# *In-utero* infection with HIV-1 associated with suppressed lymphoproliferative responses at birth

B. Lohman-Payne,*†‡ T. Sandifer,§
M. OhAinle,¶ C. Crudder,* J. Lynch,†
M. M. Omenda,* J. Maroa,*
K. Fowke,** G. C. John-Stewart†‡§ and
C. Farquhar†‡§

*Department of Paediatrics and Child Health,
University of Nairobi, Nairobi, Kenya,
Departments of †Medicine, ‡Global Health and
§Epidemiology, ¶Fred Hutchinson Cancer Research
Center, University of Washington, Seattle,
Washington, USA, and **Department of Medical
Microbiology and Infectious Diseases, University
of Manitoba, Winnipeg, Canada

## Summary

*In-utero* exposure to HIV-1 may affect the immune system of the developing child and may induce HIV-1-specific immune responses, even in the absence of HIV-1 infection. We evaluated lymphoproliferative capacity at birth among 40 HIV-1-uninfected infants born to HIV-1-infected mothers and 10 infants who had acquired HIV-1 *in utero*. Cord blood mononuclear cells were assayed using [$^3$H]-thymidine incorporation for proliferation in response to HIV-1 p55-*gag* and the control stimuli phytohaemagglutinin (PHA), *Staphylococcus* enterotoxin B (SEB) and allogeneic cells. In response to HIV-1 p55-gag, eight (20%) HIV-1-exposed, uninfected (EU) infants had a stimulation index (SI) $\geq 2$ and three (30%) *in-utero* HIV-1 infected infants had SI $\geq 2$. The frequency and magnitude of responses to HIV-1 p55-gag were low overall, and did not differ statistically between groups. However, proliferative responses to control stimuli were significantly higher in EU infants than in infants infected *in utero*, with a median SI in response to PHA of 123 [interquartile range (IQR) 77–231] *versus* 18 (IQR 4–86) between EU and infected infants, respectively ($P < 0.001$). Among infected infants, gestational maturity was associated with the strength of HIV-1 p55-gag response ($P < 0.001$); neither maternal nor infant HIV-1 viral load was associated. In summary, EU and HIV-1-infected infants mounted HIV-1-specific lymphoproliferative responses at similar rates (20–30%), and although global immune function was preserved among EU infants, neonatal immune responses were significantly compromised by HIV-1 infection. Such early lymphoproliferative compromise may, in part, explain rapid progression to AIDS and death among HIV-1-infected infants.

**Keywords:** allogenic, Candida, gestational age, p55$^{gag}$, SEB

## Introduction

HIV-1-specific cellular immunity among infants and young children develops from exposure to HIV-1, either as the result of natural infection and continuous exposure to autologous virus or through intermittent contact with maternal virus, which may occur *in utero*, during delivery or via breastfeeding. It has been proposed that development of an effective immune response may protect against HIV-1 acquisition among exposed, uninfected infants and may reduce HIV-1 disease progression among infected infants [1–3]. A South African study found that HIV-specific CD4$^+$ T cell responses in cord blood were more common among children who did not become infected compared to those who became infected [1]. Our group also observed that HIV-1-specific CD8$^+$ T cell responses at 1 month of age were associated with lower risk of subsequent HIV-1 acquisition [2]. However, the magnitude or breadth of the HIV-1-specific CD8$^+$ T cell responses did not correlate with subsequent disease progression among HIV-infected infants [3].

The prevalence and predictors of HIV-1-specific CD4$^+$ and CD8$^+$ T cell responses among HIV-1-exposed adults and older children are not well defined. In HIV-1-exposed and infected individuals, HIV-specific cellular immune responses may be associated with recent viral exposure

[4,5]. This is supported by the observation that among HIV-1-infected adults, treatment interruptions of highly active anti-retroviral therapy (HAART) are followed by increased strength and breadth of CD8+ T cell responses during viral rebound [4]. Similarly, exogenously exposed, HIV-infected virally suppressed adults with virally suppressed partners on HAART are less likely to mount an HIV-specific immune response than those with viraemic partners [5]. Despite the association of greater HIV-1-specific T cell responses with detectable viraemia, this relationship may not continue in a dose-dependent manner, with some studies demonstrating no correlation between viral load and T cell response breadth or magnitude in viraemic patients [6–8].

For infants, exposure to endogenous or maternal virus may also be an important contributing factor to frequency, breadth and magnitude of HIV-1-specific responses [1]. In this study, we compared prevalence and correlates of HIV-1-specific T cell responses in cord blood obtained from HIV-1-infected infants and HIV-1-exposed, uninfected infants. In addition, we evaluated infant immunoproliferative responses in both groups to several non-HIV antigens [phytohaemagglutinin (PHA), *Staphylococcus* enterotoxin B (SEB), allogeneic cells, *Candida albicans*] in order to understand our results in the context of the global immunoproliferative activity of cord blood from HIV-infected and uninfected infants.

## Materials and methods

### Study subjects and data collection

Infants included in this study were born to HIV-1-seropositive mothers enrolled between 1999 and 2002 in a Nairobi HIV-1 perinatal transmission trial. Maternal and infant clinical and immunological information from the larger cohort is published elsewhere [2,3,9–15]. The current study includes a subset of 40 HIV-1-exposed, uninfected (EU) infants and 10 infants who acquired HIV-1 *in utero* (HIV IU). Women were recruited during pregnancy and provided with prophylactic zidovudine starting at 34–36 weeks gestation, as per Kenya national guidelines, during the study period [16]. The study received human subjects approvals from institutional review boards at the University of Washington and Kenyatta National Hospital in Nairobi, Kenya. Written informed consent was obtained from all participating mothers on behalf of their infants. This study received ethical approval from the institutional review boards of the University of Washington and the University of Nairobi and was conducted according to the guidelines set forth by the United States Department of Health and Human Services.

Clinical and demographic data were collected from mothers at enrolment, 32 weeks gestation and delivery. At 32 weeks, maternal blood and cervical swabs were obtained for HIV-1 RNA viral load and maternal CD4 and CD8 counts were determined. Women were assessed antenatally for sexually transmitted diseases (syphilis, gonorrhoea, chlamydia and trichomonas), as well as bacterial vaginosis and *C. albicans* infection. At birth, maternal blood was drawn for HIV-1 viral load, and infant cord blood was collected for immune assays. Within 48 h of birth, infants were examined by a study physician for gestational maturity as determined by Dubowitz score, and infant venous blood was drawn for determination of infant HIV-1 status.

### Laboratory methods

To determine HIV-1 infection status of infants, nested HIV-1 Gag DNA polymerase chain reaction (PCR) was performed on dried blood filter spots, as described previously [17]. Reactions were performed in quadruplicate and $\geq 1$ positive result was considered positive for *in-utero* transmission. In the case of infants who were HIV-1 DNA-negative at birth but HIV-1 DNA-positive at 1 month, samples of plasma collected within 48 h of birth and frozen were used for HIV-1 RNA assays (Gen-Probe, San Diego, CA, USA) to determine timing of infection. Infants who were HIV-1 RNA-positive within 48 h of birth were included in the *in-utero* transmission group [17]. All 40 infants included in the EU infant group remained DNA PCR-negative at 1 month of age and remained uninfected during the study follow-up of 1–2 years.

Cord blood mononuclear cells (CBMC) were prepared from freshly collected, ethylenediamine tetraacetic acid (EDTA)-anti-coagulated cord blood. Plasma was separated by 10 min of centrifugation at $312 \times \boldsymbol{g}$. The remaining blood was diluted 1:1 with RPMI, layered on a Ficoll gradient, and centrifuged for 30 min at $385 \times \boldsymbol{g}$. CBMC were collected from the interface and cryopreserved for later use. For proliferation assays, CBMC were thawed, counted and suspended in AIM-V serum-free medium at $2 \times 10^6$ cells/ml. To reduce handling, cells were added directly to 96-well round-bottomed plates with 100 µl ($2 \times 10^5$ cells) per well. After allowing plated cells to rest overnight, antigens were added to test wells to the following final concentrations: 10 µg/ml PHA (Murex, Dartford, UK), 1 µg/ml SEB (Sigma, St Louis, MO, USA), $2 \times 10^5$ cells per well allogeneic cells, 10 µg/ml *C. albicans* antigen (Greer, Lenoir, NC, USA), 10 µg/ml HIV-1 p55-*gag* antigen (Protein Sciences Corp., Meriden, CT, USA) or left unstimulated. Whole protein antigens were chosen to allow for antigen processing and presentation on major histocompatibility complex (MHC) Class II for stimulation of CD4+ T cells. Allogeneic cells were prepared by mixing PBMC collected from five unrelated individuals of varying ethnicity followed by Caesium[151] irradiation to abolish proliferative potential, followed by cryopreservation. Tests were performed in triplicate for each antigen-sample combination. Plates were incubated at 37ºC, 5% $CO_2$ for 5 days. On the fifth day, wells

**Table 1.** Cohort characteristics.

| Variable | Exposed uninfected infants | | *In-utero*-infected infants | |
|---|---|---|---|---|
| Maternal/infant characteristics | n | Mean (s.d.) | n | Mean (s.d.) |
| Maternal age (years) | 40 | 24·6 (4·1) | 10 | 25·6 (4·8) |
| Parity | 40 | 1·3 (1·1) | 10 | 2·0 (1·2) |
| Maternal pregnancy CD4 count[†] | 39 | 472·3 (236·7) | 10 | 450·7 (267·5) |
| Maternal pregnancy CD8 count[†] | 39 | 958·7 (484·3) | 10 | 1266·8 (665·6) |
| Maternal viral load at delivery[‡] | 35 | 4·1 (1·0) | 10 | 4·6 (0·9) |
| Maternal viral load at 32 weeks[‡] | 38 | 4·7 (0·7) | 9 | 5·0 (0·6) |
| **Cervical viral load at 32 weeks[‡¶]** | **32** | **2·2 (0·9)** | **7** | **3·3 (1·3)** |
| **Days AZT during pregnancy** | **40** | **30 (15)** | **9** | **15 (20)** |
| **Dubowitz score** | **39** | **56·0 (7·3)** | **10** | **48·8 (9·6)** |
| **Birth weight (kg)** | **40** | **3·2 (0·4)** | **10** | **2·7 (0·7)** |
| Infant viral load within 48 h[‡] | 40 | n.d. | 10 | 5·4 (0·8) |
| Diagnosed during pregnancy | n | Proportion | n | Proportion |
| History of illness[§] | 40 | 0·33 | 10 | 0·30 |
| History of malaria | 40 | 0·13 | 10 | 0·10 |
| Any STI | 38 | 0·21 | 9 | 0·33 |

[†]Units are cells/μl peripheral blood. [‡]Units are $\log_{10}$ copies/ml peripheral blood or cervical mucus; n.d. = none detected; s.d. = standard deviation; AZT = zidovudine. [§]History of fever, cough, diarrhoea, weight loss, thrush or itchy skin rash during pregnancy. [¶]Bold-type characteristics were significantly different between EU infants and IU-infected infants at $P \leq 0.05$.

were spiked with 1 microCurie per well of [$^3$H]-thymidine. After overnight incubation, cells were harvested onto FilterMats, allowed to dry for at least 4 h, then prepared for scintillation counting using a Wallac MicroBeta Counter (PerkinElmer, Akron, OH, USA).

## Data analysis

Stimulation indices (SI) were calculated for each cord blood sample by dividing the median antigen-stimulated proliferation result by the median control proliferation result, as described by Reimann *et al.* [18]. SIs < 2 were considered negative and thus assigned a value of 1 for rank-based statistics [19]. We required a positive response (SI ≥ 2) to at least one positive control stimulus (PHA, SEB or allogeneic cells) to include an infant in our analyses.

Spearman's rank correlations were calculated among the SI results for the five stimuli used in the proliferation assays. Associations between strength of proliferative responses and infant infection status were assessed with Mann–Whitney *U*-tests. To test for clinical differences between negative and positive p55-*gag* responders, we used Student's *t*-tests for continuous clinical variables and Fisher's exact tests for dichotomous clinical variables. The relationships between gestational maturity as measured by Dubowitz score and proliferative response to each stimulus were further examined by single regression with log-transformed stimulation indices. A Dubowitz score <47 was considered indicative of prematurity, as a score of 47 corresponds to an estimated gestational age of 36 weeks [20].

## Results

### Cohort characteristics

Fifty live singleton births to HIV-1-infected women were selected for inclusion in this study. Forty infants were HIV-1-uninfected at the time of birth and selected randomly from among all infants HIV-1-uninfected at birth, and 10 HIV-1-infected infants were selected randomly from among those infants infected *in utero*. Mean maternal age was 24·8 years (range 19–37 years) and mean antenatal maternal CD4 count was 468 cells/μl (range 31–949). Mean plasma viral load at delivery was significantly lower than viral load at 32 weeks gestation (4·2 *versus* 4·9 $\log_{10}$ copies (c)/ml, $P$ value < 0·001) due to prophylactic zidovudine. Mean cervical viral load at 32 weeks gestation was 2·39 $\log_{10}$ copies/ml (range 0·70–4·88 $\log_{10}$ c/ml). Eleven (23%) of 47 mothers were diagnosed with a sexually transmitted infection during pregnancy and 38% of mothers were diagnosed with *C. albicans* vaginitis at 32 weeks gestation.

Eight per cent of infants were low birth weight, defined as less than 2·5 kg, and 20% of infants were premature, as defined by Dubowitz score < 47. Among the 10 IU-infected neonates, mean viral load at birth was 5·4 $\log_{10}$ c/ml (range 4·2–6·4 $\log_{10}$ c/ml) (Table 1).

The IU-infected infant group differed from the EU infant group by maternal cervical viral load, duration of zidovudine use during pregnancy, Dubowitz score and birth weight (Table 1). Cervical viral load at 32 weeks gestation was higher in mothers of IU-infected infants (mean 3·3 $\log_{10}$ *versus* 2·2 $\log_{10}$ c/ml in IU *versus* EU infants, $P = 0.01$). Maternal peripheral viral load at 32 weeks and at delivery

**Table 2.** Lymphoproliferative responses by infection status.

| Antigens | Exposed uninfected infants | | *In-utero*-infected infants | | P-value[†] |
|---|---|---|---|---|---|
| | n | Median (IQR) stimulation index | n | Median (IQR) stimulation index | |
| PHA | 40 | 123 (77–231) | 10 | 18 (4–86) | **0·002** |
| SEB | 40 | 57 (11–188) | 10 | 7 (3–35) | **0·04** |
| Allogeneic cells | 40 | 135 (54–278) | 10 | 7 (4–10) | **<0·001** |
| *Candida* | 40 | 8 (3–18) | 10 | 4 (2–7) | **0·04** |
| HIV-1 p55 | 40 | 1·2 (0·8–1·7) | 10 | 1·4 (0·9–2·1) | 0·66 |

[†]Calculated by Mann–Whitney $U$-test, $P$-values $\leq 0.05$ in bold type. PHA = phytohaemagglutinin; SEB = *Staphlyococcus* enterotoxin B.

were also higher in the infected group, but differences did not reach statistical significance. Duration of zidovudine use was shorter among mothers of IU-infected infants compared to mothers of EU infants, averaging 15 days therapy *versus* 30 days ($P = 0.01$). IU-infected infants had a lower mean Dubowitz score (49 *versus* 56, $P = 0.01$) and a lower mean birth weight (2·7 *versus* 3·2 kg, $P = 0.01$). Together these data present the impact of *in-utero* infection with HIV on the birth characteristics of infants in this cohort that may have a direct impact on the ability to mount immune responses.

## Proliferative responses to positive control antigens and *C. albicans*

All 50 infant CBMC samples had a positive response (SI ≥ 2) to at least one of the positive control antigens, and 88% of infants had a positive response to all three positive control antigens (36 of 40 EU infants and eight of 10 IU-infected infants, $P = 0.59$). Forty-four (88%) infants responded to *C. albicans*, and this proportion did not differ based on the diagnosis of maternal *C. albicans* in pregnancy (88% of infants with maternal *C. albicans* in pregnancy *versus* 86% of infants without had a positive *C. albicans* immune response, $P = 1.0$). Although the proliferative responses were above the cut-off, the IU-infected infant

group had lower proliferative responses to all three positive control antigens than the EU infant group (Table 2). Median IU-infected CBMC stimulation indices for each positive control antigen were less than 20% of those produced by EU CBMC: 18 *versus* 123 for PHA, 7 *versus* 57 for SEB and 7 *versus* 135 for allogeneic cells ($P = 0.002$, $P = 0.04$, $P < 0.001$, respectively). Response to *C. albicans* was also significantly lower among IU infants than EU infants (median SI of 4 *versus* 8, $P = 0.04$) (Table 2). If this finding, that antigen-specific clonal proliferation of cord blood mononuclear cells is reduced by 80% in HIV-1 infected infants, is generalizable, this reduced ability to respond to antigen stimulation may, in part, explain rapid disease progression in observed in untreated paediatric HIV infection.

Among the group of 40 EU infants, responses to PHA, SEB and allogeneic cells were correlated significantly. Spearman's correlation coefficients between these three stimuli were all ≥ 0·51, with $P$-values ≤ 0·001 (Table 3). Among the group of 10 IU-infected infants, allogeneic and *C. albicans* responses were correlated significantly (0·82, $P = 0.004$), while PHA and SEB responses were correlated (Spearman's correlation coefficient of 0·72), but did not reach significance. Proliferative responses to allogeneic cells did not correlate with PHA or with SEB in this group (Table 3). Within the HIV EU infants, the correlation between various strong

**Table 3.** Correlations[†] between antigen response stimulation indices in cord blood mononuclear cells (CBMC).

Exposed uninfected infants

| | PHA | SEB | Allogeneic cells | *Candida* |
|---|---|---|---|---|
| SEB | 0·57 (<0·001) | *** | *** | *** |
| Allogeneic cells | 0·70 (<0·001) | 0·51 (<0·001) | *** | *** |
| *Candida* | 0·07 (0·67) | 0·001 (0·99) | −0·02 (0·89) | *** |
| HIV-1 p55-*gag* | 0·21 (0·20) | 0·09 (0·56) | 0·19 (0·24) | 0·20 (0·21) |

*In-utero*-infected infants

| | PHA | SEB | Allogeneic cells | *Candida* |
|---|---|---|---|---|
| SEB | **0·72 (0·02)** | *** | *** | *** |
| Allogeneic cells | 0·22 (0·53) | −0·02 (0·96) | *** | *** |
| *Candida* | 0·06 (0·88) | −0·18 (0·62) | **0·82 (0·004)** | *** |
| HIV-1 p55-*gag* | −0·32 (0·36) | −0·31 (0·39) | −0·23 (0·52) | −0·34 (0·34) |

[†]Shown are Spearman's rank correlation coefficients with $P$-values in parentheses. PHA = phytohaemagglutinin; SEB = *Staphlyococcus* enterotoxin B.

**Table 4.** Characteristics of infants by proliferative response to HIV-1 p55.

| Characteristic | Responders | | Non-responders | | |
|---|---|---|---|---|---|
| | | Mean (s.d.) | | Mean (s.d.) | |
| HIV-1 infection Status | *n* | *n* (%)[†] | *n* | *n* (%) | *P*-value[†] |
| HIV-1-infected IU | | | | | |
| Dubowitz score | 3 | 41 (2·1) | 7 | 57 (1·8) | **0·001** |
| Prematurity[†] | 3 | 3 (100) | 7 | 2 (28) | **0·05** |
| Maternal pregnancy CD4 count | 3 | 270 (50) | 7 | 571 (114) | 0·06 |
| Infant viral load at 48 h | 3 | 5·9 (.65) | 7 | 5·2 (.89) | 0·2 |
| HIV-1 exposed uninfected | | | | | |
| Dubowitz score | 8 | 56 (2·1) | 31 | 56 (1·3) | 1·0 |
| Prematurity[†] | 8 | 1 (13) | 31 | 4 (13) | 1·0 |
| Maternal pregnancy CD4 count | 8 | 363 (62) | 31 | 500 (44) | 0·10 |
| Infant VL | | n.a. | | n.a. | |

[†]Prematurity defined as Dubowitz score <47; n.a. = not applicable; s.d. = standard deviation.

proliferative signals was consistent with the high SIs observed in this group; however, there were fewer correlated responses evident in the HIV IU infants.

### Proliferative response to HIV-1 p55-gag

Overall, 11 (22%) of 50 infants had a positive response (SI ≥ 2) to HIV-1 p55-*gag* antigen. Eight (20%) of 40 EU infants had an SI ≥ 2 in response to *gag* antigen, while three (30%) of 10 IU-infected infants had an SI ≥ 2 in response to *gag* antigen (*P* = 0·67). The rank distributions of *gag* stimulation indices also did not differ (*P* = 0·66) (Table 2).

To test for the possibility that the strength of proliferative responses to HIV-1 p55-*gag* or *C. albicans* was limited by general immunosuppression, we compared PHA, SEB and allogeneic cell stimulation indices between positive and negative responders to p55-*gag* and *C. albicans* antigens separately in the 40 EU and 10 IU-infected infants. We did not observe associations between positive *p55-gag* response and PHA, SEB or allogeneic stimuli-induced proliferative responses in either EU- or IU-infected infants. However, we found significantly higher allogeneic responses among *C. albicans* responders than non-responders in IU-infected infants (median SI 9 *versus* 4, *P* = 0·05), but no associations with PHA or SEB. No similar associations were seen among the EU infants.

Clinical correlates of HIV-1 p55-*gag* response differed by infant HIV infection status (Table 4). Among the group of 10 IU-infected infants, Dubowitz score trended towards an inverse association with positive p55-*gag* response. The average Dubowitz score was 52·4 among the non-responders and 40·3 among the p55-*gag* responders (*P* = 0·06). All three IU-infected *gag* responders were premature, compared to two of seven IU-infected *gag* non-responders. We did not observe a significant association between infant viral load and positive *p55-gag* response (5·2 log₁₀ c/ml among seven non-responders *versus* 5·9 log₁₀ c/ml among three responders, *P* = 0·20). For all infants there was

a trend towards a lower maternal CD4 count between HIV-1 p55-*gag* responders and non-responders. Mean maternal pregnancy CD4 counts of HIV IU-infected infant responders and non-responders were 270 *versus* 571 cells/μl, *P* = 0·06. Similarly, maternal CD4 counts of HIV EU infant responders and non-responders were 363 *versus* 504 cell/μl, *P* = 0·1. Dubowitz score, maternal viral load and other potential correlates were not associated with proliferative responses to stimuli other than p55-*gag* (data not shown).

### Discussion

We found substantial differences in global lymphoproliferative capacity between HIV-exposed uninfected and *in-utero* HIV-infected infants. Marked compromise of global lymphoproliferative responses has not been observed in adult HIV-1 infection; most studies comparing HIV-1-infected adults with seronegative adults find modest to minimal differences in proliferative responses to PHA [21–23]. When observed, the degree of diminished response varies with different stimuli, from moderate defects in response to allogeneic stimuli to severe defects in response to recall antigen [24,25]. HIV-1-infected infants have a bimodal distribution of disease progression, with ~25% of infants progressing to clinical disease within the first year of life [26,27]. The patterns of mortality and peak and set-point viraemia also differ between infants and adults; infants experience higher peak and set-point viral loads after infection than adults, and mortality rates of ~50% in the first 6 months of life if the infection is untreated [11,28,29]. We and others have investigated factors that may influence disease progression, including the phenotype of the transmitted virus [30], the increased number of target cells available for infection [31] and the ability of the infant's immune system to respond to infection [3,15,32–39]. Lower lymphoproliferative function in general and low HIV-specific lymphoproliferative responses may partially

explain the accelerated course of infant HIV-1 infection when compared with adult infection. Maternal cytokine environment may affect fetal lymphocyte development and function, facilitating fetal viral replication.

We found proliferative responses to HIV-1 p55-*gag* antigen in approximately one-quarter of infants, regardless of HIV-1 infection status, and this prevalence is comparable to that seen in other studies [2,40,41]. In adult HIV-1 infection, HIV-1-specific CD4 responses are common as a result of exposure to endogenous virus [42]. Our findings are consistent with other studies noting that neonates infected with HIV-1 appear less able to mount a prompt effective HIV-1-specific cellular immune response than adults. Ramduth *et al.* observed that 20% of acutely infected South African infants produced interferon (IFN)-γ in response to HIV-1 *gag* [38], while Thobakgale *et al.* observed no appreciable IFN-γ production by CD4 T cells in response to *gag* peptides in HIV-1 infected infants aged 2 months [37]. Although detection of HIV-1 specific responses in neonates has previously been associated with protection from vertical transmission [1], we did not observe that HIV-1-specific lymphoproliferative responses were more common in EU infants protected from infection compared to IU-infected infants. This may be due to differences in the assay readout: interleukin (IL)-2 secretion quantified by proliferation of an IL-2-dependent cell line compared with tritiated thymidine uptake. Alternatively, the induction of HIV-specific cellular responses may occur in late gestation, and be reduced in the HIV EU infants due to a greater reduction in viral load associated with duration of zidovudine prophylaxis initiated at the 32nd week of pregnancy.

This study had several limitations, including sample size and lack of CD4 count per individual. Whole protein antigens were used for preferential stimulation of $CD4^+$ T cells, and while $CD8^+$ T cells present in cord blood could potentially respond in the assay, the majority of responding T cells are expected to express CD4. The reduction in proliferative capacity, due to a reduction in T cell number or reduction in function, is a broader defect in immune capacity than previously appreciated.

Most infants had responses to *C. albicans*; while IU-infected infants had significantly lower SIs in response to *C. albicans* than EU infants, both groups' response frequency and strength were relatively high. Pabst *et al.* found that only 15% of cord blood samples from healthy, normal newborns had a stimulation index ≥ 2 to *C. albicans*, with maximum response around SI = 4 [43]. While not directly comparable, it appears that the EU infant *C. albicans* responses in our study may be substantially stronger than the HIV-1-unexposed infants in the study by Pabst *et al.* This may be due to increased antigen exposure resulting from more frequent and intense *C. albicans* infections in HIV-1-positive mothers, or to increased immune activation postulated in HIV-1-exposure or anti-retroviral drug exposure [44–46]. While lymphoproliferative response to

*Candida* is not clinically important for controlling invasive *Candida* infection in newborns, which relies upon lymphocyte adhesion functionality [47], these results offer a specific antigen comparison for the HIV-1 p55-*gag* results. Allogeneic stimulus responses were associated with *C. albicans* responses among the IU-infected infants, suggesting that immunosuppression may contribute to the lower *C. albicans* proliferative responses in IU-infected infants. The correlation between the two responses may reflect increased exposure to maternal antigens due to poor placental health.

The negative association between gestational maturity and lymphoproliferative response to p55-*gag* only in IU-infected infants was unanticipated. It is possible that IU infants with prematurity may have had longer fetal duration of infection leading to the higher immune responses. Earlier HIV-1 infection *in utero* would provide a longer window for HIV-specific immune responses to develop, while also increasing risk of preterm delivery.

In conclusion, this study presents novel findings on lymphoproliferative responses to HIV-1-specific antigen and global immune stimuli by HIV-1-exposed, uninfected and HIV-1-infected neonates. These findings suggest that neonates infected with HIV-1 *in utero* may be born with lymphoproliferative defects which, in turn, may contribute to their accelerated disease progression.

## Acknowledgements

## Disclosure

The authors have no financial or commercial conflicts of interest.

## References

1 Kuhn L, Coutsoudis A, Moodley D *et al.* T-helper cell responses to HIV envelope peptides in cord blood: protection against intrapartum and breast-feeding transmission. AIDS 2001; **15**:1–9.

2 John-Stewart GC, Mbori-Ngacha D, Lohman-Payne B *et al.* HIV-1-specific cytotoxic T lymphocytes and breast milk HIV-1 transmission. J Infect Dis 2009; **199**:889–98.

3 Lohman BL, Slyker JA, Richardson BA *et al.* Longitudinal assessment of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon responses during the first year of life in HIV-1-infected infants. J Virol 2005; **79**:8121–30.

4 Kaufmann DE, Lichterfeld M, Altfeld M *et al.* Limited durability of viral control following treated acute HIV infection. PLOS Med 2004; **2**:e36.

5 Willberg CB, McConnell JJ, Eriksson EM *et al.* Immunity to HIV-1 is influenced by continued natural exposure to exogenous virus. PLOS Pathog 2008; **4**:e1000185.

6 Feeney ME, Roosevelt KA, Tang Y *et al.* Comprehensive screening reveals strong and broadly directed human immunodeficiency virus type 1-specific CD8 responses in perinatally infected children. J Virol 2003; **77**:7492–501.

7 Addo MM, Yu XG, Rathod A *et al.* Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 2003; **77**:2081–92.

8 Betts MR, Amborzak DR, Douek D *et al.* Analysis of total human immunodeficiency virus (HIV)-specific CD4+ and CD8+ T-cell responses: relationship to viral load in untreated HIV infection. J Virol 2001; **75**:11983–91.

9 Farquhar C, VanCott TC, Mbori-Ngacha DA *et al.* Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. J Infect Dis 2002; **186**:1173–6.

10 Obimbo E, Mbori-Ngacha D, Otieno PA *et al.* Correlates of viral load in Kenyan HIV-1 infected children. 2nd International AIDS Society conference on HIV-1 pathogenesis and treatment; 13–16 July 2003, Paris, France 2003.

11 Obimbo EM, Mbori-Ngacha DA, Ochieng JO *et al.* Predictors of early mortality in a cohort of human immunodeficiency virus type 1-infected African children. Pediatr Infect Dis J 2004; **23**:536–43.

12 Otieno PA, Brown ER, Mbori-Ngacha DA *et al.* HIV-1 disease progression in breast-feeding and formula-feeding mothers: a prospective 2-year comparison of T cell subsets, HIV-1 RNA levels, and mortality. J Infect Dis 2007; **195**:220–9.

13 Lohman B, Slyker JA, Richardson BA *et al.* Infants with late breast milk acquisition of human immunodeficiency virus-1 generate interferon-gamma responses more rapidly than infants with early peripartum acquisition. Clin Exp Immunol 2009; **156**:511–7.

14 Slyker J, Lohman-Payne B, John-Stewart G *et al.* Acute cytomegalovirus infection in Kenyan HIV-1 infected infants. AIDS 2009; **23**:2173–81.

15 Slyker JA, John-Stewart G, Dong T *et al.* Phenotypic characterization of HIV-specific CD8+ T cells during early and chronic infant HIV-1 infection. PLOS ONE 2011; **6**:e20375.

16 Shaffer N, Chuachoowong R, Mock PA *et al.* Short-course zidovudine for perinatal HIV-1 transmission in Bangkok, Thailand: a randomized clinical trial. Bangkok Collaborative Perinatal HIV Transmission Study Group. Lancet 1999; **353**:773–80.

17 DeVange Panteleeff D, John GC, Nduati R *et al.* Rapid method for screening dried blood samples on filter paper for human immunodeficiency virus type 1 DNA. J Clin Microbiol 1999; **37**:350–3.

18 Reimann KA, Chernoff M, Wilkening CL, Nickerson CE, Landay A, Laboratories TAiat. Preservation of lymphoycte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1 infected donors: implications for multicenter clinical trials. Clin Diagn Lab Immunol 2000; **7**:352–9.

19 Lange CG, Xu Z, Patterson BK *et al.* Proliferative responses to HIVp24 during antiretroviral therapy do not reflect improved immune phenotype or function. AIDS 2004; **18**:605–13.

20 Dubowitz LM, Dubowitz V, Goldberg C. Clinical assessment of gestational age in the newborn infant. J Pediatr 1970; **77**:1–10.

21 Schenal M, Lo Caputo S, Fasano F *et al.* Distinct patterns of HIV-specific memory T lymphocytes in HIV-exposed uninfected individuals and in HIV-infected patients. AIDS 2005; **19**:653–61.

22 Wahren B, Bratt G, Persson C *et al.* Improved cell-mediated immune responses in HIV-1 infected asymptomatic individuals after immunization with envelope gylcoprotein gp160. J Acquir Immune Defic Syndr 1994; **7**:220–9.

23 Alimonti JB, Koesters SA, Kimani J *et al.* CD4+ T cell responses in HIV-exposed seronegative women are qualitatively distinct from those in HIV-infected women. J Infect Dis 2005; **191**:20–4.

24 Clerici M, Stocks NI, Zajac RA *et al.* Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. J Clin Invest 1989; **84**:1892–9.

25 Scarlatti G, Leitner T, Halapi E *et al.* Comparison of variable region 3 sequences of human immunodeficiency virus type 1 from infected children with the RNA and DNA sequences of the virus populations of their mothers. Proc Natl Acad Sci USA 1993; **90**:1721–5.

26 Blanche S, Tardieu M, Duliege A-M *et al.* Longitudinal study of 94 symptomatic infants with perinatally acquired human immunodeficiency virus infection. Am J Dis Child 1990; **144**:1210–5.

27 Luzuriaga K, McQuilken P, Alimenti A, Somasundaran M, Hesselton R, Sullivan JL. Early viremia and immune responses in vertical human immunodeficiency virus type 1 infection. J Infect Dis 1993; **167**:1008–13.

28 Biggar RJ, Broadhead R, Janes M, Kumwenda N, Taha TET, Cassol S. Viral levels in newborn African infants undergoing primary HIV-1 infection. AIDS 2001; **15**:1311–22.

29 Richardson BA, Mbori-Ngacha D, Lavreys L *et al.* Comparison of human immunodeficiency virus type 1 viral load in Kenyan men, women, and infants during primary and early infection. J Virol 2003; **77**:7120–3.

30 Luzuriaga K, Wu H, McManus M *et al.* Dynamics of human immunodeficiency virus type 1 replication in vertically infected infants. J Virol 1999; **73**:362–7.

31 Krogstad P, Uittenbogaart CH, Dickover R, Bryson YJ, Plaeger S, Garfinkel A. Primary HIV infection of infants: the effects of somatic growth on lymphoycte and virus dynamics. Clin Immunol 1999; **92**:25–33.

32 Luzuriaga K, Koup RA, Pikora CA, Brettler DB, Sullivan JS. Deficient human immunodeficiency virus type 1-specific cytotoxic T cell responses in vertically infected children. J Pediatr 1991; **119**:230–6.

33 Luzuriaga K, Holmes D, Hereema A, Wong J, Panicali DL, Sullivan JL. HIV-1-specific cytotoxic T lymphocyte responses in the first year of life. J Immunol 1995; **154**:433–43.

34 Pikora CA, Sullivan JL, Panicali D, Luzuriaga K. Early HIV-1 envelope-specific cytotoxic T lymphocyte responses in vertically infected infants. J Exp Med 1997; **185**:1153–61.

35 Scott ZA, Chadwick EG, Gilbson LL *et al.* Infrequent detection of HIV-1-specific, but not cytomegalovirus-specific, CD8+ T cell responses in young HIV-1-infected infants. J Immunol 2001; **167**:7134–40.

36 Lambert JS, Moye J Jr., Plaeger SF *et al.* Association of selected phenotypic markers of lymphocyte activation and differentiation with perinatal human immunodeficiency virus transmission and infant infection. Clin Diagn Lab Immunol 2005; **12**:622–31.

37 Thobakgale CF, Ramduth D, Reddy S *et al.* Human immunodeficiency virus-specific CD8+ T-cell activity is detectable from birth in the majority of *in utero*-infected infants. J Virol 2007; **81**:12775–84.

38 Ramduth D, Thobakgale CF, Mkhwanazi NP *et al.* Detection of HIV type 1 gag-specific CD4+ T clel responses in acutely infected infants. AIDS Res Hum Retroviruses 2008; **24**:265–70.

39 Tiemessen CT, Shalekoff S, Meddows-Taylor S *et al.* Cutting edge: unusual NK cell responses to HIV-1 peptides are associated with protection against maternal–infant transmission of HIV-1. J Immunol 2009; **182**:5914–8.

40 Wasik TJ, Bratosiewicz J, Wierzbicki A *et al.* Protective role of B-chemokines associated with HIV-specific Th responses against perinatal HIV transmission. J Immunol 1999; **162**:4355–64.

41 Legrand FA, Nixon DF, Loo CP *et al.* Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. PLOS ONE 2006; **1**:e102.

42 Gandhi RT, Walker BD. Immunologic control of HIV-1. Annu Rev Med 2002; **53**:149–72.

43 Pabst HF, Godel JC, Spady DW, Cho K. Transfer of maternal specific cell-mediated immunity to the fetus. Clin Exp Immunol 1987; **68**:209–14.

44 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. J Acquir Immune Defic Syndr 2006; **42**:545–53.

45 Romano MF, Buffolano W, Bisogni R *et al.* Increased CD154 expression in uninfected infants born to HIV-positive mothers exposed to antiretroviral prophylaxis. Viral Immunol 2006; **19**:363–72.

46 Hygino J, Lima PG, Filho RGS *et al.* Altered immunological reactivity in HIV-1-exposed uninfected neonates. Clin Immunol 2008; **127**:340–7.

47 Gasparoni A, Ciardelli L, Avanzini A *et al.* Age-related changes in intracellular TH1/TH2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children, and adults. Biol Neonate 2003; **84**:297–303.