HIV-exposed uninfected infants show robust memory B cell responses in spite of a delayed accumulation of memory B cells: An observational study in the first two years of life.

Eunice W. Nduati1,#, Irene N. Nkumama1, Faith K. Gambo1, Daniel M. Muema1*, Miguel G. Knight2, Amin S. Hassan1, Margaret N. Jahangir3, Timothy J. Etyang1, James A. Berkley1,2 & Britta C. Urban4

1Kenya Medical Research Institute/Wellcome Trust Research Programme, Centre for Geographic Medicine Research Coast, P.O Box 230-80108, Kilifi, Kenya, 2Nuffield Department of Clinical Medicine, University of Oxford, OX3 7FZ, Oxford, UK, 3Kilifi County Hospital, P. O. Box 9-80108, Kilifi, Kenya, 4Liverpool School of Tropical Medicine, Liverpool University, L3 5QA, Liverpool, UK

Running Head: B-cell responses in HIV exposed infants.

# Address correspondence: Eunice Wambui Nduati, Enduati@kemri-wellcome.org

*Present address KwaZulu-Natal Research Institute for Tuberculosis and HIV, Nelson R. Mandela School of Medicine, 719 Umbilo Road, Durban, 4001, South Africa
Abstract

Background

Improved HIV care has led to an increase in the number of HIV-exposed uninfected (HEU) infants born to HIV infected women. Although uninfected, these infants experience increased morbidity and mortality. One explanation may be that their developing immune system is altered by HIV-exposure predisposing them to increased post-natal infections.

Methods

We explored the impact of HIV-exposure on the B-cell compartment by determining the B-cell subset distribution, the frequency of common vaccine antigen-specific memory B cells (MBCs) and their respective antibody levels in HEU and HIV-unexposed uninfected (HUU) infants born to uninfected mothers, using flow cytometry, B-cell ELISPOT and ELISA, respectively, during the first two years of life.

Results

For the majority of the B-cell subsets there were no differences between HEU and HUU infants. However, HIV exposure was associated with a lower proportion of B cells in general and specifically MBCs, largely due to a lower proportion of unswitched memory B cells. This reduction was maintained even after correcting for age. These phenotypic differences in the MBC compartment did not affect the ability of HEU infants to generate recall responses to previously encountered antigens, or reduce the antigen-specific antibody levels at 18 months of life.

Conclusions
Although HIV-exposure was associated with a transient reduction in the proportion of MBCs, we found that the ability of HEUs to mount robust MBC and serological responses was unaffected.
Introduction

The use of highly active antiretroviral therapy (HAART), improved obstetric management and formula feeding have reduced vertical HIV infection to almost zero in the developed countries (1) with some progress being made in resource-poor countries (2). Consequently, the number of HIV exposed uninfected infants (HEUs), born to HIV infected women, has and will continue to increase, particularly in regions where HIV infection in women of childbearing age is still prevalent (3).

Increased morbidity and mortality is reported in HEUs (4-8). While these may be partly explained by increased exposure to environmental antigens and poor maternal health, it is possible that in-utero exposure to HIV antigens, antiretroviral drugs and an altered placental cytokine environment may affect the developing immune system, predisposing HEU infants to increased post-natal infections.

The impact of maternal chronic infection on fetal immunomodulation and specifically of HIV exposure has previously been reviewed (9-11). For HIV, exposure in infants has been associated with an activated, intrauterine immune environment (12, 13) and reduced T cell counts and polyfunctionality (14-17). While the available evidence has largely focused on the potential disruptions to the T-cell compartment in HEU infants, much less is known on the impact of HIV-exposure on the B-cell compartment, with a majority of the studies concentrating on serological parameters (10). Previous observations of the profound effect of HIV infection on B cells and their function (18, 19) may extend albeit subtly to HIV exposure in the absence of infection. Elevated levels of total
immunoglobulin in HEU infants compared to HIV unexposed uninfected infants (HUUs)
born of HIV uninfected mothers have been reported to persist for more than 2 years (20).
When specific antibody responses against childhood immunizations were measured,
HEUs responded with similar antibody levels as those in HUUs (21-23). However, other
studies have reported a larger proportion of non-responders to hepatitis B vaccine (24),
diminished neutralizing antibodies to poliovirus vaccine (25), lower antibody avidity (23)
and reduced opsonization for some of the pneumococcal polysaccharides of conjugate
vaccine (26) amongst HEUs. Of the few studies that have investigated the impact of HIV
exposure on B cells one reported increased B-cell apoptosis in HEUs (27), whereas others
observed higher percentage of CD19+ cells (16, 28). Recently similar proportions of B
cell subsets were reported in HEUs and HUUs at 6, 12 and 18 months (29).
Previous studies have however not associated observed phenotypes with B cell function.
In the current study we sought to investigate the impact of maternal HIV infection on the
infant’s developing B-cell compartment during the first two years of life by determining
the phenotypic composition of the B-cell compartment and associating this to the
induction and maintenance of antigen-specific memory B cells and antibodies in response
to common childhood vaccines in HEU and HUU infants.

Materials and Methods:

Study population and recruitment:
The study was conducted at the Comprehensive Care and Research Clinic (CCRC), Kilifi
County Hospital (KCH), prior to the 2012 national integration of PMTCT services with
Mother to Child Health (MCH) services. PMTCT care and testing was provided as per Kenyan guidelines (30) and as previously reported (31). In summary, the guidelines recommended that all pregnant women should be tested for HIV during their first antenatal clinic visit and that a repeat test should be offered to initially HIV negative women during the third trimester. Mothers would be placed on life-long highly active antiretroviral therapy (HAART) if their CD4 count was less than 350 cells/mm$^3$ but if higher, on prophylactic antiretroviral therapy azidothymidine (AZT) from 14 weeks of pregnancy (or at first contact with antenatal services, if later) and AZT prophylaxis continued through labour and one week after delivery. HIV exposed infants born to mothers not on HAART were prescribed nevirapine prophylaxis at birth to be continued until one week after complete cessation of breastfeeding while those with mothers on HAART, nevirapine prophylaxis stopped at six weeks of life. Infants aged less than 18 months were tested for HIV by PCR at 6 weeks after birth or at the earliest opportunity, subsequently an antibody test at 9 months (if previously PCR negative) and 18 months was performed. Infants with confirmed HIV infection at any of these test points were immediately put on HAART. All HIV-exposed infants were given prophylactic cotrimoxazole during the first 18 months of life and those testing HIV positive at any of the testing time points continued on life-long cotrimoxazole. HAART and cotrimoxazole were supplied at monthly visits. The infants also received their scheduled early childhood immunizations during these visits and their immunization cards inspected. Pairs of HIV-infected mothers and their infants (between 3 and 18 months of age) were recruited. Infants suffering from any acute infection or malnutrition at the time of recruitment were excluded from the study. Mothers contributed a single blood sample at recruitment while...
the infants were followed up longitudinally every three months until they were 24 months of age, with an upper limit of up to 30 months given to cover late follow up visits. Community controls were recruited within the same locality from cohorts under active malaria surveillance, which includes an annual cross sectional bleed (32) and from one of the sentinel dispensaries. To minimize the potential impact of malaria exposure on the B cell compartment (33, 34), only infants who had no reported episode of malaria following weekly home visits and were negative for *Plasmodium falciparum* (tested by RDT) during the annual cross sectional bleed, were selected. It was not possible to follow the community controls longitudinally and therefore infants at similar ages to the three monthly HEU follow-up time-points were recruited. The prevalence of HIV infection in adults from the study area has been estimated at 4.1% (35).

**Ethical considerations:**

Informed consent was obtained from the infants’ mothers and Ethical approval granted by the National Ethics and Review Committee, Kenya Medical Research Institute, reference 2085.

**Sample collection**

At each study related visit, a 5 ml venous blood sample was drawn and 2 ml used for immediate analysis of haematological parameters and B-cell subsets. Peripheral blood mononuclear cells (PBMC) and plasma were separated from the remaining 3 ml and
stored in liquid nitrogen and at -80°C, respectively, until use. The mothers’ single sample at recruitment was used to determine maternal viral load and CD4 counts at recruitment.

**Multiparametric flow cytometry**

B-cell subsets were described using the following monoclonal antibodies. FITC-IgM, ECD-CD19 and PC5-CD27 (Beckman Coulter), PE-CD21 and PE-Cy7-CD38 (eBioscience), PE-CD10 and APC-CD21 (BD Pharmingen). Fifty microliters of whole blood was washed, incubated with cocktails of the antibodies above and RBCs lysed. At least 80,000 lymphocytes were acquired on the Cyan ADP (Beckman Coulter) and data analysed using FlowJo software version 9.4.2 (TreeStar inc. Flowjo Africa). The gating strategy to identify different B-cell subsets is described in supplementary figure 1. B cell subsets were then represented as a proportion of the total B cell percentage in lymphocytes. Absolute B cell subset counts were determined based on the subset proportion in the total number of B cells. The total number of B cells was determined as a proportion of absolute lymphocytes counts determined from a whole blood cell count assay.

**ELISpot Assay**

Antigen specific IgG memory B cells (MBCs) against Tetanus toxoid (TT, Statens Serum Institut), measles antigen (Meridian Life Science), diphtheria toxoid (DT, Alpha Diagnostics International) and pneumococcal capsular polysaccharides (PCPs), comprising a pool of 6 common serotypes within the study region (36) (19F, 5, 1, 23F, 14
and 6B from ATCC) were quantified using a modification of a previously reported
ELISpot assay (37, 38). Briefly, 2 x 10^5 PBMCs per well were stimulated for 5 days with
2.5 μg/ml CpG oligodeoxynucleotide-2006 (Hycult biotech), 1:5000 Staphylococcus
aureus Cowan Strain protein A (Sigma) and 83 ng/ml Pokeweed Mitogen (Sigma) in flat-
bottomed 96-well culture plates. Multiscreen plates (Millipore Multiscreen) were pre-
coated with either, 5 μg/ml TT, 5 μg/ml measles antigen, 5 μg/ml DT, a pool of 6 PCPs
each at a concentration of 10 μg/ml, 10 μg/ml polyclonal sheep anti-human IgG (Binding
Site) or 1% Bovine serum albumin (BSA). Cultured PBMCs were seeded onto antigen-
coated plates at either 2 x 10^5 cells/well (antigen-specific responses) and at 2 x 10^3 or 2 x
10^5 cells/well (total IgG responses) and incubated overnight. Alkaline phosphatase
conjugated donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories)
was used as the secondary antibody. Spots were developed using BCIP/NBT (Bio-Rad)
and counted using the CTL ImmunoSpot Analyzer (Cellular Technologies). The
background was accounted for by subtracting the average number of spots in wells coated
with 1% BSA from the spots in antigen coated wells. An upper limit of three spots were
at any time detected in the wells coated with 1% BSA.

ELISA

Human IgG antibodies specific to TT, DT and a pool of 6 PCPs, described above, were
quantified using a modified ELISA protocol previously reported (39). Antibody levels
were measured after the infant’s 18th month of life to avoid maternally transferred
antibody bias. In brief, ELISA plates were coated overnight either with TT (1 μg/ml), DT
(5 μg/ml), antihuman IgG (10 ng/ml) or a pool of 6 PCPs (each at 10 μg/ml). Plasma
samples were diluted at 1:1000. Peroxidase-conjugated donkey anti-human IgG (Jackson ImmunoResearch) was used as the secondary antibody before development of the plates with o-phenylene diamine dihydrochloride (Sigma). Results were represented as arbitrary antibody units generated from a standard curve based on a “control sample” that was reactive to the antigen of interest. The “control sample” was obtained from an adult with known vaccination and reactivity status to the antigen of interest.

In addition, IgG antibody quantities against haemophilus influenzae type b (HiB) (Binding Site), measles virus IgG ELISA (Serion ESR102G) and Respiratory Syncytial virus (RSV) IgG (Serion ESR113G) were determined following the manufacturer’s instructions.

**Maternal viral load determination**

Maternal viral loads were determined at the point of infant recruitment at the International Centre for Reproductive Health, Mombasa Kenya, using a RT-qPCR test developed by the Agence Nationale de Recherches sur le SIDA (ANRS). The assay targets a conserved long terminal repeat region and has a detection limit of 300 RNA copies/ml (40).

**Sources of data and analysis**

Stata version 13.1 (Stata Corporation) was used for the Statistical analysis. To determine the impact of HIV exposure on infant immunological outcomes, linear regression models adjusted for clustering within a child were used. These models accounted for inherent correlations between repeated measurements done on the same subject, in addition to
accounting for changes with age. To avoid maternal antibody interference, antibody levels against common EPI vaccines were determined after 18 months only. Since this aspect of the study was cross-sectional, antibody levels were compared between HEU and HUU using the Wilcoxon rank-sum test. Mothers’ clinical data including age, body mass index (BMI), with a BMI of <18.5 considered as malnutrition, CD4 T-cell count and HAART use were routinely captured at the clinic. Maternal data available in the mother’s records at a time point closest to after the infant’s date of birth was included in a linear regression model to determine the impact of maternal health close to infant’s birth on the infant’s developing immune system. Only data collected within the first four months after the infant’s birth was considered. In addition, mother’s CD4 count, BMI and viral load were collected during the infant’s recruitment and were included in the linear regression model to determine the impact of mother’s health during this period on the infant’s developing immunity.

Results

Baseline characteristics of the study population

Between November 2011 and December 2012, infants born to HIV infected mothers were enrolled at the CCRC, Kilifi County Hospital. Of the 92 infants recruited, five (5.4%) tested positive for HIV, with two testing positive at six weeks while the remaining three made a first clinic visit more than two months after birth (Supplementary Figure 2). The HIV-infected infants were excluded from the study and the subsequent analysis concentrates on HIV exposed uninfected infants. Forty-three out of eighty-seven of the HEU (49.4%) were boys. Ninety-eight community controls under 30 months were
recruited for comparison. Nine of them were sampled twice having participated in two annual cross-sectional bleeds. Maternal data for the HEU’s mothers recorded closest to after the infant’s date of birth and collected during recruitment were available [Table 1]. The majority of the mothers had a BMI > 18.5 and hence were considered well nourished, both closest to after the infant’s date of birth and at recruitment. They also had CD4 counts above 400 at both time points. We were able to obtain viral load data for 78 of the mothers, of these 38/78 (48.7%) had viral loads less than 300 copies per ml, and 25/78 (32.1%) had viral loads above 5000 copies per ml. Mothers who had been on HAART for more than two years prior to the infant’s birth had a lower median viral load count at recruitment, compared to those who had not been on HAART [Table 1].

**HIV exposure is associated with a reduced proportion of unswitched MBCs.**

The first 70 HEU infants to be recruited were included in the B cell phenotypic analysis. These infants were not different from the remaining 17 who were recruited later and not included. We analysed B-cell subsets in 140 peripheral blood samples from these 70 infants (34 HEU infants contributed one sample, 36 contributed multiple samples) and 98 HUU infants (9 infants were bled at two annual cross-sectional bleeds therefore contributed two samples each) [supplementary Table 1], to determine whether HIV exposure is associated with altered B-cell subset distribution. In the univariate regression analysis, HIV exposure was associated with a significant reduction in the total B cell proportion, largely due to changes in the memory B cell (MBC) compartment [Table 2]. A reduction in resting memory B cells (CD19⁺CD10⁻CD27⁺CD21⁻) was associated with HIV exposure. This association was not observed when the whole memory B cell
population (CD19^+CD10^-CD27^+) was considered. It was largely due to changes in the unswitched MBCs (CD19^+CD10^-CD27^+IgM^-) subset while the proportion of switched MBCs (CD19^+CD10^-CD27^+IgM^+) was similar in both HEUs and HUUs. The association of HIV exposure with a lower proportion of unswitched MBCs was maintained even after adjusting for multiple testing using the bonferroni correction and correcting for age. Similarly when B cell subsets absolute counts were considered, HIV exposure was significantly associated with a reduction of unswitched MBCs absolute counts [Table 2].

Although memory B cells gradually accumulated with age, as observed from individual infant’s kinetics for those who had data from more than one time point (supplementary figure 4) and similarly when all infants were considered [Figure 1], HIV exposure resulted in a slower development of the memory B cell compartment [Figure 1]. In line with the overall reduction in unswitched memory B cells, this was true for the unswitched MBC subset while the gradual accumulation in the switched MBC compartment remained comparable to that observed in HUUs during these first two years of life.

We further determined if the mother’s data recorded closest to after the infant’s date of birth and/or at recruitment had an impact on the B-cell subset distribution observed in the HEUs. There was no association between the majority of the maternal parameters and the infant’s B-cell subset distribution, apart from the total B cell percentage that directly correlated with maternal CD4 counts but inversely with maternal BMI closest to after the infant’s date of birth and BMI at infant recruitment. Interestingly maternal viral load, although measured at the infant’s recruitment only, was significantly associated with
higher proportions of infant plasmablasts and MBC subsets but a lower proportion of naïve B cells [supplementary Table 2] suggesting a higher level of immune activation in infants of mothers with ongoing viral replication.

Recall responses to selected vaccine antigens are comparable between HEU and HUU infants

We measured recall responses to representative antigens (measles antigen, PCPs, DT, TT and against total IgG) by ELISPOT in 64 HEU infants (22 infants contributed a single sample and 18 infants contributed more than one sample) and 29 HUU infants [samples used are shown in supplementary table 1]. The samples selected for these analyses were limited by the availability of adequate cell numbers in samples from HUUs. In linear univariate analysis HIV exposure was associated with a reduction in total IgG⁺ MBC recall responses [Table 3]. This association was maintained even after adjusting for age. However HIV exposure did not perturb the generation of antigen-specific IgG MBC recall responses against measles antigen, PCPs, DT and TT, which were similar in HEU and HUU. Mothers’ data closest to after the infants’ date of birth and at recruitment were not associated with the infant’s ability to generate recall responses to previously encountered antigens [Supplementary Table 2].

IgG antibody levels at 18 months of life to selected vaccine antigens

Next, we assessed whether the plasma concentration of antibodies to vaccine antigens and common childhood infections were comparable between HEU and HUU infants. The maintenance of antibody levels to selected vaccination antigens was determined after 18
months of life in 55 and 48 plasma samples from HEU and HUU infants, respectively. Samples used are shown in supplementary table 1. A wide variation in total IgG antibody levels was observed in the HEU group compared to the HUU group [Figure 2a]. Out of four antigens (TT, DT, Hib and PCPs) against which vaccination is given in the first 16 weeks of life, anti-PCPs IgG levels were significantly higher in the HEU infants compared to HUU infants (Mann Whitney U-test p-value = 0.038) [Figure 2b, c, d, e]. Antibody levels against measles, for which infants are vaccinated at the ninth month of life, were lower in the HEU infants compared to the HUU infants (Mann Whitney U-test p-value = 0.0024) [Figure 2f]. Nevertheless, the majority of infants in both groups attained the conventionally accepted protective antibody level of 200mlU/ml (41). No differences were observed in IgG antibody levels against RSV, to which infants are naturally exposed [Figure 2g]. Of concern, approximately 50% of both HEU and HUU infants’ anti-Hib antibody levels were below a threshold required for long-term protection by 18 months of life (42) [Figure 2d]. For the two antigens, measles and PCPs, for which antibody levels between HEU and HUU infants were observed to be significantly different, a sub-analysis of antibody levels at the 18-month age category and after 18-month age categories (21 and 24 months) was done. Antibodies against PCPs showed an age related increase with levels being significantly higher in HEUs at a time when they were no longer receiving cotrimixazole prophylaxis (supplementary figure 3). Comparing antibody levels between HEU infants whose mothers at the time of the infant’s birth had been on HAART or not, (that is, besides azidothymidine (AZT) prophylaxis given as per the Kenyan guidelines at the time of the study (30)), showed no significant differences in antigen-specific antibody levels but total IgG antibody levels
were higher in infants whose mothers had received HAART (Mann Whitney U-test p-value = 0.034, data not shown). When maternal data recorded closest to after the infant’s birth and at recruitment were considered, there was no association with the infant’s ability to maintain antibodies against antigens they had been vaccinated against [Supplementary Table 2].

The total MBC percentage correlates with antigen-specific MBC numbers and antibody levels. Data on the distribution of total B cell subsets, antigen-specific MBC recall response and antibody levels to vaccine antigens were available for 64 HEU and 29 HUU. We correlated these data to determine if infants who had a higher percentage of switched MBCs were better at generating recall responses or had higher antibody levels in circulation. Infants’ recall responses to measles, PCPs and total IgG-secreting B cells directly correlated to the percentage of MBCs in circulation [Figure 3]. In addition recall responses to measles, TT, PCPs and DT directly correlated to the antibody levels against the same antigens. For the community controls with phenotypic, recall response and antibody data available, recall-responses to TT and PCPs correlated to antibody levels (Spearman correlation co-efficient rho, p-value; rho=0.600, p=<0.039 and rho=0.665, p=0.036 respectively, data not shown).

Discussion

We undertook a comprehensive analysis of the impact of HIV-exposure on the developing B-cell compartment in HIV-exposed uninfected infants. Infants born to HIV
infected mothers, even when not infected may be exposed to HIV-antigens and HAART in utero and during breast-feeding (43, 44).

Only a few studies have addressed the impact of HIV-exposure on the B-cell compartment (16, 27-29). A recent report on Malawian HEU infants showed similar B cell subsets in HEUs and HUUs during the first 18 months. In agreement, the majority of the B-cell subsets in our study were similar in HEUs and HUUs. However, we observed an association between HIV exposure and a reduced proportion of unswitched MBCs.

Although the existence of unswitched (IgM+) memory B cells was previously debated upon (45, 46), there is increasing evidence of their existence, ability to undergo secondary germinal centre reactions and receive T cell help (47). A recent study showed that unswitched memory B cells play a special role in early inflammation through their interaction with immunomodulatory neutrophils (48). Additionally, it has been suggested that unswitched memory B cells preferentially re-enter germinal centres upon antigen reactivation hence play an active role in sustaining memory, while switched memory B cells show propensity to differentiate directly into plasmocytes (48-50).

In our study setup, it is possible that MBC responses to natural antigens developed more slowly in these HEU infants due to the daily cotrimoxazole prophylaxis, routinely given, which may reduce the infant’s exposure to a broad spectrum of pathogens as is intended (30). However some studies have reported lack of reduction of pneumococcal nasopharyngeal carriage in HIV-infected children despite cotrimoxazole prophylaxis, suggesting that the direct effect on exposure to some bacterial infections could be limited.
even in HEU infants (51). Cotrimoxazole may have lead to some immunomodulatory mechanisms that may result in reduced lymphocyte proliferation (52). It is also possible that HEU infants intrinsically develop a smaller unswitched B cell compartment.

The lower proportion of IgM memory B cells in HEU infants may have clinical consequences compromising their first line humoral responses hence predisposing them to infections (53). While switched memory B cells dominate the secondary response due to their capacity to be activated in the presence of neutralizing serum immunoglobulin, it appears that once the levels of neutralizing antibodies drop, then memory is sustained by IgM reserves (50). A lower proportion of IgM MBCs in HEU infants may therefore interfere with their ability to sustain long-term memory should levels of protective antibodies fall below threshold. Although determining the clinical consequences of immunological changes in the HEUs is beyond the scope of the current study, our observation warrants further investigation if lower unswitched MBCs in HEU infants, even in the presence of similar switched MBCs to HUU infants, posses any clinical consequences.

It is encouraging that similar proportions of switched MBCs in HEU and HUU infants were observed, implying that HEUs are capable of mounting robust responses to vaccine antigens. Lower interference from maternal antibodies may have also contributed to robust responses as has been previously suggested (21). In support of the HEUs ability to mount robust vaccine responses, recall responses to previously encountered vaccination antigens were similar in HEU and HUU infants.
For a majority of the vaccine antigens against which antibody levels were determined, HEUs were able to maintain antibody levels similar to those observed in HUU infants, as previously reported in other settings (21, 22). However, we observed significantly lower antibody levels against measles antigen in the HEUs, but of importance, majority of these infants attained the recommended protective level (41). HEU infants had higher anti-pneumococcal antibody levels after the 18-month age category compared to HUUs. From previous reports, HIV infected women with opportunistic infections might be more likely to transmit these infections to their infants (54, 55). It is therefore likely that environmental exposure from an ailing mother may have led to increased exposure leading to the observed higher pneumococcal antibody levels once cotrimoxazole prophylaxis stopped at 18 months of age.

Of concern, in our study population, a large proportion of both HEU and HUU infants showed antibody levels below a threshold deemed protective against Haemophilus influenzae type b, an observation previously made (23). It is possible that MBCs rather than serological memory sustain protection (56) although sustained antibody levels at 24 months have been reported (22). This implies that antibodies may also play a role and a booster dose after early infancy may be beneficial to both HEU and HUU infants. In our study we concentrated on antibody levels it may be important for future studies to incorporate antibody functional assays, which would comprehensively ascertain if the HIV exposed infants are compromised.
Analyses of the correlation between serological and recall response data, although from a modest sample size and therefore should be interpreted with caution, suggest that infants who made good long lasting antibody responses also made better recall responses. The degree of overlap between the memory B cell compartment and long-lived antibody secreting cells, plasma cells, is difficult to determine (57) and both compartments may play important albeit different roles. Being the first two years of life, it is likely that natural exposure is still minimal and therefore more likely for antibody and memory function responses to correlate (58). Increased antigenic exposure with age may lead to a loss in this correlation.

The delayed accumulation of memory B cells in HEU infants observed in this study warrants further investigation. A recent report from Malawi showed no differences in the proportion of memory B cells between HEUs and HUUs. Although clinical care for HEU infants is similar between both settings, malaria exposure, which is also known to perturb the B-cell compartment (33), in a similar manner to HIV (18, 19), may have been different. Perturbation in the generation and maintenance of B cell memory in malaria infection (59) and distortion of the B cell compartment with the appearance of additional subsets not commonly found in healthy individuals (33, 60) have been reported. We selected HUU infants with no previously reported clinical episode of malaria based on active weekly surveillance. This ensured that they were more comparable to the HEUs who received daily cotrimoxazole prophylaxis, which although not the primary aim, protects them from malaria (61, 62). This may have increased our chances of identifying subtle immunological differences associated with HIV exposure, which may be missed in
settings where perturbations in HUUs may have already been caused by malaria exposure.

In future, studies on the potential role of HIV exposure on the infant’s developing immune system will have to be carefully designed taking into account varying environmental exposures, such as malaria endemicity and PMTCT programmes, such as, the daily use of cotrimoxazole prophylaxis, to comprehensively conclude if HIV exposure perturbs the infant's developing immune system.

Our findings describe B-cell memory vaccine responses and compliment the current body of data on serological responses that have shown sustained antibody levels in exposed infants to 2 years of age (22) and recent findings on B cell phenotypes in HEUs (29). In our study though, HIV-exposure had subtle effects on the development of the B-cell compartment and was significantly associated with a reduction in the unswitched memory B-cell proportions. Our findings also imply that maternal health may impact on the infants’ responses particularly to pneumococcal antigens. Placing mothers on life-long HAART earlier may contribute to reduced vulnerability to infections in general and benefit both maternal and infant health. Importantly, our study shows that exposed infants mount robust B-cell responses to vaccines and pathogens and it is therefore likely that these infants would be able to respond to a future HIV vaccine to prevent infection in this at risk population.

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Conflict of interests:

The authors have no competing interests, financial or otherwise.

References


activation and normal levels of endogenous antivirals are seen in healthy adolescents born of HIV-infected mothers. AIDS 21:245-248.


Figure legends

Figure 1
Distribution of a) memory B cells (CD19^+CD10^−CD27^+), b) unswitched memory B cells (CD19^+CD10^−CD27^+IgM^+), and c) switched memory B cells (CD19^+CD10^−CD27^+IgM^-) in HIV unexposed uninfected infants (HUU) – red dots and HIV exposed uninfected infants (HEU) – black dots. Straight lines show best-fit prediction of the increment in subset proportions over the two years of life. Y-axis; percentage of memory B cell subsets have been presented as the natural-log of the MBC percentages.

Figure 2
IgG antibody levels at 18 months of life to a) total IgG and to selected vaccine antigens b) tetanus toxoid protein (TT), c) diphtheria, d) haemophilus influenza type b (HiB), e) pneumococcal capsular polysaccharides (PCPs) f) measles g) Respiratory Syncytial virus (RSV). Antibody concentrations were compared between HIV unexposed uninfected infants (HUU), open circles and HIV exposed uninfected infants (HEU), closed circles. Wilcoxon rank-sum test was used and medians presented. P-values < 0.05 were considered significant. Arrows in d) and g) indicate cut-off for protective antibody concentration. Since antibody concentrations were not normally distributed, log transformed (natural-log) values of arbitrary antibody concentrations (TT, diphtheria, PCPs, total) and absolute concentrations (measles (mlU/ml), HiB (mg/L), RSV (U/ml)) have been presented.

Figure 3
Correlation of circulating antigen specific memory B cells with the percentage of switched memory B cells (first row) or IgG antibody levels against measles, tetanus toxoid protein (TT), pneumococcal capsular polysaccharides (PCPs), diphtheria and total IgG (second row). Spearman correlation co-efficients were determined and the spearman rho presented. P-values < 0.05 were considered significant.
Table 1: Mothers’ data from HIV exposed uninfected infants taken closest to after the infant’s date of birth and during the infant’s recruitment (N = 87).

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</tr>
<tr>
<td><strong>Viral load ranges (copies/ml)</strong></td>
<td><strong>Viral load ranges (copies/ml)</strong></td>
</tr>
<tr>
<td>n = 78a</td>
<td>n = 78a</td>
</tr>
<tr>
<td>&lt;300</td>
<td>48.7% (38/78)</td>
</tr>
<tr>
<td>&gt;300&lt;1000</td>
<td>7.7% (6/78)</td>
</tr>
<tr>
<td>&gt;1000&lt;5000</td>
<td>11.5% (9/78)</td>
</tr>
<tr>
<td>&gt;5000</td>
<td>32.1% (25/78)</td>
</tr>
<tr>
<td><strong>Viral load (copies/ml)</strong> categorized as per HAART use close to the infant’s birth**</td>
<td><strong>Viral load (copies/ml)</strong> categorized as per HAART use close to the infant’s birth**</td>
</tr>
<tr>
<td>Not on HAART (n = 37)</td>
<td>HAART 0 – 24 months (n = 13)</td>
</tr>
<tr>
<td>2783 [54 - 20685]</td>
<td>29 [0 – 2648]</td>
</tr>
<tr>
<td>HAART &gt;24 months (n = 20)</td>
<td>N/D</td>
</tr>
<tr>
<td>2428 [18 - 25894]</td>
<td>10 [0 – 402]</td>
</tr>
<tr>
<td>Missing HAART category (n = 8)b</td>
<td>N/D</td>
</tr>
<tr>
<td>2428 [18 - 25894]</td>
<td>N/D</td>
</tr>
</tbody>
</table>

n = represents mothers for whom data was available for a specific parameter

*a* viral load data was available for 78 of the mothers.

**HAART;** highly active antiretroviral therapy

**b** mothers for who data on the duration on HAART close to the infant’s birth was missing

[IQR] = interquartile range

N/D = not determined
Table 2: The association of HIV exposure and changes in infants’ B-cell subset percentages and absolute counts during the first two years of life.

<table>
<thead>
<tr>
<th>B-cell subset percentages</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-cell subset absolute numbers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Univariate linear regression</td>
<td>Multivariate linear regression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>HIV exposure</td>
<td>-0.139 (0.054)</td>
<td>0.012</td>
</tr>
<tr>
<td>Age</td>
<td>-0.004 (0.004)</td>
<td>0.35</td>
<td>-0.004 (0.004)</td>
</tr>
<tr>
<td>Naïve B cells</td>
<td>HIV exposure</td>
<td>-0.005 (0.017)</td>
<td>0.760</td>
</tr>
<tr>
<td>Age</td>
<td>-0.008 (0.001)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Total MBCs</td>
<td>HIV exposure</td>
<td>-0.152 (0.110)</td>
<td>0.168</td>
</tr>
<tr>
<td>Age</td>
<td>0.800 (0.062)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Resting MBCs</td>
<td>HIV exposure</td>
<td>-0.211 (0.086)</td>
<td>0.015</td>
</tr>
<tr>
<td>Age</td>
<td>1.531 (0.093)</td>
<td>&lt;0.001</td>
<td>0.812 (0.045)</td>
</tr>
<tr>
<td>Unswitched MBCs</td>
<td>HIV exposure</td>
<td>-0.337 (0.095)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.620 (0.061)</td>
<td>&lt;0.001</td>
<td>0.629 (0.057)</td>
</tr>
<tr>
<td>Switched MBCs</td>
<td>HIV exposure</td>
<td>0.131 (0.089)</td>
<td>0.140</td>
</tr>
<tr>
<td>Age</td>
<td>0.752 (0.046)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Atypical MBCs</td>
<td>HIV exposure</td>
<td>0.133 (0.081)</td>
<td>0.100</td>
</tr>
<tr>
<td>Age</td>
<td>0.009 (0.005)</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>Activated B cells</td>
<td>HIV exposure</td>
<td>-0.022 (0.077)</td>
<td>0.780</td>
</tr>
<tr>
<td>Age</td>
<td>-0.954 (0.114)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Plasmablasts</td>
<td>HIV exposure</td>
<td>0.040 (0.144)</td>
<td>0.782</td>
</tr>
<tr>
<td>Age</td>
<td>0.037 (0.009)</td>
<td>&lt;0.000</td>
<td></td>
</tr>
</tbody>
</table>

Linear regression models were used to describe the estimated change in infant’s B-cell subset percentages and counts (beta co-efficients, with the standard error indicated).

The impact of HIV exposure on B cells was determined at a significance level of p<0.05.

Subset analysis on naïve B cells, total memory B cells and plasma-blasts was performed after adjusting for multiple testing using the Bonferroni correction, at a significance level.
of p<0.02 and further subgroup analysis within the memory B cells (resting, atypical, activated, switched and unswitched memory B cells) were tested at a significance level of p<0.005. The linear regression models adjusted for clustering within an infant hence accounting for inherent correlations between repeated measurements done on the same subject. MBCs: memory B cells
Table 3: The impact of HIV exposure on infants’ memory B-cell recall responses, during the first two years of life, to antigens they had been previously vaccinated against.

Linear regression models were used to describe the estimated change in infant’s B-cell recall responses to antigens they had been previously vaccinated against (beta coefficients, with the standard error indicated). P values less than 0.05 were considered significant and indicated in bold. B-cell recall responses whose changes were significantly associated with HIV exposure were included in a multivariate regression model accounting for age. The linear regression models adjusted for clustering within an infant hence accounting for inherent correlations between repeated measurements done on the same subject.
Fig 1
Fig 2
Fig 3