 2011 30(1):45–51. [PubMed: 21173675]
8. Epalza C, Goetghebuer T, Hainaut M, et al. High incidence of invasive group B streptococcal infections in HIV-exposed uninfected infants. *Pediatrics*. Sep; 2010 126(3):e631–638. [PubMed: 20732944]
9. Slogrove A, Reikie B, Naidoo S, et al. HIV-Exposed Uninfected Infants are at Increased Risk for Severe Infections in the First Year of Life. *J Trop Pediatr*. May 3; 2012 58(6):505–508. [PubMed: 22555385]
10. Bryce J, Boschi-Pinto C, Shibuya K, Black RE, Group WHOCHER. WHO estimates of the causes of death in children. *Lancet*. Mar-Apr;2005 365(9465):1147–1152. [PubMed: 15794969]
11. Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H. Epidemiology and etiology of childhood pneumonia. *Bulletin of the World Health Organization*. May; 2008 86(5):408–416. [PubMed: 18545744]
12. Punpanich W, Groome M, Muhe L, Qazi SA, Madhi SA. Antibiotic and systemic therapies for pneumonia in human immunodeficiency virus (HIV)-infected and HIV-exposed children. *Journal of infection in developing countries*. Feb; 2012 6(2):109–119. [PubMed: 22337838]
13. AIDS Do.. Division of AIDS Table for grading the severity of adult and pediatric adverse events. 2004.
14. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. *Immunity*. Nov 16; 2012 37(5):771–783. [PubMed: 23159225]
15. Dauby N, Goetghebuer T, Kollmann TR, Levy J, Marchant A. Uninfected but not unaffected: chronic maternal infections during pregnancy, fetal immunity, and susceptibility to postnatal infections. *Lancet Infect Dis*. Feb 23; 2012 12(4):330–340. [PubMed: 22364680]
16. Black RE, Morris SS, Bryce J. Where and why are 10 million children dying every year? *Lancet*. Jun 28; 2003 361(9376):2226–2234. [PubMed: 12842379]
17. National Department of Health SA. The National Antenatal Sentinel HIV and Syphilis Prevalence Survey, South Africa. South African National Department of Health; Pretoria, South Africa: 2010.
18. Jansen K, Blimkie D, Furlong J, et al. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. *J Immunol Methods*. Jul 31; 2008 336(2):183–192. [PubMed: 18565537]
19. Blimkie D, Fortuno ES 3rd, Yan H, et al. Variables to be controlled in the assessment of blood innate immune responses to Toll-like receptor stimulation. *J Immunol Methods*. Mar 7; 2011 366(1-2):89–99. [PubMed: 21277305]
20. Engel AL, Holt GE, Lu H. The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system. *Expert review of clinical pharmacology*. Mar; 2011 4(2):275–289. [PubMed: 21643519]
21. Lee JA, Spidlen J, Boyce K, et al. MIFlowCyt: the minimum information about a Flow Cytometry Experiment. *Cytometry A*. Oct; 2008 73(10):926–930. [PubMed: 18752282]
22. Blimkie D, Fortuno ES 3rd, Thommai F, et al. Identification of B cells through negative gating-An example of the MIFlowCyt standard applied. *Cytometry A*. Jun; 2010 77(6):546–551. [PubMed: 20131398]
23. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A*. Sep 1; 2006 69(9):1037–1042. [PubMed: 16888771]
24. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology*. Jan; 1990 1(1):43–46. [PubMed: 2081237]
25. Chougnet C, Kovacs A, Baker R, et al. Influence of human immunodeficiency virus-infected maternal environment on development of infant interleukin-12 production. *J Infect Dis*. May; 2000 181(5):1590–1597. [PubMed: 10823758]

26. Schramm DB, Kuhn L, Gray GE, Tiemessen CT. In vivo effects of HIV-1 exposure in the presence and absence of single-dose nevirapine on cellular plasma activation markers of infants born to HIV-1-seropositive mothers. *Journal of acquired immune deficiency syndromes*. Aug 15; 2006 42(5):545–553. [PubMed: 16837862]
27. Velilla PA, Montoya CJ, Hoyos A, Moreno ME, Chougnet C, Rugeles MT. Effect of intrauterine HIV-1 exposure on the frequency and function of uninfected newborns' dendritic cells. *Clin Immunol*. Mar; 2008 126(3):243–250. [PubMed: 18201932]
28. Slyker JA, Lohman-Payne B, John-Stewart GC, et al. The impact of HIV-1 infection and exposure on natural killer (NK) cell phenotype in Kenyan infants during the first year of life. *Frontiers in immunology*. 2012; 3:399. [PubMed: 23293640]
29. Aghaepour N, Chattopadhyay PK, Ganesan A, et al. Early immunologic correlates of HIV protection can be identified from computational analysis of complex multivariate T-cell flow cytometry assays. *Bioinformatics*. Apr 1; 2012 28(7):1009–1016. [PubMed: 22383736]
30. Shapiro RL, Lockman S. Mortality among HIV-exposed infants: the first and final frontier. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. Feb 1; 2010 50(3):445–447. [PubMed: 20047482]
31. Nguyen M, Leuridan E, Zhang T, et al. Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. *PloS one*. 2010; 5(4):e10407. [PubMed: 20442853]
32. Belderbos ME, van Bleek GM, Levy O, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. *Clin Immunol*. Nov; 2009 133(2):228–237. [PubMed: 19648060]
33. Corbett NP, Blimkie D, Ho KC, et al. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. *PloS one*. 2010; 5(11):e15041. [PubMed: 21152080]
34. Burl S, Townend J, Njie-Jobe J, et al. Age-dependent maturation of Toll-like receptor-mediated cytokine responses in Gambian infants. *PloS one*. 2011; 6(4):e18185. [PubMed: 21533209]
35. Liscianro JG, Prescott SL, Nadal-Sims MG, et al. Ontogeny of Toll-Like and NOD-Like Receptor-Mediated Innate Immune Responses in Papua New Guinean Infants. *PloS one*. 2012; 7(5):e36793. [PubMed: 22649499]
36. Teran R, Mitre E, Vaca M, et al. Immune system development during early childhood in tropical Latin America: evidence for the age-dependent down regulation of the innate immune response. *Clin Immunol*. Mar; 2011 138(3):299–310. [PubMed: 21247809]
37. Reikie BA, Adams RC, Ruck CE, et al. Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life. *PloS one*. 2012; 7(9):e44763. [PubMed: 23028609]
38. Figueiredo CA, Alcantara-Neves NM, Veiga R, et al. Spontaneous cytokine production in children according to biological characteristics and environmental exposures. *Environmental health perspectives*. May; 2009 117(5):845–849. [PubMed: 19478971]
39. Sperling RS, Shapiro DE, McSherry GD, et al. Safety of the maternal-infant zidovudine regimen utilized in the Pediatric AIDS Clinical Trial Group 076 Study. *AIDS*. Oct 1; 1998 12(14):1805–1813. [PubMed: 9792381]
40. Bunders MJ, Bekker V, Scherpbier HJ, Boer K, Godfried M, Kuijpers TW. Haematological parameters of HIV-1-uninfected infants born to HIV-1-infected mothers. *Acta paediatrica*. Nov; 2005 94(11):1571–1577. [PubMed: 16303696]
41. Bunders M, Thorne C, Newell ML, European Collaborative S. Maternal and infant factors and lymphocyte, CD4 and CD8 cell counts in uninfected children of HIV-1-infected mothers. *AIDS*. Jul 1; 2005 19(10):1071–1079. [PubMed: 15958839]
42. Boulware DR, Meya DB, Bergemann TL, et al. Antiretroviral therapy down-regulates innate antiviral response genes in patients with AIDS in sub-saharan Africa. *Journal of acquired immune deficiency syndromes*. Dec; 2010 55(4):428–438. [PubMed: 20838227]
43. Arama C, Giusti P, Bostrom S, et al. Interethnic differences in antigen-presenting cell activation and TLR responses in Malian children during *Plasmodium falciparum* malaria. *PloS one*. 2011; 6(3):e18319. [PubMed: 21483827]

44. Kollmann TR. Variation between Populations in the Innate Immune Response to Vaccine Adjuvants. *Frontiers in immunology*. 2013; 4:81. [PubMed: 23565115]
45. Randhawa AK, Shey MS, Keyser A, et al. Association of human TLR1 and TLR6 deficiency with altered immune responses to BCG vaccination in South African infants. *PLoS Pathog*. Aug.2011 7(8):e1002174. [PubMed: 21852947]
46. M'Rabet L, Vos AP, Boehm G, Garssen J. Breast-feeding and its role in early development of the immune system in infants: consequences for health later in life. *The Journal of nutrition*. Sep; 2008 138(9):1782S–1790S. [PubMed: 18716187]
47. Peroni DG, Pescollendergg L, Piacentini GL, et al. Immune regulatory cytokines in the milk of lactating women from farming and urban environments. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. Sep; 2010 21(6):977–982. [PubMed: 20718928]
48. LeBouder E, Rey-Nores JE, Raby AC, et al. Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. *Journal of immunology*. Mar 15; 2006 176(6):3742–3752.
49. Kourtis APW, Jeffrey, Kayira Dumbani, Chasela Charles, Ellington Sascha R. Hyde Lisa, Hosseinipour Mina, van der Horst Charles, Jamieson Denise J. Health outcomes of HIV-exposed uninfected African infants. *AIDS*. Mar 13; 2013 27(5):749–759. 2013. [PubMed: 23719347]
50. team Wcs. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. *Lancet*. 2000; 355:451–455. [PubMed: 10841125]
51. Dietert RR. Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. *Journal of immunotoxicology*. Oct; 2008 5(4):401–412. [PubMed: 19404874]

Figure 1. Similar proportions of monocytes, cDC and pDC, but different proportions of B-cells in HIV-exposed uninfected versus HIV-unexposed infants

A) Example of differentiation of antigen presenting cells by flow cytometry. B) Shown are the relative proportions of live-gated B-cells, monocytes, cDC and pDC between HEU (black) and UE (grey) subjects at 2 weeks, 6 weeks, 6 months and 12 months of life. C) The relative proportion of antigen presenting cell sub-types are also shown for subjects of Mixed (grey) or African (black) descent at the respective time points. Y-axis represents the median proportion of each cell type; error bars indicate interquartile range. Mann-Whitney test was used to compare groups and p-values are shown at time points where $p < 0.05$.

Figure 2. Elevated pro-inflammatory cytokine response of antigen presenting cells in HIV-exposed uninfected versus HIV-unexposed infants

Whole blood from HEU and UE subjects was stimulated with the indicated TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF- α , IL-6 or IL-12/23p40 in A) monocytes and cDC, and B) TNF- α , IL-6 or IFN- α was detected in pDC. Y-axis represents difference cells. Mann-Whitney test was used to compare HEU and UE response at each time point and differences are signified by * ($p < 0.05$) and ** ($p < 0.01$). Unstimulated samples (near 0% of cytokine producing cells) were subtracted from stimulated samples.

Figure 3. Increased cytokine production on a per-cell basis in HIV-exposed uninfected infant versus HIV-unexposed infant antigen presenting cells

Shown are the same samples as in Figure 2, but with the difference in mean fluorescent intensity (MFI) graphed for median HEU MFI minus the median UE MFI in the respective samples for A) monocytes and cDC, for which we measured TNF- α , IL-6 and IL-12/23p40, and B) pDC, with TNF- α , IL-6 and IFN- α measured. Means for each population (y-axis) are derived from FlowJo software. Differences in MFI between HEU and UE groups as detected by Mann-Whitney test are indicated by * ($p < 0.05$) and ** ($p < 0.01$). measured.

Figure 4. Summary of differences in functional responses between HIV-exposed uninfected vs. HIV-unexposed infants

Whole blood samples were stimulated with TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF- α , IL-6 and IL-12/23p40 in monocytes, and cDC. TNF- α , IL-6 and IFN- α was detected in pDC. Cell groups were subdivided on expression of 1, 2 or 3 cytokines in various permutations, and each functional group was compared between HEU and UE APC cell types for percent responder cells. A) Summary of the number of comparisons for monocytes (grey), cDC (black) and pDC (dashed) between HEU and UE groups ($p < 0.05$). For every comparison, the HEU functional that were statistically different group responded more strongly than their UE counterparts. B) Specific functional subgroups with significantly different responses ($p < 0.05$) between infants. Specific p-values from Mann-Whitney test are shown.

Table 1
Maternal and infant demographic, clinical and housing characteristics

Shown are maternal and infant characteristics adapted from Slogrove et al., 2012⁹, and data pertaining to housing and childcare conditions collected at the 6 month time point. Comparison between HEU and UE groups were performed by student's t-test.

Maternal characteristics [*]	Total (55)	HEU (27)	UE (28)	<i>p-value</i>
Age (in years)–mean (SD)	26.6 (6.3)	25.9 (6.8)	27.3 (5.7)	0.4
Completed secondary education (%)	15 (27)	6 (22)	9 (32)	0.6
Smoked during pregnancy (%)	14 (25)	3 (11)	11 (39)	0.17
Consumed alcohol during pregnancy (%)	4 (7)	0 (0)	4 (14)	0.11
Antenatal CD4 count in cells μL^{-1} –median (range)	337 (131–673)			

Infant Characteristics [*]	Total (55)	HEU (27)	UE (28)	<i>p-value</i>
Antiretroviral exposure ^a (%)	23 (85)			
Male (%)	22 (40)	7 (26)	15 (54)	0.35
Ethnicity				0.001
African ^b (%)	30 (55)	22 (81)	8 (29)	
Mixed or Caucasian ^c (%)	25 (45)	5 (19)	20 (71)	
Birthweight–mean (95% CI) (g)	2966 (2857–3075)	2945 (2866–3024)	2986 (2830–3142)	0.7
Breastfeeding (%)	29 (53)	1 (4)	28 (100)	<0.001
Growth at 6months– <i>n</i>	Total (47)	HEU (25)	UE (22)	
WAZ (SD)	–0.25 (1.07)	+0.17 (0.95)	–0.73 (1.16)	0.03
LAZ (SD)	–0.64 (1.26)	–0.44 (1.05)	–0.87 (1.61)	0.42
Growth at 12 months	Total (44)	HEU (23)	UE (21)	
WAZ (SD)	–0.09 (1.16)	+0.26 (1.13)	–0.47 (1.09)	0.18
LAZ (SD)	–0.20 (1.29)	+0.10 (1.09)	–0.53 (1.43)	0.22

Infant Care and Housing Conditions	Total (46)	HEU (26)	UE (20)	<i>p-value</i>
Mother as primary caregiver (%)	35 (76)	19 (73)	16 (80)	0.812
Formal housing (%)	28 (61)	11 (42)	17 (85)	0.007
Number of people in the household–mean (SD)	4.76 (2.47)	3.92 (2.25)	5.76 (2.39)	0.010
Number of people sharing infants room–mean (SD)	2.76 (1.37)	2.28 (1.14)	3.35 (1.42)	0.008
Access to running water (%)	28 (61)	11 (42)	17 (85)	0.007

SD, standard deviation; WAZ, weight-for-age Z-score; LAZ, length-for-age Z-score

^{*} Maternal and Infant Characteristics adapted from Slogrove et al.⁹

^a ARV exposure as PMTCT prophylaxis (*n*=19) or from mother with lifelong combination ARV therapy (*n*=4)

^b African included infants of Xhosa speaking South African (*n*=27), Malawian (*n*=2) and Zimbabwean (*n*=1) descent

^c All mixed ethnicity except 1 Caucasian UE