Title: Contrasting Patterns of Serologic and Functional Antibody Dynamics to *Plasmodium falciparum* Antigens in a Kenyan Birth Cohort

Running title: Dynamics of Infant Antimalarial Antibody Responses

Arlene E. Dent,[a,b,#] Indu Malhotra,[a] Xuelie Wang,[c] Denise Babineau,[c*] Kee Thai Yeo,[b] Timothy Anderson,[d§] Rhonda J. Kimmel,[a] Evelina Angov,[e] David E. Lanar,[e] David Narum,[f] Sheetij Dutta,[e] Jack Richards,[g] James G. Beeson,[g,h] Brendan S. Crabb,[g] Alan F. Cowman,[i] Toshihiro Horii,[j] Eric Muchiri,[k] Peter L. Mungai,[a,k] Christopher L. King,[a] James W. Kazura,[a]

Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA[a]; Department of Pediatrics, Rainbow Babies and Children's Hospital, Cleveland, OH, USA[b]; Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH, USA[c]; School of Medicine, Case Western Reserve University, Cleveland, OH, USA[d]; Malaria Vaccine Branch, Walter Reed Army Institute of Research, Silver Springs, MD, USA[e]; Laboratory of Malaria Immunology and Vaccinology, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA[f]; Burnet Institute, Melbourne, Australia[g]; Department of Microbiology, Monash University, Melbourne, Australia[h]; Walter and Eliza Hall of Institute of Medical Research, Parkville, Australia[i]; Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan[j]; Division of Vector Borne and Neglected Tropical Diseases, Ministry of Public Health and Sanitation, Nairobi, Kenya[k]

[#]Address correspondence to Arlene Dent, arlene.dent@case.edu

*Present address: Rho Federal Systems Division, Chapel Hill, NC, USA

[§]Present address: Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh PA, USA

Abstract

IgG antibodies to *Plasmodium falciparum* (Pf) are transferred from the maternal to fetal circulation during pregnancy, wane after birth, and are subsequently acquired in response to natural infection. We examined the dynamics of malaria antibody responses of 84 Kenyan infants from birth to 36 months of age by i) serology, ii) variant surface antigen (VSA) assay, iii) growth inhibitory activity (GIA) and iv) invasion inhibition assays (IIA) specific for merozoite surface protein 1 (MSP1) and sialic acid dependent invasion pathway. Maternal antibodies in each of these four categories were detected in cord blood and decreased to their lowest level by approximately 6 months of age. Serologic antibodies to three pre-erythrocytic and ten blood stage antigens subsequently increased, reaching peak prevalence by 36 months. In contrast, antibodies measured by VSA, GIA, and IIA remained low even up to 36 months. Infants sensitized to Pf *in utero,* defined by cord blood lymphocyte recall responses to malaria antigens, acquired antimalarial antibodies at the same rate as those who were not sensitized *in utero,* indicating that fetal exposure to malaria antigens did not affect subsequent infant antimalarial responses. Infants with detectable serologic antibodies at 12 months of age had an increased risk of Pf infection during the subsequent 24 months. We conclude that serologic measures of antimalarial antibodies by children ≤36 months of age represent biomarkers of malaria exposure rather than protection, and that functional antibodies develop after 36 months of age in this population.

Introduction

Naturally acquired immunity to malaria develops slowly over time in children in malaria endemic areas as a consequence of repeated infections (1). Antibodies play a key role in this immunity as demonstrated by passive antibody transfer from immune adults to children with clinical malaria resulting in reduction of symptoms and parasitemia (2, 3). Very young infants <6 months old are relatively protected from clinical malaria, a phenomenon thought to be mediated primarily by maternal IgG antibodies transferred to the fetus in the last trimester of pregnancy. High levels of fetal hemoglobin and nutritional factors may also contribute to decreased malaria susceptibility during early infancy (4-6). Maternal IgG antibodies detectable in cord blood progressively decrease, leaving infants older than approximately four to six months of age vulnerable to Pf infection and symptomatic malaria. With repeated infections and increasing age, young infants subsequently acquire IgG antibodies directed against many Pf antigens. The exact antigenic targets of these antibodies, their relative rates of development, and how they function to mediate protection from infection and symptomatic malaria are incompletely understood.

Antimalarial IgG antibodies may potentially mediate protection through multiple functions, e.g. blocking sporozoite invasion of hepatocytes and merozoite invasion of erythrocytes, opsonizing merozoites and infected erythrocytes expressing variant surface antigens on their surface for phagocytosis, and fixation and activation of complement on the merozoite surface with resultant parasite lysis. An increasing number of Pf antigens have been identified as relevant to naturally acquired immunity, and thus, are considered potential vaccine targets (7-9). Evaluation of infant antibody responses to Pf has relied mainly on serologic assays, with some studies indicating that such antibodies are associated with protection from infection and symptomatic malaria (10, 11), while others conclude that they are biomarkers of exposure which, when elevated, are associated prospectively with an increased risk of malaria (6, 12-14). Measurements of alternative functional antibody activities such as the variant

surface antigen (VSA) assay, growth inhibitory activity (GIA), and invasion inhibitory assays (IIA) that reflect impaired interaction of merozoite ligands with the erythrocyte surface membrane have been developed (15-20). There have been few studies of VSA antibodies focused on infants in malaria endemic areas (21). Antibodies that inhibit the growth of Pf *in vitro* have been used to assess vaccine efficacy in animal models and malaria-naïve human volunteers (22-26). Pf GIA has been associated with protection from infection in children in some studies, but this has not been a consistent finding (15, 27, 28).

The objective of our study was to advance knowledge on the breadth and dynamics of various infant antimalarial antibody responses and determine whether specific antigens and functional antibody responses may be prioritized during the development of naturally acquired immunity in early childhood. Infants born in Msabmweni, Kenya, from 2006 to 2009 were followed every six months from birth to 36 months. Plasma from the study participants was examined for the presence and magnitude of serologically determined IgG antibodies directed against multiple pre-erythrocytic and blood stage antigens over time. In addition, we measured IgG antibodies to VSA expressed by three different Pf laboratory-adapted isolates – 3D7, a widely used reference isolate; BFD06, isolated from an adult traveler returning from Burkina Faso presenting to the hospital with severe malaria (29); and Msam06, isolated from a child presenting with acute uncomplicated malaria at Msambweni District Hospital, Kenya (30). We evaluated GIA with D10 and W2mef parasites and the acquisition of invasion-inhibitory antibodies directed against MSP1-19 (16, 31) and sialic acid dependent invasion pathways (32).

Methods

Study population and ethical approval

Healthy, pregnant mothers were recruited from antenatal clinics at Msambweni District Hospital, Coast Province, Kenya from 2006 to 2009 as previously described (33). Malaria endemicity at the time was in transition from moderate transmission in 2007 to low transmission in 2009 (34).

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Per Kenya Ministry of Health national policy, women received intermittent preventative treatment for malaria with sulfadoxine-pyrimethamine beginning in the second trimester as well as iron, folic acid, and bed nets as part of routine care. Full-term healthy neonates were enrolled in the study. Cord blood was collected after delivery and blood collected from the infants (by venipuncture) every 6 months until 36 months of age. All infants with data for this study were born to HIV negative mothers, and all women provided written, informed consent. The study was approved by the Institutional Review Boards at the Kenya Medical Research Institute and University Hospitals Case Medical Center.

Samples and sample preparation

Cord blood was collected in heparinized bags from placentas of full term deliveries (35). Plasma was stored at -20^{o}C. Cord blood mononuclear cells (CBMC) were isolated using Ficoll-Paque PLUS (GE Healthcare, NJ) density gradient centrifugation and cryopreserved in 90% fetal bovine serum plus 10% dimethyl sulfoxide (Sigma-Aldrich, MO) (35). Heparinized blood from infants was centrifuged and plasma stored at -20^{o}C.

In utero sensitization to malaria antigens

Freshly isolated CBMC were used to evaluate cytokine production in response to known T cell epitopes within the C-terminal 83 kDa fragment of Merozoite Surface Protein1 (MSP1), the 42 kDa fragment of recombinant MSP1 (MSP1-42 FVO and MSP1-42 3D7), and PfP0 (a Pf ribosomal phosphoprotein (36)) as previously described (35). A newborn was considered to be sensitized to malaria antigens *in utero* when one of the following three conditions were met: 1) by IFN-γ ELISPOT, there were >4 cytokine-secreting cells/10^6 CBMC in response to MSP1 peptides/MSP1-42/PfP0 and no secreting cells were detected in negative control wells containing media alone; 2) by IFN-γ ELISPOT, in cases where cytokine-secreting cells were observed in negative control wells, the number of spots generated by MSP1 driven CBMC was 2-fold greater than control wells; 3) by ELISA for IFN-γ, IL-2, IL-5 or IL-13, net cytokine production by CBMC in response to MSP1 peptides/MSP1-42/PfP0 was at least 2-fold greater

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than that of negative control wells. (35) If these criteria were not met, the newborn was categorized as not sensitized.

Diagnosis of infection by blood smear and PCR

All blood samples were examined for Pf parasites. Thick and thin blood smears were prepared, stained with 5% Giemsa, and examined by light microscopy for Pf-infected erythrocytes. A slide was deemed negative when no parasites were seen after counting microscopic fields containing at least 200 leukocytes. After Ficoll processing of cord blood and infant blood samples, DNA from 200µL of the erythrocyte pellet was extracted using QIAamp 96 DNA blood kit (Qiagen, Valencia, CA). The DNA was subjected to a Pf specific PCR/Ligase Detection Reaction-Fluorescence Microsphere Assay as previously described (37). Pf infections (n=39) were detected in asymptomatic pregnant women during this time and extracted DNA was utilized for $MSP1_{19}$ haplotype determination (see below).

Serologic IgG and IgM antibodies to Pf antigens measured by Luminex® multiplex assay

Recombinant antigens tested included the following proteins: Liver stage antigen 1 (LSA1 (38); Circumsporozoite protein (CSP (39)); Cell-traversal protein for ookinetes and sporozoites (PfCelTOS (40)); Serine repeat antigen 5 (SERA5; SE50 (41), SE36 (42)); Merozoite surface protein 1, 42 kDa fragment ($MSP1_{42}$ 3D7 (43), FVO (44), and FUP (45)); Erythrocyte binding antigen (EBA) 140 (46), EBA175 (47), EBA181 (48)); and Apical membrane antigen 1 (AMA1 3D7 (49) and FVO (50)). The proteins AMA1, PfCelTOS, CSP, and MSP1 alleles were all GMP quality proteins and therefore had no host cell contamination. We did not find that there was high reactivity of the responses to the other antigens compared with these GMP quality proteins. These antigens were selected as previous cohort studies have indicated that antibodies against them have been generally associated with protective immunity, are targets of acquired invasion-inhibitory antibodies, and are vaccine candidates (17, 51-54). Carboxylated microspheres (Luminex, Austin, TX) were coupled to the proteins using the manufacturer's protocol and as described (55-57). Antigen-specific IgG was detected by incubating 1,000 beads of each

153 antigen per well with 1:1000 plasma dilution in a final volume of 100µL. Antigen-specific IgM
154 was detected using the same incubation techniques and a 1:100 plasma dilution. Plasma
155 samples from four North American malaria naïve adults were used as negative controls for each
156 plate. A pool of Kenyan adult plasma was used as a positive control on all plates to ensure
157 assay performance and minimal plate to plate variation. For IgG antibody responses, the mean
158 fluorescence intensity (MFI) of individual Kenyan plasma samples was normalized to the mean
159 MFI of the negative controls to obviate plate to plate variations. A positive value was assigned if
160 the normalized value was >1.5 fold over malaria naïve controls. For IgM, a positive value was
161 considered if the normalized value was >5 fold over negative controls. All positive values were
162 also greater than the mean plus 3 SD of the value of the individual negative control plasma
163 samples.

164 *Growth Inhibition Assays*

165 D10 (D10-PfM3' (16)) and W2mef parasites were utilized in GIAs as previously described (15).
166 Briefly, ring-stage parasites were synchronized twice by sorbitol lysis (5% D-sorbitol (Sigma, St.
167 Louis, MO)) and allowed to mature to late trophozoite/schizont stages. Parasites were cultured
168 at 4% hematocrit in RPMI-1640 supplemented with 25mg/mL HEPES, 2 mg/mL sodium
169 bicarbonate, 0.5% Albumax II (Gibco, Grand Island, NY), 2.4mM L-glutamine, 0.08 mg/ml
170 gentamicin, and 0.2mM hypoxanthine. Cultures were maintained at 37°C in an atmosphere of
171 5% CO_2, 1% O_2 and 94% N_2. Purified parasites were adjusted to 0.5% infected erythrocytes
172 with a final 2% hematocrit, 1:10 plasma dilution (not heat inactivated at 56°C and thus,
173 containing complement proteins required for activation by classical and alternate pathways,
174 although freezer storage could make complement function suboptimal), and 100µL final volume
175 in 96-well flat-bottom microtiter plates. The cultures were incubated for 26 hours to allow for
176 schizont rupture and merozoite invasion (monitored by microscopy to ensure full schizont
177 rupture). Twenty-five µL of resuspended cultures were removed, fixed with 0.25%
178 gluteraldehyde in PBS for 45 minutes, and placed in 10x SYBR Green I (Molecular Probes,

Eugene, OR) in 400µL 1x PBS for >24 hours at 4°C to stain parasite DNA. Stained cells were examined with a BD LSR II flow cytometer to collect data from a minimum of 5 x 10^4 cells. Becton-Dickinson FACS Diva 5.01 was used to collect and FlowJo 8.5.1 to analyze cytometry data. The mean parasitemia for duplicate wells was used to determine the percent GIA calculated with the following equation: 100 - (test plasma parasitemia/non-immune plasma parasitemia x 100). Plasma samples from four North Americans who had never been exposed to malaria were pooled as the "non-immune" plasma controls.

Target-specific Invasion Inhibition Assays

Methods to quantify MSP1-19 IIA and sialic acid dependent invasion IIA (Sial Dep IIA) were as described previously (16, 32, 58). Briefly, for the MSP1-19 specific IIAs, D10-PfM3' and an isogenic D10-PcMEGF parasite line in which the *P. chabaudi* orthologue replaces the *P. falciparum* MSP1-19 region were tested in parallel. Greater inhibition of D10-PfM3' compared to D10-PcMEGF parasites is interpreted as inhibitory antibodies targeting Pf-MSP1-19. For the Sial Dep IIA, W2mef isolate and W2mef with genetic deletion of EBA175 (ΔEBA175) isolate were tested in parallel. W2mef invades predominantly via sialic acid dependent invasion pathways and W2mefΔEBA175 invades via sialic acid independent pathways. Greater inhibition of W2mef parental versus W2mefΔEBA175 is interpreted as inhibitory antibodies to sialic acid dependent invasion (32). For both assays, ring-stage parasites were synchronized twice by sorbitol lysis and allowed to mature to late trophozoite/schizont stages. Parasites were adjusted to 4% hematocrit with 0.5% Pf infected erythrocytes, and 50µL aliquots were placed in 96-well, flat-bottom microtiter plates with an equal volume of 1:5 prediluted plasma in culture medium (final plasma dilution 1:10, final volume 100µL). The same batch of prediluted plasma was added to the two parasite lines in the same assays. The cultures were incubated for 26 hours to allow for schizont rupture and merozoite invasion. 25µL of resuspended cultures was removed, fixed with 0.25% gluteraldehyde in PBS for 45 minutes, and placed in 10x SYBR Green I (Molecular Probes, Eugene, OR) in 400µL 1x PBS for >24 hours at 4 °C (15, 58). Stained cells

were examined with a BD LSR II flow cytometer to collect data from a minimum of 5 x 10^4 cells using Becton-Dickinson FACS Diva 5.01. Ring-stage parasitemia was calculated by quantifying singly infected erythrocytes plus multiply infected erythrocytes (quantified as having two intracellular rings)/total erythrocytes according to flow cytometry gating previously described (15, 58). FlowJo 8.5.1 was used to analyze cytometry data. The mean number ring-stage parasitemia for duplicate wells was calculated and results expressed as a percentage of the ring-stage parasitemia of non-immune control plasma (derived from four North Americans who had never been exposed to malaria) in parallel cultures. The percentage change of invasion inhibition antibodies specifically attributable to MSP1-19 antibodies (MSP1-19 IIA) or Sial Dep IIA was calculated by subtracting the percentage of invasion of the parent Pf strain (D10-PfM3' or W2mef) relative to non-immune controls from the percent invasion of mutated Pf strain (D10-PcMEGF or W2mefΔEBA175) relative to non-immune controls. A positive response was defined as ≥5% inhibition attributable to MSP1-19 IIA or Sial Dep IIA.

Antibodies to variant surface antigens

Anti-VSA IgG antibodies were measured by flow cytometry as previously described with minor modifications (59, 60); these antibodies appear to predominantly target the infected erythrocyte surface antigen Pf erythrocyte membrane protein 1 (PfEMP1) (19). Three Pf isolates were used: i) 3D7, a widely used reference isolate, and antibodies to this isolate were previously associated with protection to malaria in Kenyan children (19, 61); ii) BFD06, which was isolated from an adult traveler with acute severe malaria returning from Burkina Faso in 2006 (29); and iii) Msam 06, which was isolated in 2006 from a child with acute uncomplicated malaria in Msambweni, Kenya, the study site for this cohort (30). Pf isolates from the two acute malaria patients were adapted to *in vitro* culture. Parasites were grown in group O erythrocytes, synchronized, harvested at the late trophozoite stage and cryopreserved. All plasma samples were processed at the same time for each individual parasite line. Positive control plasma consisted of pooled plasma from eight malaria immune Kenyan adults, and negative control

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plasma consisted of pooled plasma from four malaria naïve North Americans. Thawed parasites were adjusted to 0.2% hematocrit. Two µL of heat-inactivated test plasma was added to each well of a U-bottom 96 well microtiter plate. Thirty-eight µL of the adjusted thawed parasites were added to each well (final plasma dilution 1:20) and incubated for 60 minutes at room temperature. Between each incubation step, cells were washed three times with PBS/0.1% casein. 40µL of 1:100 diluted polyclonal rabbit anti-human IgG (Dako, Carpinteria, CA) was added and incubated for 30 minutes at room temperature, followed by 40µL of 1:100 diluted Alexa-Fluor-647-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR) with 10x SYBR Green I incubated for 30 minutes at room temperature. Cells were resuspended in 200µL PBS/0.1% casein and examined with a BD LSR II flow cytometer. Infected erythrocytes were differentiated from non-infected erythrocytes by SYBR Green fluorescence. For quantification of the Alexa-Fluor, the geometric mean fluorescent intensity (GeoMFI) of each population was used. The magnitude of VSA reactivity was calculated as GeoMFI of infected erythrocytes minus GeoMFI of non-infected erythrocytes. A positive response was defined as GeoMFI greater than the mean plus 3 SD of the North American negative controls.

$MSP1_{19}$ haplotype detection

DNA was extracted from 200 µL of venous blood using QIAamp DNA blood mini kit (Qiagen Corp, Valencia, CA). PCR amplification using $MSP1_{19}$ specific and Pf small subunit rRNA specific primers, the Ligase Detection Reaction – Fluorescent Microsphere Assay (LDR-FMA), and haplotype assignment based on allele-specific mean fluorescence intensity were performed as previously described (55, 62). Importantly, if four alleles (Q, E, KNG, and TSR) were detected in a single sample, we conservatively assumed that only two haplotypes were present. Therefore, the maximum number of haplotypes assigned to any infection was two.

Statistical analysis

We estimated the probability of positive antibody responses over time using restricted cubic splines, and used generalized linear mixed models to estimate the probability of anti-malaria

antibody responses over time among newborns sensitized and not sensitized to MSP1 *in utero*. A generalized estimation equation regression model was used to estimate the rate of change in the probability of presence of each antibody response over time and to assess if the mean rate of change in the probability of detecting serological antibodies after 12 months of age was different than the mean rate of change in the probability of detecting VSA, GIA, and IIA antibodies after 12 months of age. Cox proportional hazards regression models were fit to investigate the association between antibody responses at birth or at 12 months of age and the incidence of Pf infection during the follow up time period.

Results

IgG antibody magnitude and prevalence in cord blood

We report the malarial antibody dynamics of 84 infants who had approximately 4.3 blood samples per participant with a mean follow-up time of 29 months (minimum 4 months, maximum 39 months, and median 33 months). The presence and magnitude of cord blood maternal IgG antibodies are shown in Table 1. Serologic antibodies were common, with anti-AMA1 (3D7 and FVO) having the highest prevalence (97.4%) and magnitude, and antibodies to PfCelTOS and SERA5 (SE50) having the lowest prevalence (14.1-19.2%) and magnitude. VSA reactive antibodies were moderately prevalent in cord blood (47.4-69.2%). GIAs and Sial Dep IIA were very low in cord blood with virtually no MSP1-19 IIA detected (6.1%).

Antigens utilized in serologically measured antibodies were selected to reflect the circulating allele frequencies in the population. For example, MSP1 is the most abundant protein found on the merozoite surface and a vaccine candidate. As the merozoite invades the erythrocyte, MSP1 is processed into several fragments, of which the C-terminal 19 kDa fragment remains on the merozoite surface during invasion (63-65). $MSP1_{19}$ is composed of 98 highly conserved amino acids with the exception of residues 1644 (E/Q), 1691(T/K), 1700 (S/N), and 1701 (R/G). Non-synonymous changes at these positions result in four predominant

haplotypes: ETSR (3D7/PNG-MAD20), EKNG (FUP/Uganda-PA), QKNG (FVO/Wellcome), and QTSR (Indo) (66-69). We found that the frequency of circulating $MSP1_{19}$ haplotypes in this region was 44% EKNG (FUP), 39% QKNG (FVO), 8% ETSR (3D7) and 0% QTSR. Therefore, the $MSP1_{19}$ FUP, FVO and 3D7 alleles of the recombinant proteins used should reflect the circulating alleles at the time. Additionally, antibodies to $MSP1_{19}$ haplotypes are thought to be broadly cross-reactive (55). The frequency of AMA1 alleles was not measured for this cohort. However, a study conducted in 2000 measured Pf AMA1 haplotype frequencies in nearby Kilifi, found that there were78 unique haplotypes in the area, but that antibodies to AMA1 3D7, AMA1 FVO, and AMA1 HB3 were highly correlated (70). Thus using the AMA1 3D7 and FVO alleles in the assays should reflect the circulating alleles at the time of this study.

IgG antibody prevalence in the longitudinal infant cohort plasma

Examples of raw data results for the various antibody assays over time are shown in Supplementary Figure 1. To visualize the complex patterns of antibody responses more clearly, we plotted the probability of the presence of each response over time using restricted cubic splines. Figure 1 illustrates the probability of detecting serologically measured antibody responses over time. In general, maternal antibodies against each recombinant antigen measured in cord blood waned to a nadir by 6 to 9 months of age. The probability of having IgG antibodies to each antigen then increased over time, and generally returned to the prevalence observed in cord blood by 36 months of age. Antibodies to AMA1 (3D7 and FVO) were of the highest magnitude in cord blood and did not wane as rapidly as other antibodies. Antibodies to PfCelTOS and SERA5 were essentially absent from cord blood, with infants and young children gradually acquiring IgG antibodies to these antigens over 36 months.

VSA antibodies waned by 6-9 months of age and were not (re)acquired during infancy and early childhood (Figure 2A). W2mef GIA, though not highly prevalent in cord blood, waned by 6 months of age and subsequently (re)appeared at a low rate while D10 GIA had a

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consistently negligible prevalence (Figure 2B). Sial Dep IIA prevalence was overall higher than MSP1-19 IIA, but both were low throughout infancy (Figure 2C).

We used a generalized estimation equation regression model to estimate the rate of change in the probability of the presence of each antibody response over time using cord blood antibody levels as the baseline or comparator group. Due to the presence of nonlinear relationships in the curves, segmented linear spline terms were used to provide separate estimates of the odds ratios per one month change in age within the first 6 months after birth and after 6 months of age. The exception for this analysis was for serologically measured AMA1 antibodies, where we used 12 months of age as the cutoff. The magnitude of cord blood antibodies against AMA1 was high and infant catabolism of these reached a nadir at 12 months; thus we compared the rate of waning to the rate of aquistion based around this time point. Additionally, we tested whether there was a significant difference between rates of change of antibody responses before and after 6 months of age. Table 2 presents this analysis for each antibody response as it relates to age ≤ 6 months and > 6 months. As an example, during the first 6 months after birth, each month there was an associated 25% odds reduction in the presence of EBA181 antibodies (Odds Ratio 0.75, 95%CI 0.68-0.82; p <0.001), while after 6 months of age, each month there was an associated 6% higher odds for the presence of EBA181 antibodies (Odds Ratio 1.06, 95%CI 1.03-1.08; p <0.001). Similar results were observed for the other serologically measured antibody responses. With regards to VSA, taking VSA BFD 2006 reactive IgG as an example, during the first 6 months after birth, each month of age was associated with a 24% reduced odds of detecting this antibody (Odds Ratio 0.76, 95%CI 0.69-0.84; p <0.001), and after 6 months of age this odd did not significantly increase (Odds Ratio 1.01, 95%CI 0.99-1.03; p =0.33). Similar results were observed for the other Pf isolates. In general, serologically measured responses waned by 6-12 months of age and subsequently increased to reach their highest prevalence by 36 months. In contrast, VSA, GIA, and IIA antibodies waned by 6-9 months of age and were not acquired to any great extent

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during infancy and early childhood. Supplemental Figure 2 illustrates the difference in acquisition/dynamics of serologically measured antibodies vs. VSA, GIA, and IIA antibodies.

Based on the antibody dynamics observed, a generalized estimation equation regression model was used to assess if the mean rate of change in the probability of having serologic anti-malaria antibodies after 12 months was different than the mean rate of change in the probability of detecting VSA, GIA, and IIA antibodies. Averaging over 13 serological antibody responses, the probability of detecting antibodies increased significantly after 12 months of age. Each month of age was associated with a 5% higher odds of detecting the antibodies (Odds Ratio 1.05, 95%CI 1.02-1.08; p =0.002). Averaging over the seven VSA, GIA, and IIA antibody responses, the probability of detecting antibodies did not change significantly over time after 12 months (Odds Ratio 0.99, 95%CI 0.98-1.00; p =0.16). The mean rate of change in the probability of detecting 13 serological antimalarial antibody responses after 12 months was significantly different than the mean rate of change in the probability of detecting 7 VSA, GIA, and IIA antibodies after 12 months (p <0.001). Thus, infants acquired serologically measured antibodies but not VSA, GIA, and IIA antibodies after 12 months of age.

While the prevalence of serologic antibody responses at 36 months of age was similar to cord blood prevalence, the magnitude of antibody responses was considerably lower. Cord blood levels of antibodies against $MSP1_{42}$ (3D7, FVO, FUP), AMA1 (3D7, FVO), LSA1, EBA175, SE50, and CSP were significantly higher than in 36 month old young children (Figure 3A and 3B). The levels of the three VSA antibodies were also significantly higher in cord blood than in 36 month old young children (Figure 3C). The GIA/IIA antibodies, however, were low in both groups (Figure 3D). The only exception to this trend was serologically measured antibodies against PfCelTOS that, in cord blood, had a median of 1 whereas the 36 month infants had a median of 3.1 fold increase relative to malaria naïve North American negative controls (p<0.0001, Mann Whitney test, Figure 3B). It is unknown whether this increase has

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biological relevance, although it is noted that the magnitude of response is considerably lower compared to other antigens.

Fetal sensitization to malaria and acquisition of IgG antibodies

We hypothesized that newborns that were sensitized to malaria antigens *in utero* would have antibody responses to multiple antigen targets of broader diversity (serologic and functional) at earlier time points during infancy compared to newborns that were not sensitized. In this cohort, 76 infants had complete sensitization and antibody data. Thirty neonates were classified as "sensitized" and 46 neonates were classified as "not sensitized" based on CBMC recall responses to malaria antigens as measured by IFN-γ ELISPOT and cytokine production. We found no statistical difference in either the prevalence or magnitude of IgG antibodies in cord blood regardless of "sensitized" or "not sensitized" categorization. Examination of longitudinal data revealed no difference in the rate of change (waning or acquisition) for any antimalarial antibodies between sensitized and not sensitized infants/young children over time (Supplemental Table 1). Thus, fetal sensitization to malaria antigens did not affect subsequent infant acquisition of any antimalarial antibody measured in this cohort.

Infants with serologically measured antimalarial antibody responses were more likely to incur Pf infection

We examined the association between cord blood and infant antibodies and the risk of Pf infection. The first occurrence of infection was measured by PCR, blood smear, and/or ≥6 positive antimalarial IgM responses to the 13 tested antigens. IgM positivity was used as a marker of recent infection. If an infant had IgM antibodies at one time point, invariably it was absent at the following time point, as has been demonstrated by others (6, 10, 11). Thirty infants had Pf infections detected by 36 months of age. Infections were detected in seven infants younger than 12 months of age. With respect to the prevalence of maternal antimalarial IgG antibodies in cord blood, there was no difference between infants who incurred malaria infections during the entire follow up period and those who did not. The paucity of malaria

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infections in infants younger than 12 months prohibited further analyses regarding sensitization status or characterization of antibody responses.

To exclude confounding maternal antibody responses, we examined the risk of Pf infection after 12 months of age as related to infant antibody responses at the 12 month time point. Sixty-seven infants had 12 month antibody data. Within this subset, malaria infections were detected in 17 infants in the subsequent 24 months of follow-up. Using a Cox proportional hazards regression model, we found that infants with serologically measured antibodies at 12 months were more likely to incur malaria infections than infants who were seronegative (Table 3). Specifically, 12 month old infants who had IgG antibodies to CSP, SERA5, MSP1-42 (FVO), MSP1-42 (FUP), EBA140, EBA175, AMA1 (3D7) or AMA1 (FVO) had a statistically significant increased risk of infection (hazard ratio range 2.64-6.21) compared to infants with negative serology at 12 months of age. No increased risk was associated with VSA/GIA/IIA antibodies at 12 months, though the prevalence of these antibodies was low.

Discussion

Early infancy is a critical time in the development of immunity to malaria in children born in malaria endemic areas. Whereas a relative degree of protection from Pf infection and symptomatic malaria is thought to exist from birth to approximately 6 months of age (4-6, 71-73), subsequent exposure to mosquito borne transmission during early infancy is accompanied by the absence of *de novo* synthesized fetal hemoglobin and the catabolic loss of maternal IgG antibodies that have passed from the maternal to fetal circulation during the last trimester of pregnancy. More generally, the infant immune system is immature until at least 2 years of age (74). Our prospective study of infants born in a malaria endemic area of coastal Kenya from 2006 to 2009 was performed to understand in more detail the interplay between the loss of malaria antigen-specific maternal malarial antibodies present at birth and the subsequent acquisition of infant antibodies that result from natural exposure to Pf. In addition to measuring

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411 serologic IgG antibodies to Pf antigens from birth through early infancy, as several other studies
412 have reported (6, 12, 75), we performed several assays that reflect functional antibody
413 responses that include antibody binding to VSAs expressed on the surface of Pf infected
414 erythrocytes, GIA, and IIA specific for MSP1 and sialic acid dependent erythrocyte invasion by
415 merozoites. The main conclusions from our study indicate that i) serologic measures of maternal
416 IgG antibodies to pre-erythrocytic and blood stage antigens wane by 6 months after birth and
417 reappear over the following 24 to 36 months as a consequence of natural malaria exposure; ii)
418 increased levels of serologic antibodies at 12 months of age are predictive of an increased
419 subsequent risk of Pf infection; iii) VSA and functional antibody responses mediated by maternal
420 antibodies in cord blood disappear within six months after birth and, unlike serologically
421 determined antibodies, remain low up to 36 months of age; iv) *in utero* sensitization to Pf is not
422 associated significantly with enhanced antibody responses following the loss of maternal IgG
423 antibodies.

424 Our observations related to serologic maternal malaria IgG antibodies present at birth
425 (Table 1) indicate that antigenic targets of such antibodies are expressed by both pre-
426 erythrocytic (CSP, LSA1, PfCeltos) and blood stage parasites (e.g. MSP1, EBA140, EBA175,
427 EBA181, AMA1, SERA5). These antibodies decreased significantly by six months after birth,
428 and then gradually increased up to age 36 months. Maternal IgG antibodies detectable in cord
429 blood have previously been reported to be directed against ring-infected erythrocyte surface
430 antigen (RESA), CSP, MSP1-19, MSP3, AMA1, EBA175 and glutamate rich protein (GLURP)
431 (6, 11-13, 75). Studies of other birth cohorts in sub-Saharan Africa have reported the waning of
432 maternal malarial IgG antibodies by six to nine months of age. Following this loss of maternal
433 antibodies, the level of serologically detectable malaria specific IgG antibodies gradually
434 increased up to 36 months (Figure 1). However, these newly acquired antibodies were not
435 associated with protective immunity but an increased prospective risk of infection, most likely
436 due to increased exposure. Elevated levels of malaria IgG antibodies has previously been

found to be a biomarker of increased malaria risk during early infancy in a birth cohort study from Ghana (6). Although we did not compare antibody levels in young infants with those of older children in the Kenyan study population described here, a recent study by Stanisic et al (14) did so in cohorts of 1-4 and 5-14 year old Papua New Guinean children. Results of this study in PNG indicate that one of the reasons why antibody responses in young infants represent biomarkers of malaria exposure rather than protection from malaria is related to failure of antibody responses to reach a critical "protective" level, as determined by serology, until age 4 years or older. Mathematical models of antibody half-lives in cohorts of younger and older African children suggest that antibodies in younger children have shorter half-lives than those of older children, and that this difference in half-lives may be related to differing populations of long lived and short lived antibody secreting cells in the two age groups (76). Although not measured in this study, IgG subclasses may affect the longevity of circulating antibodies. In general, malaria infection induces predominantly IgG1 and IgG3 isotypes to various Pf specific antigens (14, 52, 77-81). In contrast to serologic measures of antibody responses, our data indicate that functional assays of antibody activity are, overall, weak at birth, decrease by 6 months, and do not reappear by 36 months of age (Figure 2). We have previously shown in different infants examined from the same cohort that GIA (D10, 3D7, W2mef, Msam 06) decreased in infants over time until 12 months of age (30). In the present study, only W2mef GIA increased transiently at 18 months of age, but had low prevalence by 36 months of age. This indicates that, if boosted, the resultant antibodies were short lived in these 18 month old young children. It may be that in this infant cohort, antibodies to merozoite antigens did not reach sufficiently high levels to mediate substantial invasion-inhibitory activity. Prior studies have suggested that GIA antibodies are not readily boosted by increasing exposure to malaria (15, 27). However, in young children GIA antibodies showed some association with malaria exposure transmission level (27). VSA antibodies were moderately prevalent (47-69%) in cord blood indicating that mothers, who were children themselves during

463 a time of higher malaria transmission in coastal Kenya (34), had VSA antibodies transferred to
464 their fetuses in the third trimester. This infant cohort with lower malaria exposure, on the other
465 hand, failed to develop much VSA antibody. Of note, a trend toward higher prevalence of VSA
466 antibodies to BFD06, a parasite taken from a patient with severe malaria, was noted in infants
467 approximately 24 months of age. Others have proposed that children develop VSA antibodies
468 to parasites expressing VSA associated with severe disease earlier in childhood than VSA
469 associated with mild or moderate disease (82). These findings are similar to those of
470 Vestergaard et al (21) who showed that infants residing in a low malaria transmission region of
471 Tanzania had low prevalence and magnitude of VSA antibodies compared to infants residing in
472 regions of high transmission. Nhabomba et al. (11) also found a lack of VSA antibody
473 acquisition in infants up to two years of age in Mozambique. Conversely, the parasite isolates
474 used in our study may not have been an accurate representation of the circulating parasites
475 from the region, despite one isolate coming from a child with non-severe acute malaria from this
476 study cohort. Antibodies to VSA are known to be highly isolate-specific among children (83,
477 84), and may be short-lived (85). Therefore, the prevalence of antibodies to any one isolate may
478 be low in young children, as we found here. With respect to antibodies that function to impair
479 merozoite invasion of erythrocytes, we used two assays that assess antibodies to the 19 kD C
480 terminal region of MSP1 and antibodies that target sialic acid dependent invasion pathways (16,
481 32). MSP1 is involved with the initial low affinity binding of the merozoite to the erythrocyte, with
482 the MSP1-19 portion of the cleaved MSP1 being retained as the merozoite invades (86). A
483 secondary interaction is then required with ligands of the EBA family and Pf reticulocyte-binding
484 homologs (PfRh) proteins (87). The variable expression of these proteins facilitates the
485 merozoite invading through roughly grouped phenotypes labeled “sialic acid dependent” or
486 “sialic acid independent”, and variation in their use facilitates evasion of acquired antibodies
487 (32). W2mef generally invades through a sialic acid dependent pathway. When EBA175 is
488 genetically deleted (W2mefΔEBA175), it invades through a sialic acid independent pathway.

Thus, plasma that contains antibodies that bind to EBA175, and other ligands of sialic acid dependent invasion, may inhibit the invasion of W2mef but not W2mefΔEBA175 parasites. EBA140, EBA181 and PfRh1 may also participate in the sialic acid dependent pathway (32). With respect to both MSP1-19 and sialic acid dependent IIA, we found that antibodies with these activities were low to negligible at birth and were not detectable at 36 months. These findings are discordant with serology, as both $MSP1_{42}$ and EBA175 antibodies were detectable at birth and progressively increased in infants between age 12 and 36 months. This could be explained by antibodies to merozoite antigens being at levels below a threshold concentration to effectively inhibit invasion, or antibodies targeting non-functional epitopes. This discordance between serologic and functional measures of antibody responses has been described in other studies (31, 88), and highlights the challenge of validating *in vitro* assays relevant to malaria pathogenesis *in vivo*. Recent studies have identified several such potential functional assays that include evaluating antibodies that opsonize merozoites for phagocytosis or fix complement to inhibit invasion and lyse merozoites (89, 90). Additionally, competitive ELISAs for EBA175 (91) and refined assays examining AMA-1 complex responses (92) are in development.

With respect to individual covariates that might impact the acquisition of antibody responses by young infants, we examined the relationship between *in utero* sensitization to malaria and post-natal serologic and functional antibody responses. Studies we conducted in this area of coastal Kenya from 2000 to 2003 have shown that 45-60% of newborns were sensitized *in utero* as determined by the cord blood T cell cytokine responses to malaria antigens(35). This sensitization was associated with rapid acquisition of MSP1-19 IIA relative to newborns that were not sensitized (88). However, in the 2006-2009 birth cohort reported here, we found no association of *in utero* T cell sensitization and accelerated development of antibody responses in young infants. We speculate that this discrepancy is likely related to changes in malaria exposure during the two different time periods. While transmission was stable and

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relatively high from 2000 to 2003, it decreased significantly from 2007 to 2009 as a result increasing use of insecticidal bed nets and other public health interventions (34).

In conclusion, results of our study highlight several issues pertinent to the development of naturally acquired immunity during the first three years after birth. Firstly, while serologic measures of malaria antigen specific antibodies are clearly indicative of exposure to Pf, they are unlikely to be relevant to protective immunity as opposed to malaria exposure in young infants. In this context, a limitation of our study is that we did not perform active surveillance for symptomatic malaria, and thus our results can only be linked with susceptibility to Pf infection. Secondly, results of various birth cohort studies may vary according to prevailing levels of malaria endemicity during gestation, e.g. maternal malaria exposure and maternal antibodies transferred to the fetus, as well as malaria exposure experienced by infants after the waning of maternal antibodies. Thirdly, our results underscore the need for additional functional antibody assays, e.g. phagocytosis of antibody opsonized merozoites and complement fixation, that have been found to correlate with protection from symptomatic malaria (89, 90). Achieving the latter goal will be challenging given the likely complexity and redundancy of host immune and non-immune mechanisms underlying naturally acquired immunity to malaria.

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881 Table 1: Magnitude and prevalence of IgG antibodies in cord blood

Assay/Antigen tested/Pf Isolate	Sample size	Mean(SD)/ Median(IQR)	Frequency Positive (Percentage)
Serology (fold increase in MFI over negative controls)			
LSA1	78	24.7(42.6) 4.7(1.6,27.8)	59 (75.6%)
CSP	78	29.5(39.7) 11.4(3.3,40.0)	68 (87.2%)
PfCelTOS	78	0.6(1.4) 0.0(0.0,0.0)	15 (19.2%)
SE50	78	0.6(2.1) 0.0(0.0,0.0)	11 (14.1%)
SE36	78	3.1(6.9) 0.0(0.0,2.6)	34 (43.6%)
$MSP1_{42}$ (3D7)	78	9.3(11.9) 5.8(0.0,12.0)	58 (74.4%)
$MSP1_{42}$ (FVO)	78	6.5(9.5) 2.3(0.0,10.6)	46 (59.0%)
$MSP1_{42}$ (FUP)	78	12.2(13.8) 6.8(2.4,16.6)	68 (87.2%)
EBA140	78	8.7(19.6) 1.7(0.0,7.3)	41 (52.6%)
EBA175	78	9.9(14.7) 3.7(0.0,14.5)	49 (62.8%)
EBA181	78	9.6(17.9) 2.7(0.0,8.6)	51 (65.4%)
AMA1 (3D7)	78	81.0(46.4) 88.1(48.4,101.6)	76 (97.4%)
AMA1 (FVO)	78	96.7(61.2) 113.9(39.5,131.2)	76 (97.4%)
Variant Surface Antigen Assay (Geometric mean MFI)			
BFD 2006	79	40.2(56.4) 17.0(11.0,46.0)	42 (53.8%)
Msambweni 2006	79	37.4(40.9) 21.0(13.0,44.0)	54 (69.2%)
3D7	79	24.2(28.0) 13.5(8.0,30.0)	37 (47.4%)
Growth and Invasion Inhibition Assays (Percent Inhibition)			
Sialic Acid Dep IIA	84	2.8(7.5) 0.0(0.0,0.0)	12 (14.6%)
W2mef GIA	84	11.3(15.6) 6.0(0.0,19.6)	31 (37.8%)
MSP1-19 IIA	84	0.8(2.7) 0.0(0.0,0.0)	5 (6.1%)
D10 GIA	84	4.2(8.5) 0.0(0.0,4.6)	18 (22.0%)

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884 Table 2: IgG antibody rates of change before and after 6 months of age

Assay/Antigen tested/Pf isolate	Time period	Odds Ratio (95%CI) / 1 month	p	P difference between rates of change [a]
Serology				
LSA1	Before 6 months [b]	0.72 (0.65,0.80)	**<0.001**	**<0.001**
	After 6 months [c]	1.04 (1.02,1.07)	**<0.001**	
CSP	Before 6 months	0.64 (0.57,0.72)	**<0.001**	**<0.001**
	After 6 months	1.04 (1.02,1.06)	**<0.001**	
PfCelTOS	Before 6 months	1.16 (1.04,1.28)	**<0.001**	0.08
	After 6 months	1.05 (1.03,1.07)	**<0.001**	
SE50	Before 6 months	1.20 (1.07,1.34)	**0.0025**	**0.03**
	After 6 months	1.04 (1.02,1.06)	**<0.001**	
SE36	Before 6 months	0.88 (0.80,0.98)	**0.01**	**0.003**
	After 6 months	1.04 (1.03,1.06)	**<0.001**	
$MSP1_{42}$ (3D7)	Before 6 months	0.75 (0.68,0.82)	**<0.001**	**<0.001**
	After 6 months	1.04 (1.02,1.06)	**<0.001**	
$MSP1_{42}$ (FVO)	Before 6 months	0.79 (0.73,0.87)	**<0.001**	**<0.001**
	After 6 months	1.05 (1.03,1.08)	**<0.001**	
$MSP1_{42}$ (FUP)	Before 6 months	0.66 (0.58,0.74)	**<0.001**	**<0.001**
	After 6 months	1.05 (1.03,1.08)	**<0.001**	
EBA140	Before 6 months	0.85 (0.77,0.92)	**<0.001**	**<0.001**
	After 6 months	1.06 (1.04,1.09)	**<0.001**	
EBA175	Before 6 months	0.76 (0.70,0.84)	**<0.001**	**<0.001**
	After 6 months	1.05 (1.03,1.07)	**<0.001**	
EBA181	Before 6 months	0.75 (0.68,0.82)	**<0.001**	**<0.001**
	After 6 months	1.06 (1.03,1.08)	**<0.001**	
AMA1 (3D7)	Before 12 months	0.76 (0.71,0.81)	**<0.001**	**<0.001**
	After 12 months	1.03 (1.01,1.06)	**0.02**	
AMA1 (FVO)	Before 12 months	0.79 (0.75,0.83)	**<0.001**	**<0.001**
	After 12 months	1.04 (1.01,1.07)	**0.003**	
Variant Surface Antigen Assay				
BFD 2006	Before 6 months	0.76 (0.69,0.84)	**<0.001**	**<0.001**
	After 6 months	1.01 (0.99,1.03)	0.33	
Msambweni 2006	Before 6 months	0.60 (0.53,0.68)	**<0.001**	**<0.001**
	After 6 months	1.02 (0.98,1.05)	0.35	
3D7	Before 6 months	0.63 (0.55,0.73)	**<0.001**	**<0.001**
	After 6 months	1.01 (0.97,1.05)	0.63	

Assay/Antigen tested/Pf isolate	Time period	Odds Ratio (95%CI) / 1 month	p	P difference between rates of change [a]
Growth and Invasion Inhibition Assays				
Sialic Acid Dep IIA	Before 6 months	1.18 (1.04,1.34)	**0.01**	**0.02**
	After 6 months	0.98 (0.93,1.04)	0.56	
W2mef GIA	Before 6 months	0.77 (0.68,0.89)	**<0.001**	**<0.001**
	After 6 months	1.05 (1.00,1.10)	0.07	
MSP1-19 IIA	Before 12 months	1.03 (0.94,1.13)	0.53	0.31
	After 12 months	0.93 (0.81,1.08)	0.35	
D10 GIA	Before 12 months	0.86 (0.78,0.95)	0.004	0.07
	After 12 months	1.05 (0.92,1.19)	0.491	

[a] Difference between rates of change between birth to 6 (or 12) months of age and between 6 months of age and 36 months of age (Fisher's exact test). [b] Compared to cord blood responses. [c] Compared to 6 month responses.

889 Table 3: Association between the presence IgG antibodies at 12 months of age and first
890 occurrence of a malaria infection after 12 months of age.

Assay/Antigen tested	N	N Malaria Events	Hazard Ratio (95% Confidence Interval)	p
Serology				
LSA1	67	17	1.84(0.71, 4.77)	0.21
CSP	67	17	**2.64(1.00, 6.97)**	**0.05**
PfCelTOS	67	17	2.67(0.94, 7.61)	0.07
SE50	67	17	**4.51(1.46, 13.9)**	**0.009**
SE36	67	17	2.35(0.89, 6.19)	0.08
$MSP1_{42}$ (3D7)	67	17	1.90(0.73, 4.99)	0.19
$MSP1_{42}$ (FVO)	67	17	**4.37(1.52, 12.6)**	**0.006**
$MSP1_{42}$ (FUP)	67	17	**3.13(1.14, 8.57)**	**0.03**
EBA140	67	17	**3.27(1.19, 8.95)**	**0.02**
EBA175	67	17	**3.12(1.18, 8.25)**	**0.02**
EBA181	67	17	1.76(0.68, 4.56)	0.25
AMA1 (3D7)	67	17	**2.98(1.07, 8.33)**	**0.04**
AMA1 (FVO)	67	17	**6.21(1.67, 23.1)**	**0.006**
Variant Surface Antigen Assay				
BFD 2006	67	17	2.43(0.78, 7.56)	0.13
Msambweni 2006	67	17	1.00(0.13, 7.60)	1.00
3D7	67	17	2.45(0.56, 10.8)	0.23
Growth and Invasion Inhibition Assays				
Sialic Acid Dep IIA	65	17	1.64(0.60, 4.47)	0.34
W2mef GIA	65	17	1.52(0.49, 4.74)	0.47
MSP1-19 IIA	65	17	1.56(0.36, 6.84)	0.55
D10 GIA	65	17	1.49(0.20, 11.3)	0.70

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Figure Legends

Figure 1. Serological responses in infants over time. (A) Detection probability (y axis) of each antibody in infants over time (x axis, months). Responses to specific antigens are indicated in each plot box. **(B)** Magnitude of antibodies in cord blood for each indicated antigen expressed as fold increase relative to negative control North Americans (NAm) (mean + SEM).

Figure 2. Infant VSA and W2mef GIA antibodies over time. (A) Detection probability (y axis) of each VSA measured antibody response in infants over time (x axis, months). VSA responses to each Pf isolate are indicated. Bar graph to the right of the plot show the geometric mean fluorescence intensity (GeoMFI) for VSA antibodies in cord blood (mean + SEM). **(B)** Detection probability (y axis) of W2mef GIA antibody response in infants over time (x axis, months). Bar graph to the right of the plot shows the percentage of growth inhibition of W2mef GIA responses in cord blood (mean + SEM).

Figure 3. Antibodies in cord blood (open circles) compared to 36 month young children (open triangles). (A) Dot plots of serologic antibody responses (fold increase relative to North Americans (NAm)) to specified antigens measured in cord blood and 36 month old young children. **(B)** Dot plot of serologic antibody responses to another set of antigens measured in cord blood and 36 month old young children. Note the smaller y axis compared to **(A)**. **(C)** GeoMFI of VSA responses measured in cord blood and 36 month old young children. **(D)** Percent growth inhibition of GIA and IIA antibody responses measured in cord blood and 36 month old young children. (*p=0.04, **p=0.0005, ***p=0.0002, ****p<0.0001; horizontal bar when visible represents median values).