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. Nduati[1,#], Irene N. Nkumama[1], Faith K. Gambo[1], Daniel M. Muema[1,*], Miguel G. Knight[2], Amin S. Hassan[1], Margaret N. Jahangir[3], Timothy J. Etyang[1], James A. Berkley[1,2] & Britta C. Urban[4]

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

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

45

Clinical and Vaccine Immunology

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 10^2 or 2 x 10^3 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

Clinical and Vaccine Immunology

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

248 population ($CD19^+CD10^-CD27^+$) was considered. It was largely due to changes in the
249 unswitched MBCs ($CD19^+CD10^-CD27^+IgM^+$) subset while the proportion of switched
250 MBCs ($CD19^+CD10^-CD27^+IgM^-$) was similar in both HEUs and HUUs. The association
251 of HIV exposure with a lower proportion of unswitched MBCs was maintained even after
252 adjusting for multiple testing using the bonferroni correction and correcting for age.
253 Similarly when B cell subsets absolute counts were considered, HIV exposure was
254 significantly associated with a reduction of unswitched MBCs absolute counts [Table 2].
255
256 Although memory B cells gradually accumulated with age, as observed from individual
257 infant's kinetics for those who had data from more than one time point (supplementary
258 figure 4) and similarly when all infants were considered [Figure 1], HIV exposure
259 resulted in a slower development of the memory B cell compartment [Figure 1]. In line
260 with the overall reduction in unswitched memory B cells, this was true for the unswitched
261 MBC subset while the gradual accumulation in the switched MBC compartment
262 remained comparable to that observed in HUUs during these first two years of life.
263
264 We further determined if the mother's data recorded closest to after the infant's date of
265 birth and/or at recruitment had an impact on the B-cell subset distribution observed in the
266 HEUs. There was no association between the majority of the maternal parameters and the
267 infant's B-cell subset distribution, apart from the total B cell percentage that directly
268 correlated with maternal CD4 counts but inversely with maternal BMI closest to after the
269 infant's date of birth and BMI at infant recruitment. Interestingly maternal viral load,
270 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

Clinical and Vaccine Immunology

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 2 b, 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

Clinical and Vaccine Immunology

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

Clinical and Vaccine Immunology

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.

Clinical and Vaccine Immunology

386

387 For a majority of the vaccine antigens against which antibody levels were determined,
388 HEUs were able to maintain antibody levels similar to those observed in HUU infants, as
389 previously reported in other settings (21, 22). However, we observed significantly lower
390 antibody levels against measles antigen in the HEUs, but of importance, majority of these
391 infants attained the recommended protective level (41). HEU infants had higher anti-
392 pneumococcal antibody levels after the 18-month age category compared to HUUs. From
393 previous reports, HIV infected women with opportunistic infections might be more likely
394 to transmit these infections to their infants (54, 55). It is therefore likely that
395 environmental exposure from an ailing mother may have led to increased exposure
396 leading to the observed higher pneumococcal antibody levels once cotrimoxazole
397 prophylaxis stopped at 18 months of age.

398

399 Of concern, in our study population, a large proportion of both HEU and HUU infants
400 showed antibody levels below a threshold deemed protective against Haemophilus
401 influenzae type b, an observation previously made (23). It is possible that MBCs rather
402 than serological memory sustain protection (56) although sustained antibody levels at 24
403 months have been reported (22). This implies that antibodies may also play a role and a
404 booster dose after early infancy may be beneficial to both HEU and HUU infants. In our
405 study we concentrated on antibody levels it may be important for future studies to
406 incorporate antibody functional assays, which would comprehensively ascertain if the
407 HIV exposed infants are compromised.

408

Clinical and Vaccine Immunology

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.

Funding information:

This work was supported by the Wellcome Trust (Grants No: WT095068 to EWN, WT089351MA to ASH, WT083579MA to JAB and WT079082 to BCU). The clinic is run by the Kenyan Government. The funders had no role in the study design, data collection and interpretation, or decision to submit the work for publication.

Acknowledgement:

We thank the study participants for taking part in this study, the field workers (Jefwa Kithunga, Anne Njogu, Salma Said and Magaret Lozi), the hospital staff for their dedicated patient care and keeping hospital records and Dr. Francis Ndungu for reviewing the manuscript. We are also grateful to TreeStar inc. Flowjo Africa for free FlowJo software access. This study is published with the permission of the Director of the Kenya Medical Research Institute (KEMRI).

Conflict of interests:

The authors have no competing interests, financial or otherwise.

References

1. **Nesheim S, Harris LF, Lampe M.** 2013. Elimination of perinatal HIV infection in the USA and other high-income countries: achievements and challenges. Curr Opin HIV AIDS **8:**447-456.
2. **UNAIDS.** 2011. World AIDS day report: How to get to zero: Faster. smarter. Better. Joint United Nations Programme on HIV/AIDS,
3. **Shapiro RL, Lockman S.** 2010. Mortality among HIV-exposed infants: the first and final frontier. Clin Infect Dis **50:**445-447.
4. **Marinda E, Humphrey JH, Iliff PJ, Mutasa K, Nathoo KJ, Piwoz EG, Moulton LH, Salama P, Ward BJ, Group ZS.** 2007. Child mortality according to maternal and infant HIV status in Zimbabwe. Pediatr Infect Dis J **26:**519-526.
5. **Slogrove A, Reikie B, Naidoo S, De Beer C, Ho K, Cotton M, Bettinger J, Speert D, Esser M, Kollmann T.** 2012. HIV-Exposed Uninfected Infants are at

Increased Risk for Severe Infections in the First Year of Life. J Trop Pediatr doi:fms019 [pii]10.1093/tropej/fms019.

6. **Taron-Brocard C, Le Chenadec J, Faye A, Dollfus C, Goetghebuer T, Gajdos V, Labaune JM, Perilhou A, Mandelbrot L, Blanche S, Warszawski J, France RN, Sud Sida HIVHEPFCOCOSG.** 2014. Increased Risk of Serious Bacterial Infections Due to Maternal Immunosuppression in HIV-Exposed Uninfected Infants in a European Country. Clin Infect Dis **59:**1332-1345.

7. **Mussi-Pinhata MM, Freimanis L, Yamamoto AY, Korelitz J, Pinto JA, Cruz ML, Losso MH, Read JS, National Institute of Child H, Human Development International Site Development Initiative Perinatal Study G.** 2007. Infectious disease morbidity among young HIV-1-exposed but uninfected infants in Latin American and Caribbean countries: the National Institute of Child Health and Human Development International Site Development Initiative Perinatal Study. Pediatrics **119:**e694-704.

8. **von Mollendorf C, von Gottberg A, Tempia S, Meiring S, de Gouveia L, Quan V, Lengana S, Avenant T, du Plessis N, Eley B, Finlayson H, Reubenson G, Moshe M, O'Brien KL, Klugman KP, Whitney CG, Cohen C, for the Group for Enteric R, Meningeal Disease Surveillance in South A.** 2015. Increased Risk for and Mortality From Invasive Pneumococcal Disease in HIV-Exposed but Uninfected Infants Aged <1 Year in South Africa, 2009-2013. Clin Infect Dis doi:10.1093/cid/civ059.

9. **Dauby N, Goetghebuer T, Kollmann TR, Levy J, Marchant A.** 2012. Uninfected but not unaffected: chronic maternal infections during pregnancy, fetal immunity, and susceptibility to postnatal infections. Lancet Infect Dis **12:**330-340.

10. **Afran L, Garcia Knight M, Nduati E, Urban BC, Heyderman RS, Rowland-Jones SL.** 2014. HIV-exposed uninfected children: a growing population with a vulnerable immune system? Clin Exp Immunol **176:**11-22.

11. **Mofenson LM.** 2015. New Challenges in the Elimination of Pediatric HIV Infection: The Expanding Population of HIV-Exposed but Uninfected Children. Clin Infect Dis doi:10.1093/cid/civ064.

12. **Hygino J, Vieira MM, Guillermo LV, Silva-Filho RG, Saramago C, Lima-Silva AA, Andrade RM, Andrade AF, Brindeiro RM, Tanuri A, Guimaraes V, de Melo Bento CA.** 2011. Enhanced Th17 phenotype in uninfected neonates born from viremic HIV-1-infected pregnant women. J Clin Immunol **31:**186-194.

13. **Economides A, Schmid I, Anisman-Posner DJ, Plaeger S, Bryson YJ, Uittenbogaart CH.** 1998. Apoptosis in cord blood T lymphocytes from infants of human immunodeficiency virus-infected mothers. Clin Diagn Lab Immunol **5:**230-234.

14. **Clerici M, Saresella M, Colombo F, Fossati S, Sala N, Bricalli D, Villa ML, Ferrante P, Dally L, Vigano A.** 2000. T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to HIV. Blood **96:**3866-3871.

15. **Vigano A, Saresella M, Schenal M, Erba P, Piacentini L, Tornaghi R, Naddeo V, Giacomet V, Borelli M, Trabattoni D, Clerici M.** 2007. Immune

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

16. **Kakkar F, Lamarre V, Ducruet T, Boucher M, Valois S, Soudeyns H, Lapointe N.** 2014. Impact of maternal HIV-1 viremia on lymphocyte subsets among HIV-exposed uninfected infants: protective mechanism or immunodeficiency. BMC Infect Dis **14:**236.
17. **Kidzeru EB, Hesseling AC, Passmore JA, Myer L, Gamieldien H, Tchakoute CT, Gray CM, Sodora DL, Jaspan HB.** 2014. In-utero exposure to maternal HIV infection alters T-cell immune responses to vaccination in HIV-uninfected infants. AIDS **28:**1421-1430.
18. **Moir S, Fauci AS.** 2014. B-cell exhaustion in HIV infection: the role of immune activation. Curr Opin HIV AIDS **9:**472-477.
19. **Amu S, Ruffin N, Rethi B, Chiodi F.** 2013. Impairment of B-cell functions during HIV-1 infection. AIDS **27:**2323-2334.
20. **Bunders M, Pembrey L, Kuijpers T, Newell ML.** 2010. Evidence of impact of maternal HIV infection on immunoglobulin levels in HIV-exposed uninfected children. AIDS Res Hum Retroviruses **26:**967-975.
21. **Jones CE, Naidoo S, De Beer C, Esser M, Kampmann B, Hesseling AC.** 2011. Maternal HIV infection and antibody responses against vaccine-preventable diseases in uninfected infants. JAMA **305:**576-584.
22. **Reikie BA, Naidoo S, Ruck CE, Slogrove AL, de Beer C, la Grange H, Adams RC, Ho K, Smolen K, Speert DP, Cotton MF, Preiser W, Esser M, Kollmann TR.** 2013. Antibody responses to vaccination among South African HIV-exposed and unexposed uninfected infants during the first 2 years of life. Clin Vaccine Immunol **20:**33-38.
23. **Gaensbauer JT, Rakhola JT, Onyango-Makumbi C, Mubiru M, Westcott JE, Krebs NF, Asturias EJ, Fowler MG, McFarland E, Janoff EN.** 2014. Impaired Haemophilus influenzae type-b Transplacental Antibody Transmission and Declining Antibody Avidity through the First Year of Life Represent Potential Vulnerabilities for HIV-Exposed but Uninfected Infants. Clin Vaccine Immunol doi:10.1128/CVI.00356-14.
24. **Abramczuk BM, Mazzola TN, Moreno YM, Zorzeto TQ, Quintilio W, Wolf PS, Blotta MH, Morcillo AM, da Silva MT, Dos Santos Vilela MM.** 2011. Impaired humoral response to vaccines among HIV-exposed uninfected infants. Clin Vaccine Immunol **18:**1406-1409.
25. **Sanz-Ramos M, Manno D, Kapambwe M, Ndumba I, Musonda KG, Bates M, Chibumbya J, Siame J, Monze M, Filteau S, Gompels UA, team Cs.** 2013. Reduced Poliovirus vaccine neutralising-antibody titres in infants with maternal HIV-exposure. Vaccine **31:**2042-2049.
26. **Madhi SA, Adrian P, Cotton MF, McIntyre JA, Jean-Philippe P, Meadows S, Nachman S, Kayhty H, Klugman KP, Violari A, Comprehensive International Program of Research on AST.** 2010. Effect of HIV infection status and anti-retroviral treatment on quantitative and qualitative antibody responses to pneumococcal conjugate vaccine in infants. J Infect Dis **202:**355-361.

27. **Miyamoto M, Pessoa SD, Ono E, Machado DM, Salomao R, Succi RC, Pahwa S, de Moraes-Pinto MI.** 2010. Low CD4+ T-cell levels and B-cell apoptosis in vertically HIV-exposed noninfected children and adolescents. J Trop Pediatr **56:**427-432.
28. **Borges-Almeida E, Milanez HM, Vilela MM, Cunha FG, Abramczuk BM, Reis-Alves SC, Metze K, Lorand-Metze I.** 2011. The impact of maternal HIV infection on cord blood lymphocyte subsets and cytokine profile in exposed non-infected newborns. BMC Infect Dis **11:**38.
29. **Longwe H, Phiri KS, Mbeye NM, Gondwe T, Jambo KC, Mandala WL.** 2015. Proportions of CD4+, CD8+ and B cell subsets are not affected by exposure to HIV or to Cotrimoxazole prophylaxis in Malawian HIV-uninfected but exposed children. BMC Immunol **16:**50.
30. **NASCOP NASCP.** 2009. Guidelines for Prevention of Mother to Child Transmission (PMTCT) of HIV/AIDS in Kenya. National AIDS & STI Control Programme,
31. **Nduati EW, Hassan AS, Knight MG, Muema DM, Jahangir MN, Mwaringa SL, Etyang TJ, Rowland-Jones S, Urban BC, Berkley JA.** 2015. Outcomes of prevention of mother to child transmission of the human immunodeficiency virus-1 in rural Kenya-a cohort study. BMC Public Health **15:**1008.
32. **Mwangi TW, Ross A, Snow RW, Marsh K.** 2005. Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. J Infect Dis **191:**1932-1939.
33. **Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, Kayentao K, Ongoiba A, Doumbo OK, Pierce SK.** 2009. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol **183:**2176-2182.
34. **Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, Bejon P, Crompton PD, Marsh K, Ndungu FM.** 2013. Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion. J Immunol **190:**1038-1047.
35. **Kimanga DO, Ogola S, Umuro M, Ng'ang'a A, Kimondo L, Murithi P, Muttunga J, Waruiru W, Mohammed I, Sharrif S, De Cock KM, Kim AA, Group KS.** 2014. Prevalence and incidence of HIV infection, trends, and risk factors among persons aged 15-64 years in Kenya: results from a nationally representative study. J Acquir Immune Defic Syndr **66 Suppl 1:**S13-26.
36. **Abdullahi O, Karani A, Tigoi CC, Mugo D, Kungu S, Wanjiru E, Jomo J, Musyimi R, Lipsitch M, Scott JA.** 2012. Rates of acquisition and clearance of pneumococcal serotypes in the nasopharynges of children in Kilifi District, Kenya. J Infect Dis **206:**1020-1029.
37. **Crotty S, Aubert RD, Glidewell J, Ahmed R.** 2004. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. J Immunol Methods **286:**111-122.
38. **Muema DM, Macharia GN, Hassan AS, Mwaringa SM, Fegan GW, Berkley JA, Nduati EW, Urban BC.** 2015. Control of Viremia Enables Acquisition of Resting Memory B Cells with Age and Normalization of Activated B Cell Phenotypes in HIV-Infected Children. J Immunol **195:**1082-1091.

Clinical and Vaccine Immunology

39. **Ndungu FM, Olotu A, Mwacharo J, Nyonda M, Apfeld J, Mramba LK, Fegan GW, Bejon P, Marsh K.** 2012. Memory B cells are a more reliable archive for historical antimalarial responses than plasma antibodies in no-longer exposed children. Proc Natl Acad Sci U S A **109:**8247-8252.

40. **Rouet F, Chaix ML, Nerrienet E, Ngo-Giang-Huong N, Plantier JC, Burgard M, Peeters M, Damond F, Ekouevi DK, Msellati P, Ferradini L, Rukobo S, Marechal V, Schvachsa N, Wakrim L, Rafalimanana C, Rakotoambinina B, Viard JP, Seigneurin JM, Rouzioux C.** 2007. Impact of HIV-1 genetic diversity on plasma HIV-1 RNA Quantification: usefulness of the Agence Nationale de Recherches sur le SIDA second-generation long terminal repeat-based real-time reverse transcriptase polymerase chain reaction test. J Acquir Immune Defic Syndr **45:**380-388.

41. **Cutts FT.** 1993. The immunological basis for immunization series. Module 7: measles. World Health Organization, Geneva, Switzerland.

42. **Peltola H, Kayhty H, Virtanen M, Makela PH.** 1984. Prevention of Hemophilus influenzae type b bacteremic infections with the capsular polysaccharide vaccine. N Engl J Med **310:**1561-1566.

43. **Van de Perre P, Simonon A, Hitimana DG, Dabis F, Msellati P, Mukamabano B, Butera JB, Van Goethem C, Karita E, Lepage P.** 1993. Infective and anti-infective properties of breastmilk from HIV-1-infected women. Lancet **341:**914-918.

44. **Shapiro RL, Ndung'u T, Lockman S, Smeaton LM, Thior I, Wester C, Stevens L, Sebetso G, Gaseitsiwe S, Peter T, Essex M.** 2005. Highly active antiretroviral therapy started during pregnancy or postpartum suppresses HIV-1 RNA, but not DNA, in breast milk. J Infect Dis **192:**713-719.

45. **Klein U, Rajewsky K, Kuppers R.** 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med **188:**1679-1689.

46. **Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, Plebani A, Kumararatne DS, Bonnet D, Tournilhac O, Tchernia G, Steiniger B, Staudt LM, Casanova JL, Reynaud CA, Weill JC.** 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood **104:**3647-3654.

47. **Tangye SG, Good KL.** 2007. Human IgM+CD27+ B cells: memory B cells or "memory" B cells? J Immunol **179:**13-19.

48. **Seifert M, Przekopowitz M, Taudien S, Lollies A, Ronge V, Drees B, Lindemann M, Hillen U, Engler H, Singer BB, Kuppers R.** 2015. Functional capacities of human IgM memory B cells in early inflammatory responses and secondary germinal center reactions. Proc Natl Acad Sci U S A **112:**E546-555.

49. **Dogan I, Bertocci B, Vilmont V, Delbos F, Megret J, Storck S, Reynaud CA, Weill JC.** 2009. Multiple layers of B cell memory with different effector functions. Nat Immunol **10:**1292-1299.

50. **Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK.** 2011. Different B cell populations mediate early and late memory during an endogenous immune response. Science **331:**1203-1207.

51. **Mwenya DM, Charalambous BM, Phillips PP, Mwansa JC, Batt SL, Nunn AJ, Walker S, Gibb DM, Gillespie SH.** 2010. Impact of cotrimoxazole on carriage and antibiotic resistance of Streptococcus pneumoniae and Haemophilus influenzae in HIV-infected children in Zambia. Antimicrob Agents Chemother **54:**3756-3762.
52. **Church JA, Fitzgerald F, Walker AS, Gibb DM, Prendergast AJ.** 2015. The expanding role of co-trimoxazole in developing countries. Lancet Infect Dis **15:**327-339.
53. **Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter HH, Berner R, Peters A, Boehm T, Plebani A, Quinti I, Carsetti R.** 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. J Exp Med **197:**939-945.
54. **Mofenson LM, Oleske J, Serchuck L, Van Dyke R, Wilfert C, Cdc, National Institutes of H, Infectious Diseases Society of A.** 2004. Treating opportunistic infections among HIV-exposed and infected children: recommendations from CDC, the National Institutes of Health, and the Infectious Diseases Society of America. MMWR Recomm Rep **53:**1-92.
55. **McNally LM, Jeena PM, Lalloo U, Nyamande K, Gajee K, Sturm AW, Goldblatt D, Tomkins AM, Coovadia HM.** 2005. Probable mother to infant transmission of Pneumocystis jiroveci from an HIV-infected woman to her HIV-uninfected infant. AIDS **19:**1548-1549.
56. **Heath PT, Bowen-Morris J, Griffiths D, Griffiths H, Crook DW, Moxon ER.** 1997. Antibody persistence and Haemophilus influenzae type b carriage after infant immunisation with PRP-T. Arch Dis Child **77:**488-492.
57. **Shlomchik MJ, Weisel F.** 2012. Germinal center selection and the development of memory B and plasma cells. Immunol Rev **247:**52-63.
58. **Amanna IJ, Carlson NE, Slifka MK.** 2007. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med **357:**1903-1915.
59. **Langhorne J, Ndungu FM, Sponaas AM, Marsh K.** 2008. Immunity to malaria: more questions than answers. Nat Immunol **9:**725-732.
60. **Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, Skinner J, Virtaneva K, Sturdevant DE, Porcella SF, Doumbo OK, Doumbo S, Kayentao K, Ongoiba A, Traore B, Sanz I, Pierce SK, Crompton PD.** 2015. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. Elife **4**.
61. **Longwe H, Jambo KC, Phiri KS, Mbeye N, Gondwe T, Hall T, Tetteh KK, Drakeley C, Mandala WL.** 2015. The effect of daily co-trimoxazole prophylaxis on natural development of antibody-mediated immunity against P. falciparum malaria infection in HIV-exposed uninfected Malawian children. PLoS One **10:**e0121643.
62. **Church JA, Fitzgerald F, Walker AS, Gibb DM, Prendergast AJ.** 2015. The expanding role of co-trimoxazole in developing countries. Lancet Infect Dis doi:10.1016/S1473-3099(14)71011-4.

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

734 Correlation of circulating antigen specific memory B cells with the percentage of
735 switched memory B cells (first row) or IgG antibody levels against measles, tetanus
736 toxoid protein (TT), pneumococcal capsular polysaccharides (PCPs), diphtheria and total
737 IgG (second row). Spearman correlation co-efficients were determined and the spearman
738 rho presented. P-values < 0.05 were considered significant.
739
740

741 **Table 1:** Mothers' data from HIV exposed uninfected infants taken closest to after the

742 infant's date of birth and during the infant's recruitment (N = 87).

		Mother's characteristics close to infant's birth	Mother's characteristics at infant's recruitment
Median age in years [IQR]		29.2 [25.4 – 34.2] (n = 87)	30.1 [26.1 – 35.1] (n = 87)
Body Mass Index Median [IQR]		21.5 [19.5 – 23.3] (n = 72)	21.2 [19.5 – 23.0] (n = 74)
CD4 counts/mm^3 Median [IQR]		410 [292 - 640] (n = 53)	457 [322 - 603] (n = 75)
Viral load ranges (copies/ml) n = 78[a]	<300		48.7% (38/78)
	>300<1000		7.7% (6/78)
	>1000<5000	N/D	11.5% (9/78)
	>5000		32.1% (25/78)
Viral load (copies/ml) categorized as per HAART use close to the infant's birth Median [IQR]	Not on HAART (n = 37)		2783 [54 - 20685]
	HAART 0 – 24 months (n = 13)		29 [0 – 2648]
	HAART >24 months (n = 20)	N/D	10 [0 – 402]
	Missing HAART category (n = 8)[b]		2428 [18 - 25894]

743

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

745 [a] viral load data was available for 78 of the mothers.

746 HAART; highly active antiretroviral therapy

747 [b] mothers for who data on the duration on HAART close to the infant's birth was missing

748 [IQR] = interquartile range

749 N/D = not determined

750

751 **Table 2:** The association of HIV exposure and changes in infants' B-cell subset

752 percentages and absolute counts during the first two years of life.

		B-cell subset percentages				B-cell subset absolute numbers			
		Univariate linear regression		Multivariate linear regression		Univariate linear regression		Multivariate linear regression	
		β-coefficient (Std Error)	P value	β-coefficient (Std Error)	P value	β-coefficient (Std Error)	P value	β-coefficient (Std Error)	P value
B cells	HIV exposure	**-0.139 (0.054)**	**0.012**	**-0.138 (0.055)**	**0.013**	-0.063 (0.089)	0.482		
	Age	-0.004 (0.004)	0.35	-0.004 (0.004)	0.378	-0.0126 (0.0067)	0.061		
Naïve B cells counts	HIV exposure	-0.005 (0.017)	0.760			-0.019 (0.098)	0.850		
	Age	**-0.008 (0.001)**	**<0.001**			**-0.020 (0.007)**	**0.004**		
Total MBCs	HIV exposure	-0.152 (0.110)	0.168			-0.054 (0.122)	0.657		
	Age	**0.800 (0.062)**	**<0.001**			**-1.430 (0.131)**	**0.000**		
Resting MBCs counts	HIV exposure	**-0.211 (0.086)**	**0.015**	**-0.259 (0.065)**	**<0.001**	-0.191 (0.124)	0.124		
	Age	**1.531 (0.093)**	**<0.001**	**0.812 (0.045)**	**<0.001**	**-1.351 (0.161)**	**<0.001**		
Unswitched MBCs counts	HIV exposure	**-0.337 (0.095)**	**<0.001**	**-0.359 (0.081)**	**<0.001**	**-0.287 (0.138)**	**0.039**	**-0.364 (0.128)**	**0.005**
	Age	**0.620 (0.061)**	**<0.001**	**0.629 (0.057)**	**<0.001**	**-0.466 (0.067)**	**<0.001**	**-0.493 (0.061)**	**<0.001**
Switched MBCs counts	HIV exposure	0.131 (0.089)	0.140			0.201 (0.126)	0.111		
	Age	**0.752 (0.046)**	**<0.001**			**-1.34 (0.173)**	**<0.001**		
Atypical MBCs counts	HIV exposure	0.133 (0.081)	0.100			0.130 (0.116)	0.266		
	Age	0.009 (0.005)	0.103			-0.038 (0.019)	0.052		
Activated B cells counts	HIV exposure	-0.022 (0.077)	0.780			-0.050 (0.106)	0.634		
	Age	**-0.954 (0.114)**	**<0.001**			**-0.374 (0.064)**	**<0.001**		
Plasmablasts	HIV exposure	0.040 (0.144)	0.782			0.0370 (0.164)	0.823		
	Age	**0.037 (0.009)**	**<0.000**			**-0.335 (0.103)**	**0.001**		

753 Linear regression models were used to describe the estimated change in infant's B-cell

754 subset percentages and counts (beta co-efficients, with the standard error indicated).

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

756 Subset analysis on naïve B cells, total memory B cells and plasma-blasts was performed

757 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

764 **Table 3:** The impact of HIV exposure on infants' memory B-cell recall responses, during
765 the first two years of life, to antigens they had been previously vaccinated against.
766

		Univariate		Multivariate	
		Beta Coefficient (Standard Error)	**P value**	**Beta Coefficient (Standard Error)**	**P value**
PCPs	HIV exposure	0.713 (0.685)	0.302		
	Age	0.0813 (0.068)	0.235		
Measles	HIV exposure	0.309 (1.033)	0.766		
	Age	0.024 (0.197)	0.904		
Tetanus Toxoid	HIV exposure	-0.195 (1.049)	0.853		
	Age	0.059 (0.074)	0.431		
Diphtheria	HIV exposure	0.708 (0.727)	0.334		
	Age	-0.022 (0.056)	0.700		
Total IgG	HIV exposure	**-44.44 (20.88)**	**0.037**	**-48.145 (19.14)**	**0.014**
	Age	**7.268 (1.212)**	**0.000**	**7.418 (1.266)**	**0.000**

767

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

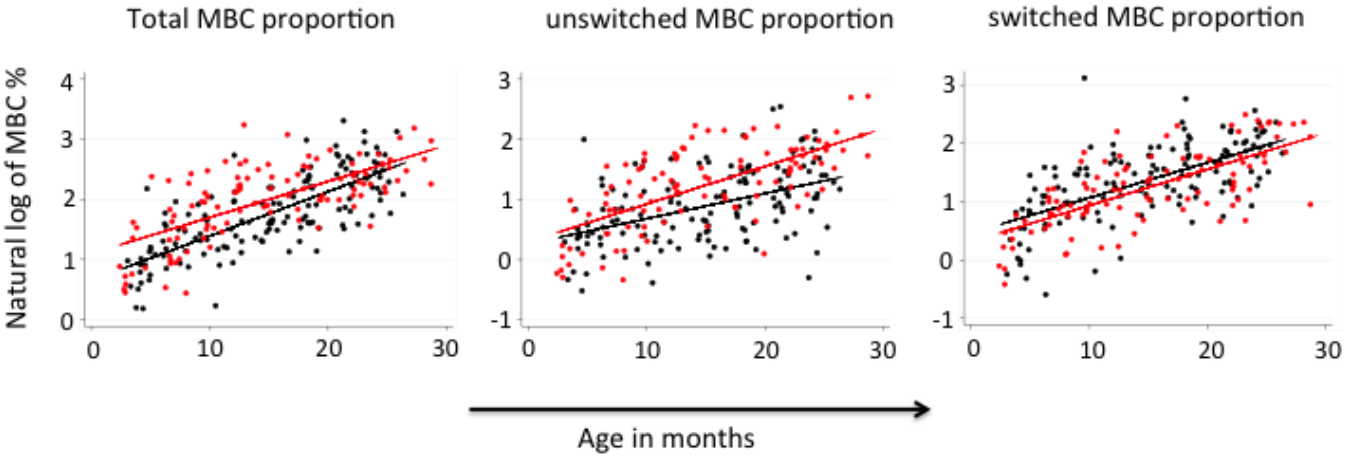

Fig 1

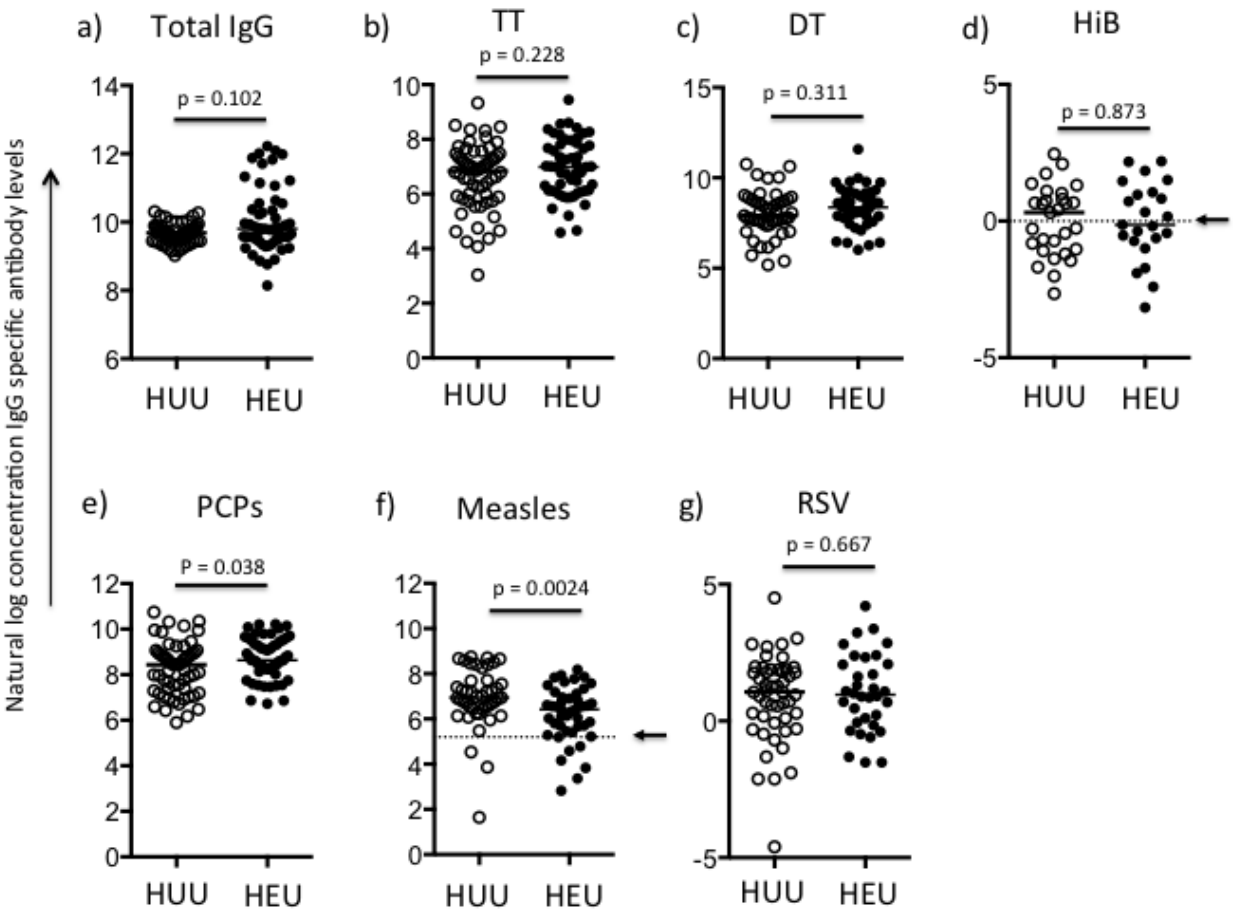

Fig 2

Clinical and Vaccine Immunology

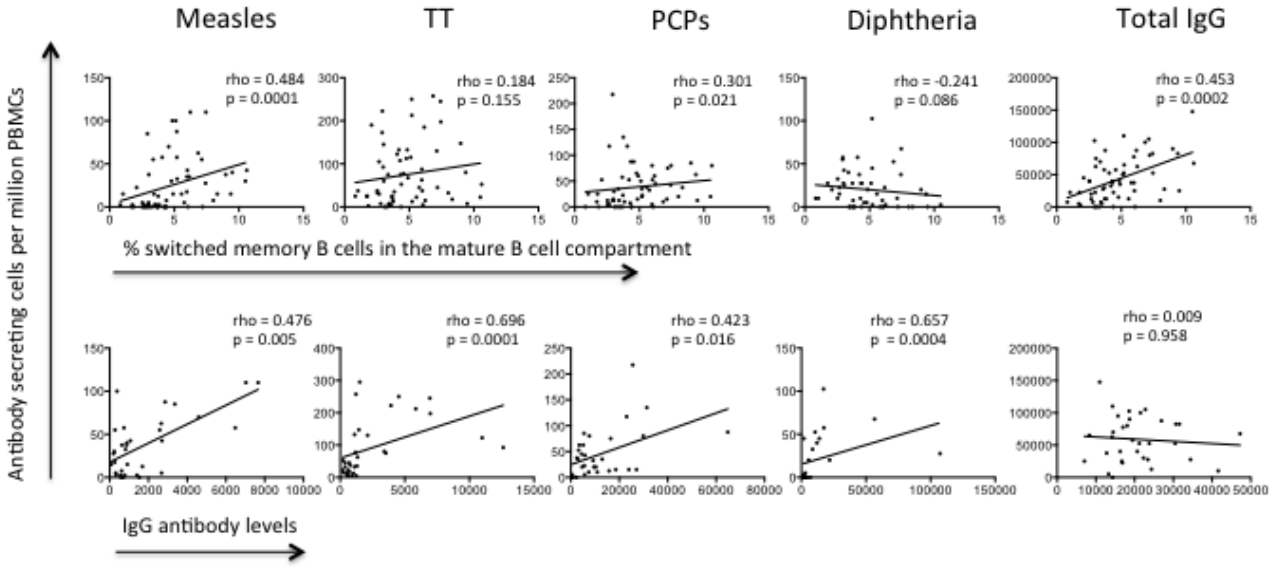

Fig 3