

A Malaria-Resistant Phenotype with Immunological Correlates in a Tanzanian Birth Cohort Exposed to Intense Malaria Transmission

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Abstract. Malaria incidence is highly heterogeneous even in areas of high transmission, although no conclusive evidence exists that innate or naturally acquired resistance can prevent infection over an extended period of time. This longitudinal study examined immunoparasitological evidence for a malaria-resistant phenotype in which children do not develop malaria despite an extended period of exposure to parasites. Within a birth cohort followed from 2002 to 2006 in Muheza, Tanzania, an area of intense transmission, children ($N = 687$) provided blood smears biweekly during infancy and monthly thereafter. Maternal and childhood characteristics were obtained, cord-blood cytokines were measured, and antibody responses were assayed as measures of stage-specific exposure. Sixty-three (9.2%) children had no blood smear-positive slides over 2 years of follow-up (range: 1–3.5 years) and were identified as malaria resistant. Malaria-resistant children were similar to other children with respect to completeness of follow-up and all maternal and childhood characteristics except residence area. Antibody seroprevalence was similar for two sporozoite antigens, but malaria-resistant children had a lower antibody seroprevalence to merozoite antigens merozoite surface protein 1 (5.4% versus 30.2%; $P < 0.0001$) and apical membrane antigen 1 (7.2% versus 33.3%; $P < 0.0001$). Malaria-resistant children had higher cytokine levels in cord blood, particularly interleukin-1 β . In summary, a subset of children living in an area of intense transmission was exposed to malaria parasites, but never developed patent parasitemia; this phenotype was associated with a distinct cytokine profile at birth and antibody profile during infancy. Further research with malaria-resistant children may identify mechanisms for naturally acquired immunity.

INTRODUCTION

Malaria is a major cause of mortality in Africa with an estimate of nearly 438,000 deaths per year, over 90% of which occur in children < 5 years of age.¹ In areas of high transmission, the burden of severe malaria is greatest during late infancy, whereas mild malaria episodes occur throughout childhood.^{2,3} The increased risk in young children has been ascribed to an underdeveloped immune response once maternal antibodies wane^{2,4}; however, the mechanisms of protective immunity are not yet clear.² While malaria infection and morbidity are highly heterogeneous in a community, even within areas of intense transmission,^{5,6} no conclusive evidence exists that innate or naturally acquired resistance can prevent infection.^{2,7,8} Variations in susceptibility to *Plasmodium falciparum* among children remain largely unexplained.⁹

Several studies have recently demonstrated overdispersion in the number of clinical episodes of malaria, whereby a subgroup of children bore a much higher burden of malaria than other children living in similar conditions.^{5,10,11} Conversely, studies have rarely explored characteristics of children on the other end of the distribution among whom, despite similar malarial conditions, episodes of clinical malaria or positive blood smears were rare or nonexistent. Such subgroups have been previously identified in various transmission settings in sub-Saharan Africa.^{5,6,8,10,12} The aim of this study was to identify immunological markers of a subset of children

with a potential malaria-resistant phenotype from a longitudinal birth cohort study in northeast Tanzania.

MATERIALS AND METHODS

The Mother–Offspring Malaria Study (MOMS) project was a longitudinal birth cohort study in Muheza District, Tanzania, conducted between 2002 and 2006 in an area of intense malaria transmission.¹³ The estimated entomological inoculation rate in this area around the time of the study was 400 infective mosquito bites per year,¹⁴ the cumulative incidence of malaria infection in this cohort was 2.4 infections per child-year, and the median age at the first severe malaria episode was 38.7 weeks.³ MOMS population and data collection details have been published previously.^{3,13,15} Briefly, healthy mothers (no chronic or debilitating illness; age range: 18–45 years) admitted to Muheza Designated District Hospital for delivery were enrolled and provided written informed consent. Exclusion criteria for this analysis of the birth cohort included stillbirth, twin birth, human immunodeficiency virus (HIV) infection, sickle cell disease, and early neonatal death. Children in the birth cohort ($N = 882$) were examined at birth, every 2 weeks during the 1st year and every 4 weeks thereafter. Sick children were monitored by physicians at the hospital or by weekly surveillance by mobile clinics.¹⁵ Those children who were followed for at least a year (≥ 52 weeks) ($N = 687$) comprised the analytic population for this study (mean follow-up: 2.4 years [range: 1.0–3.7 years]). This study was approved by the Tanzanian (National Institute for Medical Research, Medical Research Coordinating Committee) and U.S. (Western Institutional Review Board) ethical review boards.

Trained nurses administered questionnaires to mothers, and placental and fetal cord blood samples were collected

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immediately after delivery as previously described.^{13,16} At each follow-up visit, participants were seen in the clinic or by village health workers. Infant blood smears were collected via finger- or heel-prick at all routine visits and at the time of any illness. Venous blood was collected at 3 and 6 months and then approximately every 6 months thereafter. Children with parasitemia who developed symptoms of malaria were treated by a study clinician.

Laboratory methods. Blood smears from placental and peripheral blood were stained in 10% Giemsa, washed, and then examined using light microscopy at 100× magnification.¹³ Parasitemia was defined as any parasite detected after counting at least 200 white blood cells. Ten thousand red cells were examined in the thin smear before determining that the slide was parasite negative. For the thick blood smear, 100 high-power fields were examined before concluding that a peripheral blood slide was negative.

Yeast-expressed merozoite surface protein 1 (MSP1_QTSP) was obtained from MR4 (Manassas, VA), and Nathalie Scholler of the Fred Hutchinson Cancer Research Center kindly provided yeast-expressed human mesothelin as a negative control protein.

We used *Escherichia coli* Rosetta host strains and pET28 vector under T7 promoter (Novagen, Billerica, MA) to express full-length human dihydrofolate reductase (DHFR) (hDHFR; amino acids [aa] 1–187) as negative control protein, as well as *P. falciparum* strain 3D7 antigens circumsporozoite protein (CSP) (PlasmoDB.org gene ID PF3D7_0304600/PFC0210c; aa 21–371) and apical membrane antigen 1 (AMA1) (PF3D7_1133400/PF11_0344; aa 24–543), as 6X His-tagged recombinant proteins. All proteins were purified to > 90% purity on Ni-NTA His•Bind® Resin per manufacturer's instructions (Novagen) based on Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels; hDHFR and AMA1 were purified from inclusion bodies, and CSP was purified from both inclusion bodies and the soluble fraction. All proteins from inclusion bodies were refolded using a column-refolding strategy, with on-column refolding using serial reduction of urea concentration from 8 M to 0 M to yield soluble protein.

We used wheat germ extract and EU-E01-His-TEV-MCS vector under SP6 promoter (CellFree Sciences, Matsuyama, Ehime, Japan) to express human DHFR (hDHFR; aa 3–188) as negative control protein, as well as *P. falciparum* strain 3D7 antigens sporozoite and liver stage asparagine-rich protein/sporozoite asparagine-rich protein 1 (SLARP/SAP1) (PF3D7_1147000/PF11_0480; aa 2744–2935) and liver specific protein 1 (LISP1) (PF3D7_1418100/PF14_0179; aa 1631–2002), as 6X His-tagged recombinant proteins. All proteins were expressed at large scale using the CellFree Sciences system, then purified with Ni-NTA His•Bind Resin per manufacturer's instructions (Novagen); anti-His antibody Western blots of proteins separated on SDS-PAGE gels were performed to confirm purification of recombinant protein of appropriate size.

Reactivity of plasma samples collected at weeks 24, 48, 76, 100, and 124 was measured by Luminex analysis as previously reported.¹⁷ Briefly, Luminex microspheres of varying fluorescence intensity were covalently coupled with different antigens according to the manufacturer's protocol, then lyophilized, sealed under nitrogen gas, and stored at

–80°C. At the time of assay, microspheres were reconstituted, diluted, and dispensed into wells on a 96-well microtiter plate, to which diluted plasma samples were added. After incubation and washing, microspheres were analyzed on a BioPlex 200 instrument (Bio-Rad, Irvine, CA) to measure IgG antibody responses to various antigens, expressed as median fluorescence intensities after subtracting reactivity to control protein. Data were transformed using the natural log due to skewness, and seroreactivity was defined as an antibody level greater than the mean + 2 × standard deviation of nonimmune control volunteers (U.S. adults, AB sera, $N = 20$).

As described previously, cytokine assays were performed on thawed plasma samples using a multiplex, bead-based platform (BioPlex; Bio-Rad) and custom-made assay kits.¹⁶ All analytes for a single sample were performed on the same day, and levels were adjusted to account for dilution in anti-coagulant. Detection limits for these analytes were interleukin (IL)-1 β , 0.01 pg/mL; IL-5, 0.02 pg/mL; IL-6, 1.45 pg/mL; IL-10, 0.02 pg/mL; tumor necrosis factor (TNF)- α , 0.10 pg/mL; TNF-RI, 1.58 pg/mL; TNF-RII, 0.21 pg/mL; and interferon (IFN)- γ , 0.04 pg/mL.

Maternal anemia was defined as hemoglobin level of < 11 g/dL, whereas fetal anemia was defined as cord blood hemoglobin level of < 12.5 g/dL.¹⁸ Childhood anemia was defined as Hgb < 8 g/dL and iron deficiency was defined as ferritin level < 30 ng/mL when C-reactive protein was < 8.2 ng/mL, or ferritin level < 70 ng/mL when C-reactive protein level was > 8.2 μ g/mL.¹⁵ Assays for hemoglobin, ferritin, C-reactive protein, sickle cell trait, and α -thalassemia have been detailed previously.^{15,16}

Data analysis. To be considered malaria resistant, a participant had to have been followed for at least 1 year and to never have had a detectable parasitemia by blood smear. Malaria-resistant children were then compared with children who were also followed for at least a year, but who had at least one positive blood smear during follow-up. Comparisons between these groups were tested using χ^2 tests for categorical variables and Student's t tests or Mann–Whitney tests for continuous variables. Antibody seroprevalence comparisons were assessed through generalized estimating equations (GEE) to account for within-person repeat measurements (binomial distribution, autoregressive correlation). Tertiles were calculated for each cord cytokine level based on the distribution in the whole cohort, with those values below the detection limit placed in the lowest tertile. Multivariate logistic regression was used to estimate associations between cytokine levels and malaria-resistant status controlling for covariates. Sensitivity analyses included running final models within participants residing only around Muheza Township, and after including village location as a clustering variable in GEE logistic models. Statistical analysis was done with SAS 9.3 (SAS Institute Inc., Cary, NC). Statistical significance was assessed at $P < 0.05$.

RESULTS

Overall, 63 (9.2%) children were determined to be malaria resistant (Table 1). These children had no positive blood smears over an average of 2 years of follow-up (range: 1–3.5 years), for a total of 125 person-years. Malaria-resistant children were similar to nonresistant children in terms of completeness of follow-up and mean number of

TABLE 1

Clinical characteristics of malaria-resistant and nonresistant children, the Mother–Offspring Malaria Study, 2002–2006

Characteristic	Malaria resistant	Nonresistant
Sample size	63	624
Mean duration of follow-up (range), weeks*	103.0 (52–184)	126.6 (52–192)
Median (IQR) time (weeks) to first infection	NA	30 (18–52)
Blood smear positive by 6 months	NA	277 (44.4%)
Blood smear positive by 12 months	NA	471 (75.5%)
Blood smear positive by 18 months	NA	548 (87.8%)
Blood smear positive by 24 months	NA	588 (94.2%)
Number (%) of gaps in routine follow-up†	137 (5.4%)	1,610 (4.9%)
Median time (range) for gap in follow-up, year 1 (weeks)	4.0 (3–8)	4.0 (3–22)
Median time (range) for gap in follow-up, year 2+ (weeks)	8.0 (5–12)	7.0 (5–48)
Mean number (95% CI) of BSneg walk-in visits	5.0 (4.0, 5.9)	4.7 (4.4, 5.0)
Mean number (95% CI) of BSneg visits with fever ($\geq 38^{\circ}\text{C}$)	1.4 (1.0, 1.9)	1.2 (1.1, 1.3)
Mean number (95% CI) of BSneg visits with diarrhea	0.7 (0.5, 0.9)	0.8 (0.7, 0.9)
Number (%) ever used malaria medication at study visit	3 (4.8%)	594 (95.2%)
Number (%) ever used malaria medication at home	4 (6.4%)	125 (20.0%)

BSneg = blood smear negative; CI = confidence interval; IQR = interquartile range.

* $P < 0.05$.

†When the time between visits was > 2 weeks in the 1st year and > 4 weeks in the following years, then it was counted as a gap in follow-up.

visits with nonmalaria fever and diarrhea. Malaria-resistant children had a shorter duration of follow-up compared with nonresistant children (103.0 weeks [52–184 weeks] versus 126.6 weeks [52–192 weeks]; $P < 0.0001$). Since a large majority of nonresistant children (75%) acquired their first parasitemia episode during infancy, this duration difference most likely did not lead to misclassification.

Malaria-resistant children were similar to nonresistant children for most maternal and childhood characteristics including birth season, bednet use, and hemoglobin AS genotype (Table 2). A higher proportion of malaria-resistant children were living around Muheza Township versus surrounding villages. Muheza Township residents did not differ from residents in other villages in terms of completeness or duration of follow-up, season of birth, or fetal anemia (data not shown). Bednet use among mothers from around Muheza Township (62.2%) was lower than use in Magila-Mkumbaa village (73.2%), and was greater than use in Bwembwera, Potwe (40.0%). As reported previously,³ children living around Muheza Township were less likely to develop severe malaria; however, average parasite density during infection did not differ between individuals living in villages versus the township.

Longitudinal serological data were available through 124 weeks for both malaria-resistant children (53 participants, 111 total measures, median = 2 measures [range: 1–5] per person) and nonresistant children (553 participants, 1,194 measures, median = 2 measures [range: 1–7] per person) (Table 3). Adjusting for within-child correlations over time (GEE), antibody seroprevalence was not significantly different between malaria-

resistant children and nonresistant children for CSP and SLARP antigens. Nonresistant children had a higher overall antibody seroprevalence to LISP1 than malaria-resistant children ($P = 0.008$). Seroprevalence of preerythrocytic-stage antigens were greater at week 100+ than they were at week 48 for both groups. After removing the antibody data from week 24 which may be affected by maternal antibodies,^{2,4} among nonresistant children, the differences between week 48 and week ≥ 100 were significant for SLARP (27.9% versus 44.0%; $P < 0.0001$), LISP1 (17.1% versus 39.6%; $P < 0.0001$), and CSP (32.4% versus 41.2%; $P = 0.003$). Among malaria-resistant children, these increases with time were of similar magnitude but were not significant likely due to the smaller sample size (Table 3).

Malaria-resistant children had a lower antibody seroprevalence to MSP1 (5.4% versus 30.2%; $P < 0.0001$) and AMA1 (7.2% versus 33.3%; $P < 0.0001$) antigens compared with nonresistant children. Seroprevalence to the merozoite antigen AMA1 increased over time (week 48: 25.4% versus week ≥ 100 : 36.5%; $P < 0.002$) in nonresistant children but not in malaria-resistant children, whereas MSP1 fluctuated over the course of the study for all participants. Preerythrocytic and merozoite antigen seroreactivity analyses were rerun after removing those visits in which nonresistant children had

TABLE 2

Maternal and child characteristics by malaria-resistant status, The Mother–Offspring Malaria Study, 2002–2006

Characteristic	Malaria resistant	Nonresistant
Maternal factors		
Maternal age, years	26.8	26.3
Placental malaria	5 (7.9%)	87 (13.9%)
Primigravid mother	10 (15.9%)	170 (27.2%)
Maternal anemia	31 (58.5%)	269 (52.3%)
Maternal IPT use	51 (86.4%)	520 (88.1%)
Village*		
Magila-Mkumbaa	3 (4.8%)	120 (19.2%)
Muheza Township	51 (81.0%)	256 (41.0%)
Mkanyageni, Mkuzi	6 (9.5%)	140 (22.4%)
Bwembwera, Potwe	3 (4.8%)	108 (17.3%)
Bednet use	36 (70.6%)	342 (60.8%)
Child factors		
Male sex	32 (50.8%)	320 (51.3%)
Birth season†		
Low	40 (63.5%)	345 (55.3%)
High	23 (36.5%)	279 (44.7%)
Birth weight, kg	3.22 (0.49)	3.19 (0.43)
HbS		
AA	54 (85.7%)	519 (84.4%)
AS	9 (14.3%)	96 (15.6%)
Alpha Thal		
a2/a2	26 (44.8%)	276 (47.3%)
a2/a3.7	28 (48.3%)	238 (40.8%)
a3.7/a3.7	4 (6.9%)	70 (12.0%)
Fetal anemia (cord levels)	12 (23.1%)	168 (32.1%)
Percentage (95% CI) of visits—iron deficient‡§	62.8% (52.6,73.0)	62.0% (58.9,65.1)
Percentage (95% CI) of visits—iron deficient‡§ (Hgb < 8)‡	1.2% (0.8,1.6)	1.2% (1.0,1.4)

CI = confidence interval; HbS = sickle cell trait; Hgb = hemoglobin; IPT = intermittent preventive treatment; Thal = thalassemia.

* $P < 0.05$; Univariate analysis.

†High transmission season was between May and October.

‡Percentage of iron deficient/anemia-positive visits out of the number of visits where measured. Does not include subsequent blood smear-positive visits.

§Iron deficiency was defined as ferritin level < 30 ng/mL when C-reactive protein was < 8.2 ng/mL, or ferritin level < 70 ng/mL when C-reactive protein level was > 8.2 $\mu\text{g}/\text{mL}$.

TABLE 3

Number (%) of participants seroprevalent for stage-specific malaria antigens by malaria-resistant status and time, the Mother–Offspring Malaria Study, 2002–2006

Antigens	Week 24	Week 48	Week 76	Week ≥ 100	<i>P</i> trend for time†	Total occurrences
Sporozoite and liver stage						
CSP						
Nonresistant	98 (29.3)	93 (32.4)	96 (37.7)	131 (41.2)	0.009	418 (35.0)
Malaria resistant	15 (41.7)	9 (27.3)	11 (52.4)	9 (42.9)	0.121	44 (39.6)
SLARP						
Nonresistant	58 (17.4)	80 (27.9)	100 (39.2)	140 (44.0)	<.0001	378 (31.7)
Malaria resistant	6 (16.7)	15 (45.5)	8 (38.1)	10 (47.6)	0.821	39 (35.1)
Late liver stage						
LISP1*						
Nonresistant	58 (17.4)	49 (17.1)	67 (26.3)	126 (39.6)	< 0.0001	300 (25.1)
Malaria resistant	2 (5.6)	5 (15.2)	1 (4.8)	7 (33.3)	0.210	15 (13.5)
Merozoite stage						
MSP1*						
Nonresistant	84 (25.2)	94 (32.8)	92 (36.1)	91 (28.6)	0.482	361 (30.2)
Malaria resistant	2 (5.6)	1 (3.0)	3 (14.3)	0 (0.0)	0.744	6 (5.4)
AMA1*						
Nonresistant	120 (35.9)	73 (25.4)	88 (34.5)	116 (36.5)	0.0002	397 (33.3)
Malaria resistant	6 (16.7)	0 (0.0)	2 (9.5)	0 (0.0)	0.017	8 (7.2)

AMA1 = apical membrane antigen 1; CSP = circumsporozoite protein; LISP1 = liver specific protein 1; MSP1 = merozoite surface protein 1; SLARP = sporozoite and liver stage asparagine-rich protein. Seroprevalent cut-point defined as mean + 2 × standard deviation of log-transformed level from a nonimmune U.S. adult (*N* = 20).

**P* < 0.05, differences between malaria-resistant and nonresistant children tested in unadjusted generalized estimating equation (GEE) models (binomial distribution, autoregressive correlation matrix).

†*P* test for trend: Week entered as ordinal variable (week 48 – week ≥ 100), modeled in GEE models (Autoregressive correlation matrix).

concurrent parasitemia (*N* = 236 [20%] visits) and results were similar (data not shown) to those in Table 3. When seroreactivity analyses were repeated among participants residing around Muheza Township only, results were also similar (data not shown).

Malaria-resistant children had a nearly 2-fold increased odds of having higher levels of the cord cytokines IL-1β, IL-6, TNF-α, and TNF-RI compared with nonresistant children (Table 4), although associations were significant only for IL-1β (tertile 2 versus tertile 1, odds ratio = 2.7, 95% confidence interval = 1.3, 5.8). Effect sizes for tertile 2 and tertile 3 versus tertile 1 were of similar magnitude for all cytokines suggesting the possibility that cytokine levels above a certain

threshold may lead to increased protection throughout early life. Cord levels of IL-5, IL-10, IFN-γ, and TNF-RII were not associated with malaria-resistant status. When models were repeated within children residing around Muheza Township, malaria-resistant children had higher odds of being in the highest tertile, but results were not significant (data not shown). In GEE models controlling for village as a clustering variable, the results were similar (data not shown).

DISCUSSION

Among a birth cohort of children in an area of intense malaria transmission, we observed a malaria-resistant phenotype whereby a subgroup of children never developed patent parasitemia. Nine percent of the children in this cohort had no positive blood smears over a cumulative total of 125 person-years of active follow-up. These children were similar to nonresistant children in terms of completeness of follow-up, nonmalaria illness, and all maternal and childhood characteristics other than area of residence. Both groups of children had similar seroprevalence of sporozoite-stage antibody responses suggesting similar intensity of exposure. Conversely, malaria-resistant children had significantly lower seroprevalence of liver-stage specific antibody response to LISP-1 and merozoite-specific antibody response to AMA1 and MSP1 antigens throughout early childhood. Levels of cord-blood inflammatory cytokines at birth were higher among malaria-resistant children suggesting that the differences between these children and nonresistant children may be related in part to immunological profiles determined in utero.

Malaria morbidity can be highly heterogeneous within hyperendemic areas.^{5,6} To better target malaria control efforts, studies often focus on the characteristics of individuals with a high burden of malaria.^{10,11,19} However, several previous cohort studies conducted in a range of transmission settings have also identified never-infected or asymptomatic subgroups.^{5,6,8,10,12} For example, Mwangi and others, in a study in Kenyan children, identified a subgroup that had no clinical

TABLE 4

Associations between cord-blood cytokines and malaria-resistant status in early childhood, the Mother–Offspring Malaria Study, 2002–2006

Characteristic	Unadjusted	Adjusted*
	OR (95% CI)	OR (95% CI)
IL-1β		
Tertile 1	Ref	Ref
Tertile 2	2.7 (1.3, 5.8)	2.5 (1.1, 5.3)
Tertile 3	2.1 (0.9, 4.5)	1.9 (0.9, 4.2)
IL-6		
Tertile 1	Ref	Ref
Tertile 2	1.3 (0.6, 2.7)	1.5 (0.7, 3.1)
Tertile 3	1.7 (0.9, 3.4)	1.7 (0.9, 3.5)
TNF-α		
Tertile 1	Ref	Ref
Tertile 2	1.8 (0.9, 3.8)	1.9 (0.9, 4.0)
Tertile 3	1.9 (0.9, 4.0)	1.8 (0.9, 3.8)
TNF-RI		
Tertile 1	Ref	Ref
Tertile 2	1.7 (0.8, 3.4)	1.7 (0.8, 3.4)
Tertile 3	1.7 (0.8, 3.4)	1.6 (0.8, 3.3)

CI = confidence interval; IL = interleukin; OR = odds ratio; TNF = tumor necrosis factor.

*Model adjusted for bednet use (3-level variable, yes-no-missing) and parity (2-level variable, primigravid vs. secondi-multigravid).

malaria episodes and were parasite negative at all cross-sectional surveys over a period of up to 5 years,¹⁰ whereas Tran and others described a subgroup of individuals in Mali who remained uninfected throughout the transmission season despite intense transmission pressure and active surveillance at the study site.⁸ Genetic and/or immunological factors related to parasitemia control and immunity may contribute to the observed differences in malaria susceptibility. Examples of this include the protection conferred by the hemoglobin AS genotype, or the distinct immunological responses among the Fulani versus their neighbors of different ethnic backgrounds,^{20,21} However, none of these genetic backgrounds are known to completely prevent infection. In the HIV/acquired immunodeficiency syndrome literature, the identification of elite viremia controllers has led to the identification of potential vaccine targets.^{22,23} Further study into children particularly resilient to malaria may identify mechanisms of natural immunity acquisition.

Longitudinal seroreactivity data from a panel of established preerythrocytic and merozoite antigens helped elucidate malaria transmission patterns within this population. Malaria-resistant and nonresistant children had a similar antibody seroprevalence to sporozoite and early liver-stage antigens (CSP and SLARP),^{24,25} and seroprevalence was greater at week 100+ than week 48, suggesting similar transmission pressure over the course of the study. Seroprevalence is a measure of cumulative exposure, and antibody levels, including those reactive to preerythrocytic antigens, can be used as indicators of transmission intensity.^{26–30} Differences in results between LISP1 and the other preerythrocytic-stage antigens could be due to differences in the timing of antigen expression, as LISP1 is expressed in late liver stage, and is important in the egress of parasites from liver cells.³¹ The antigens AMA1 and MSP1 are expressed by merozoites that develop during late liver stage or blood stage, and whole parasite vaccines that arrest in the liver can induce antibodies against both merozoite antigens.³² They have been used in many cohort studies as correlates of blood-stage parasite exposure, and are vaccine targets.³³ Here, malaria-resistant children had a much lower seroprevalence for both AMA1 (7.2% versus 33.3%) and MSP1 (5.4% versus 30.2%) than nonresistant children.

Studies in endemic areas have failed to demonstrate evidence of naturally acquired sterile immunity to *P. falciparum*, even after many years of infection.² In this study, malaria-resistant children failed to develop patent parasitemia over the course of intense active follow-up, but acquired similar antibody responses to sporozoite antigens, suggesting a similar degree of parasite exposure as other children. In contrast, malaria-resistant children had lower antibody responses to late liver-stage and merozoite antigens, suggesting their immunity might be killing parasites in late liver stages, before antibodies to blood-stage antigens could develop. However, differentiating between immunity at these two stages has proven difficult,^{8,34} and serological patterns are not yet established as a definitive method to do so. Resistance in these children is evident from early infancy, suggesting that their immunity is innate or acquired quickly, rather than slowly over many years as is seen with immunity that controls blood-stage infections. Our results are consistent with evidence that a small fraction of the population does not develop patent parasitemia across all age spectra.^{8,12}

Malaria-resistant children tended to have higher levels of cord-blood pro-inflammatory mediators, most predominantly the cytokine IL-1 β . Interleukin-1 β has also been associated with protection from severe malaria within this cohort.¹⁶ Pro-inflammatory cytokines may be protective against malaria by helping to control blood-stage parasites,³⁵ inhibiting intra-hepatocytic development³⁶ and enhancing the cellular and humoral immune response.³⁷ Malaria during pregnancy may lead to changes in fetal innate immune function possibly through transplacental exposure to malaria antigens.^{38,39} Conversely, exposure to such products might also produce a tolerant phenotype, namely a low production of cytokine levels in response to malarial antigens, which has been shown to be associated with malaria infection later in childhood.³⁸ The cord-blood cytokine results observed in this study, although modest, suggest that the origin of the malaria-resistant phenotype may be related in part to an immunologic profile present at birth.

An important limitation of this study was that parasitemia was defined by blood smear microscopy; therefore, the possibility of subpatent infection among malaria-resistant children cannot be ruled out. Polymerase chain reaction can detect parasitemias of much lower magnitude than microscopy,^{40,41} and has detected low parasitemia even among children with prolonged periods without a positive blood smear.⁴⁰ However, submicroscopic parasitemia may mean less complex infections,⁴¹ increased control and clearance,⁴² and may reflect an effective antimalarial immune response overall.³⁹ Therefore, although submicroscopic infections cannot be ruled out for malaria-resistant children, this still implies that these children had a remarkable ability to suppress parasitemia to subpatent levels.

The intensity of surveillance was a strength of this study. The cohort was actively followed through the peak risk period for severe malaria in an area of intense malarial transmission. Comprehensive follow-up included routine visits with blood smears every 2 weeks during infancy, and every 4 weeks thereafter. Free transportation and patient care were provided for participants at clinics when malaria symptoms arose, reducing the likelihood of undocumented malaria episodes. Longitudinal serology data which included antibody responses to well-known blood- and preerythrocytic-stage antigens helped to better characterize the patterns of malaria infection in this cohort. Although it is difficult to prove definitively that malaria-resistant children were truly infected, available evidence suggests that these children were living in a setting of intense malaria transmission, and were as similarly exposed to malaria as other children in the cohort. For example, the median age at the first severe malaria episode in this cohort was 39 weeks, the entomological inoculation rate around the time of this study was high, and these children had similar levels of preerythrocytic-stage antibody responses to nonresistant children.

Our study identified a subgroup of children living in a highly endemic area who appear to have been exposed to malaria but never developed patent parasitemia. Antibody profiles confirmed that these children were acquiring infections, but that they appeared to have better control of infection possibly at the preerythrocytic stage. Differences in cytokine levels at birth may have contributed to the phenotype. Further research with malaria-resilient children may inform the understanding of acquisition of natural immunity to malaria.

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