

# Humoral and Cellular Immunity to *Plasmodium falciparum* Merozoite Surface Protein 1 and Protection From Infection With Blood-Stage Parasites

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**Background.** Acquired immunity to malaria develops with increasing age and repeated infections. Understanding immune correlates of protection from malaria would facilitate vaccine development and identification of biomarkers that reflect changes in susceptibility resulting from ongoing malaria control efforts.

**Methods.** The relationship between immunoglobulin G (IgG) antibody and both interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10) responses to the 42-kD C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (MSP1<sub>42</sub>) and the risk of (re)infection were examined following drug-mediated clearance of parasitemia in 94 adults and 95 children in an area of holoendemicity of western Kenya.

**Results.** Positive IFN- $\gamma$  enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunosorbent spot assay (ELISPOT) responses to MSP1<sub>42</sub> 3D7 were associated with delayed time to (re)infection, whereas high-titer IgG antibodies to MSP1<sub>42</sub> 3D7 or FVO alleles were not independently predictive of the risk of (re)infection. When IFN- $\gamma$  and IL-10 responses were both present, the protective effect of IFN- $\gamma$  was abrogated. A Cox proportional hazard model including IFN- $\gamma$ , IL-10, MSP1<sub>42</sub> 3D7 IgG antibody responses, hemoglobin S genotype, age, and infection status at baseline showed that the time to blood-stage infection correlated positively with IFN- $\gamma$  responses and negatively with IL-10 responses, younger age, and asymptomatic parasitemia.

**Conclusions.** Evaluating combined allele-specific cellular and humoral immunity elicited by malaria provides a more informative measure of protection relative to evaluation of either measure alone.

**Keywords.** human immunity; Merozoite Surface Protein 1; humoral immunity; cellular immunity; interferon-gamma; interleukin-10; sickle cell trait; longitudinal study; observational study.

Globally, falciparum malaria remains a major cause of childhood mortality and morbidity. Considerable reduction in malaria transmission has been achieved

through use of insecticidal bed nets and indoor spraying [1]. Protective immunity to blood-stage *Plasmodium falciparum*, the parasite stage underlying malaria pathogenesis, develops in children and is maintained in adults as a consequence of periodic boosting manifested as asymptomatic low-density parasitemia [2]. Anticipating future decreases in malaria transmission, understanding the mechanisms underlying protective immunity is pertinent to identifying markers predictive of age-related shifts in malaria susceptibility and developing vaccines to blood-stage *P. falciparum*.

Validating correlates of immune protection in malaria-endemic populations is challenging because both humoral

Received 8 November 2012; accepted 9 January 2013; electronically published 28 March 2013.

Presented in part: American Society of Tropical Medicine and Hygiene Annual Meeting, November 7–11, 2004, Miami Beach, Florida, USA. Abstract 947.

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**The Journal of Infectious Diseases** 2013;208:149–58

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DOI: 10.1093/infdis/jit134

and cellular immunity are induced and because only a limited number of candidate antigens have been studied to date. Most have described the association between the malarial immunoglobulin G (IgG) or IgG subclass antibodies and susceptibility to clinical malaria [3]. Antibodies are clearly important since transfer of IgG from immune African adults to children and nonimmune adults experiencing acute malaria rapidly reduces parasitemia and abrogates fever [4, 5]. These antibodies are directed against a breadth of blood-stage antigens, which, in combination and after achieving a critical threshold level, correlate with decreased susceptibility to high-density parasitemia and clinically mild malaria [6, 7]. The mechanisms by which such antibodies mediate protective immunity are incompletely resolved [2]. With respect to cellular immunity, studies of rodent malaria [8], malaria-naive humans exposed to sporozoites and liver-stage *P. falciparum* [9] or to a small number of infected erythrocytes followed by drug cure [10], and studies of residents of malaria endemic areas [11] indicate that T-cell cytokine responses, particularly interferon  $\gamma$  (IFN- $\gamma$ ) responses, correlate with protection from blood-stage infection and malaria illness [12, 13].

Merozoite surface protein 1 (MSP1) has been considered as a blood-stage vaccine candidate [14, 15]. Although a clinical trial of the C-terminal 42-kDa region of the MSP1 (MSP1<sub>42</sub>) 3D7 allele showed no impact on the susceptibility to mild malaria in Kenyan children [16], improved immunogenicity and efficacy may be possible by using viral vectors [17] and different adjuvants [18, 19], allowing MSP1 to remain a candidate for a multistage vaccine [20]. IgG antibody and T-cell cytokine responses are directed to epitopes contained within fragments of MSP1<sub>42</sub>, MSP1<sub>19</sub>, and MSP1<sub>33</sub>, that are generated by endogenous parasite proteases prior to invasion of the erythrocyte [15]. Studies of mouse malaria and malaria-naive human volunteers experimentally inoculated with blood-stage *P. falciparum* suggest that T-cell cytokine responses to epitopes contained within MSP1<sub>33</sub> mediate protection from parasitemia independent of antibodies [10, 21, 22]. T-helper cell responses that support anti-MSP1 antibody production by B cells may also be generated [23, 24]. Polymorphisms of MSP1<sub>19</sub> and MSP1<sub>33</sub> are common in areas of endemicity [25], raising concern that variant-specific immunity needs to be considered when studying MSP1 and other polymorphic blood-stage antigens as targets of acquired and vaccine-induced immunity [26]. We addressed these issues by conducting an observational study of adults and children living in a malaria-holoendemic area of Kenya in 2003, prior to widespread introduction of insecticide bed nets and control strategies. IgG antibody, IFN- $\gamma$ , and interleukin 10 (IL-10) responses specific to MSP1<sub>42</sub> were evaluated as correlates of delay in the time to (re)infection and association with clinical malaria following drug-mediated cure of blood-stage parasitemia.

## METHODS

### Study Participants and Design

Asymptomatic healthy adults (n = 101) and children (n = 100) from Kanyawegi in Nyanza Province, Kenya, were enrolled in July 2003 at the beginning of a seasonal peak in malaria transmission. Women were excluded if they self-reported to be pregnant. Venous blood was collected for immunologic studies prior to administration of a weight-adjusted 6-dose regimen of artemether-lumefantrine that all participants received at baseline (week 0) regardless of malaria status determined by blood smear.

Blood samples were collected weekly by finger stick for 11 consecutive weeks to determine the time to (re)infection. Giemsa-stained thick and thin smears were scored as negative when no asexual-stage *P. falciparum* or other malaria species was observed after microscopic inspection that included  $\geq 200$  leukocytes. Parasite density was expressed as the number of parasites per microliter of blood, on the assumption of a leukocyte count of 8000 per  $\mu\text{L}$  blood. Parasite clearance following artemether-lumefantrine administration at baseline was defined by a malaria-negative blood smear 2 weeks later. If the blood smear was positive at week 2, these individuals were excluded from the remaining analysis. Passive surveillance for clinical malaria was performed and defined by a positive blood smear, as well as an axillary temperature of  $\geq 37.5^\circ\text{C}$  and malaria symptoms [7]. Asymptomatic blood-stage infections during weeks 2–11 were not treated with antimalarial drugs, consistent with 2003 Kenya Ministry of Health guidelines. Malaria-naive adults (n = 20) from Cleveland, Ohio, were screened to evaluate nonspecific immune responses.

### Ethical Approval

Ethical approval was obtained from the Institutional Review Board at the University Hospitals Case Medical Center and from the Kenya Medical Research Institute Ethical Review Committee. Adults signed a written informed consent form in the local language (ie, English or Duhluo). Parents or guardians signed for minors <18 years old.

### Recombinant MSP1<sub>42</sub> and MSP1<sub>33</sub> Peptide T-Cell Epitopes

Recombinant MSP1<sub>42</sub> corresponding to the dimorphic allelic variants PNG-MAD20 (3D7 strain, E-TSR haplotype) and Wellcome-K1 (FVO strain, Q-KNG haplotype) were expressed in *Escherichia coli* and used for peripheral blood mononuclear (PBMC) cytokine recall assays and IgG antibody assays [20]. Synthetic M2 peptide (GISYEEKVLAKYKDDLE; MSP1 residues 1467–1483, SigmaGenosys, Gainesville, TX) corresponding to a region of MSP1<sub>33</sub> 3D7 was selected as an immunodominant T-cell epitope [27].

## Cytokine Recall Assays

Enzyme-linked immunosorbent spot assays (ELISPOT) and enzyme-linked immunosorbent assays (ELISAs) were performed as previously described [28, 29] by incubating PBMCs for 84 hours with 5 µg/mL MSP1<sub>42</sub> 3D7, MSP1<sub>42</sub> FVO, or M2 3D7 peptide. IFN-γ and IL-10 ELISA responses were considered positive if the culture supernatant contained ≥20 pg/mL following stimulation with malaria antigen. An IFN-γ ELISPOT response was considered positive if the number of spot-forming units (SFU) in the stimulated well was significantly greater than that in the unstimulated background well, using a *P* value of < .05, calculated by  $\chi^2$  analysis of two proportions, with adjustment for small sample size. The mean number of SFU per 10<sup>6</sup> PBMCs was <5 in unstimulated wells for all study participants. The magnitude of IFN-γ ELISPOT responses was determined by subtracting the number of SFU in the unstimulated well from that for the well containing malaria antigen. Malaria-naive controls (*n* = 20) showed no IFN-γ or IL-10 responses to recombinant MSP1<sub>42</sub> or M2 peptide by ELISA or ELISPOT.

## IgG Antibody ELISA

IgG antibodies to MSP1<sub>42</sub> 3D7 and FVO were measured by ELISA, as previously described [7]. Plasma from 9 malaria-naive North American adults was pooled and used as a negative control, and plasma from 20 Kenyan adults was pooled and used to create a standard curve. Individual antibody responses were expressed in arbitrary units (AU) and categorized into quartiles. Individuals with high-AU IgG responses (ie, those with an AU in the upper 2 quartiles; defined as high-titer antibodies) were considered positive, high-responders for the survival analysis.

## Hemoglobin S Genotype

Genomic DNA was extracted from blood using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA). The sickle hemoglobin mutation was detected by polymerase chain reaction amplification, followed by *Bsu* 361 digestion, as described elsewhere [30].

## Statistical Analysis

Differences in the frequency of immunological variables were compared across age groups (ie, adult aged ≥18 years vs children aged 1–14 years), using McNemar  $\chi^2$  tests. Median cytokine recall responses were compared across age groups for adults and subsets of children 10–14, 5–9, and 1–4 years old, using the Kruskal-Wallis nonparametric method.

To proceed from a stepwise analytical approach, we first conducted survival analyses (for 9 weeks, from weeks 2–11), using dichotomized variables for antibodies, cytokine responses, hemoglobin AA/AS genotype, and parasitemia prior to treatment one at a time, in keeping with similar studies [3]. To explore synergistic and antagonistic immune responses, survival analyses were then conducted with a combination of 2 variables,

such as IFN-γ and IL-10 or IFN-γ and IgG antibody. The time to (re)infection was determined with a Kaplan-Meier log-rank test for survival analysis, using SAS 9.2 (SAS Institute, Cary, NC). Multiple factors that simultaneously might influence time to (re)infection were next evaluated. Cox regression models were used to estimate the association between these variables or combination of variables and the risk for malaria where proportional hazards assumptions were met. Covariates in the analysis included, IFN-γ, IL-10, IgG antibody, HbAS/AA genotype, malaria status prior to treatment and age (continuous). Finally, we correlated baseline antibody and cytokine responses with susceptibility to clinical malaria.

## RESULTS

### Study Population Characteristics

Entomologic inoculation rates in July 2003 were estimated to be >300 cases per person per year [31]. Thirty-four adults had asymptomatic parasitemia at baseline, and 40 remained blood smear negative during the 11-week follow-up period (Table 1). One adult failed to clear blood-stage infection 2 weeks after treatment because of noncompliance with directly observed antimalarial treatment. Six were lost to follow-up, resulting in 94 adults eligible for analysis. Eighty-two of 100 children had blood-stage *P.*

**Table 1. Study Population Characteristics**

Characteristic	Adults ( <i>n</i> = 101)	Children ( <i>n</i> = 100)
Age, y, mean (range)	39.6 (18–79)	7.7 (1–14)
Parasitemic yet asymptomatic prior to treatment <sup>a</sup>	34	82
Hemoglobin level, g/dL, mean	12.6	11.4
Parasite density, parasites/µL blood, mean (range) <sup>a</sup>	148 (80–5120)	3740 (80–48 000)
Cleared blood-stage parasites 2 weeks after treatment, by blood smear	100	100
Remained aparasitemic during 12-wk follow-up period <sup>a</sup>	40	8
Time to reinfection, wk, median <sup>a</sup>	7.4	5.3
Lost to follow-up	6	5
Required treatment for symptomatic malaria <sup>a</sup>	3	23
Frequency of hemoglobin AS genotype, %	30	30
Total included in analysis of immune correlates	94	95

<sup>a</sup> Significant differences were observed when comparing adults to children for median time to (re)infection, parasite prevalence, mean parasite density at baseline, number who remained aparasitemic during the follow-up period and for those who had symptomatic malaria infections (*P* < .0001).

**Table 2. Frequency of Naturally Acquired Immune Responses to *Plasmodium falciparum* Merozoite Surface Protein 1 (MSP1) in Adults and Children**

Assay, Variable	Adults Aged ≥18 y	Children Aged 1–14 y	<i>P</i> <sup>a</sup>	Children Aged 10–14 y	Children Aged 5–9 y	Children Aged 1–4 y
<b>IFN-γ ELISA</b>						
Subjects, no.	90	88		22	44	22
Stimulus, %						
PHA	100.0	98.0	.500	100	95	100
M2 peptide	30.7	13.6	<b>.007</b>	13.4	18.2	12.5
MSP1 <sub>42</sub> 3D7	62.5	34.1	<b>.001</b>	45.4	27.3	36.4
MSP1 <sub>42</sub> FVO	47.7	26.1	<b>.003</b>	36.4	25.0	18.2
<b>IFN-γ ELISPOT</b>						
Subjects, no.	94	91		19	46	26
Stimulus, %						
PHA	95.0	91.0	.447	86.4	93.9	89.7
M2 peptide	9.5	4.4	.152	5.3	3.8	7.7
MSP1 <sub>42</sub> 3D7	37.9	26.4	.126	21.1	21.7	38.5
MSP1 <sub>42</sub> FVO	9.5	2.2	<b>.036</b>	0.0	2.2	3.8
<b>IL-10 ELISA</b>						
Subjects, no.	90	88		22	44	22
Stimulus, %						
PHA	100.0	100.0	NS	100.0	100.0	100.0
MSP1 <sub>42</sub> 3D7	59.1	44.6	.058	42.9	42.9	55.0
MSP1 <sub>42</sub> FVO	21.6	16.9	.434	4.8	16.7	30.0
<b>IgG antibody ELISA</b>						
Subjects, no.	90	88		22	44	22
Stimulus, %						
MSP1 <sub>42</sub> 3D7	49.5	25.0	<b>.003</b>	22.7	28.6	20.7
MSP1 <sub>42</sub> FVO	36.6	28.0	.190	22.7	34.7	20.7

Bold values denote significant results.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon γ; IgG, immunoglobulin G; IL-10, interleukin 10; MSP1<sub>42</sub>, 42-kD C-terminal fragment of *Plasmodium falciparum* MSP1; NS, not significant.

<sup>a</sup> Adults vs children.

*falciparum* at baseline, with higher average parasite densities than adults (Table 1). Only 8 children did not have a positive blood smear during the follow-up period, and the average time to (re)infection was significantly shorter, at 5.3 weeks, compared with 7.4 weeks for adults. When children were reinfected, they tended to have 2.5-fold higher parasite densities as compared to reinfected adults. Five children were lost to follow-up, leaving 95 children included in the analysis. Twenty-three children developed symptomatic malaria during the 12-week observation period. The frequency of sickle cell trait was 30% for both adults and children. No individuals were homozygous for hemoglobin S.

### MSP1<sub>42</sub> IFN-γ and IL-10 Responses

To appreciate differences in cellular immunity to MSP1<sub>42</sub> between adults and children, we compared the baseline prevalence of IFN-γ and IL-10 responses (Table 2). Higher frequencies of IFN-γ ELISA responses among adults, compared with children, were

observed for M2 3D7 peptide ( $P = .007$ ), MSP1<sub>42</sub> FVO ( $P = .003$ ), and MSP1<sub>42</sub> 3D7 ( $P < .001$ ), with responses to 3D7 more common than those to FVO. Analysis of children grouped according to age 10–14, 5–9, and 1–4 years revealed that the frequency of IFN-γ ELISA responses had a positive, age-related trend for MSP1<sub>42</sub> FVO and 3D7, but neither was statistically significant. IFN-γ ELISPOT results showed a lower proportion of responders than IFN-γ recall measured by ELISA (eg, 9.5% vs 47.7% for MSP1<sub>42</sub> FVO;  $P < .0001$ , by the Fisher exact test). In general, adults had more positive responses to malaria antigens, as measured by IFN-γ ELISPOT; however, only responses to MSP1<sub>42</sub> FVO ( $P = .036$ ) were significantly higher in adults as compared to children. As was the case for IFN-γ ELISA, the frequency of positive ELISPOT responses was greater when PBMCs were stimulated with MSP1<sub>42</sub> 3D7 than with MSP1<sub>42</sub> FVO (Table 2). With respect to IL-10, responses to both MSP1<sub>42</sub> FVO and 3D7 showed no significant differences according to age; however, IL-10

responses were more common to 3D7 as compared to the FVO variant (59.1%–44.6% and 21.6%–16.9%, respectively).

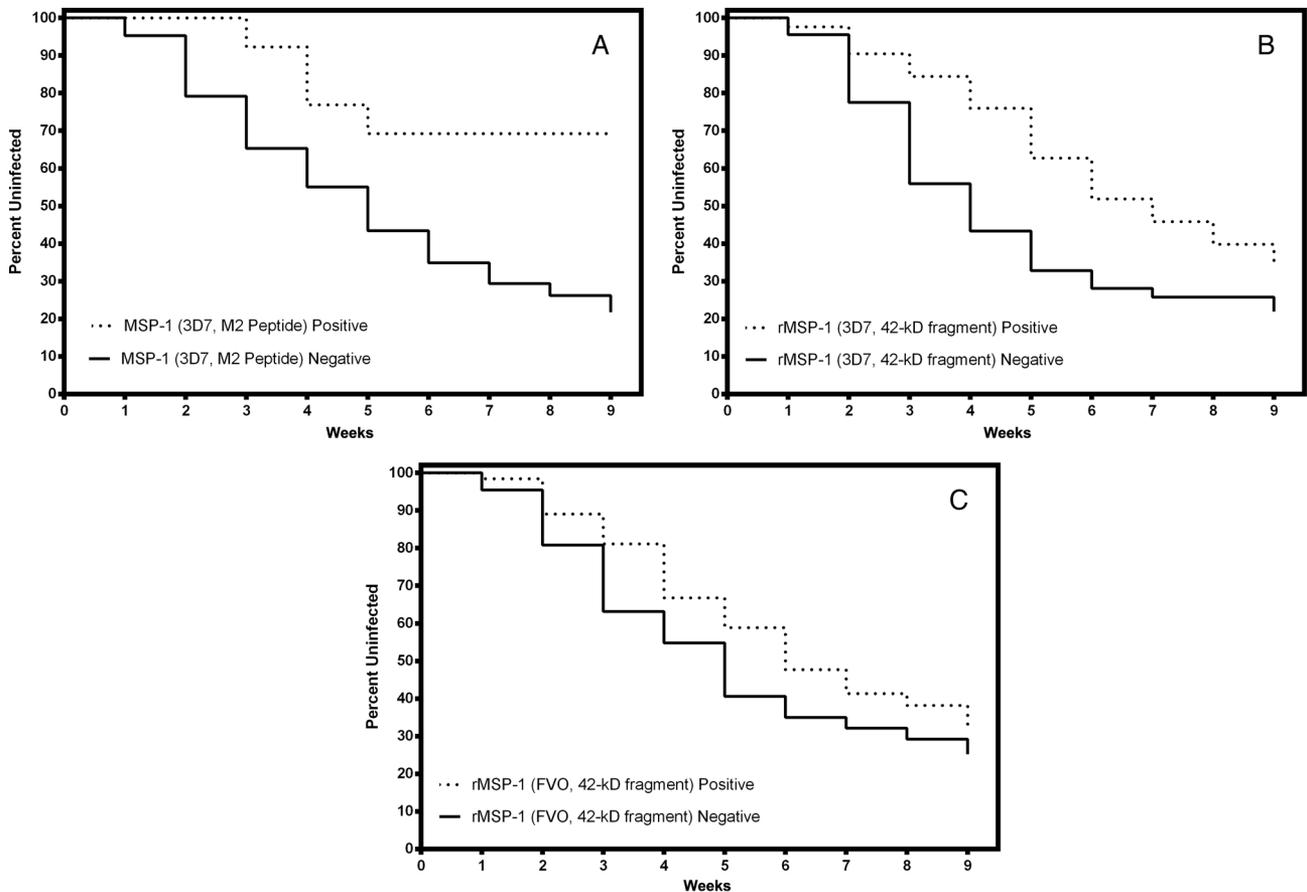
We next compared the magnitude of IFN- $\gamma$  responses among adults and children to address whether a threshold level is important for immune protection against malaria. The amount of IFN- $\gamma$  produced by adults versus children for MSP1<sub>42</sub> 3D7 was similar (median level, 150 and 110 pg/mL, respectively). However, IFN- $\gamma$  concentrations were negatively related to age, with the youngest children secreting relatively more IFN- $\gamma$  in response to MSP1<sub>42</sub> 3D7 ex vivo stimulation (median levels for children aged 10–14, 5–9, and 1–4 years, 49, 123 and 160 pg/mL, respectively;  $P = .006$ , by the Kruskal-Wallis test; [Supplementary Figure 1](#)). MSP1<sub>42</sub> FVO-driven IFN- $\gamma$  production was uniformly low across all age groups ([Supplementary Figure 1](#)). With respect to IFN- $\gamma$  ELISPOT, the level was similar among children and adults who responded to MSP1<sub>42</sub> 3D7 (median level, 60 SFU per 10<sup>6</sup> PBMC). The median value for IFN- $\gamma$  ELISPOT responses to MSP1<sub>42</sub> FVO was one-half that of MSP1<sub>42</sub> 3D7 (data not shown).

### MSP1<sub>42</sub> IgG Antibody Responses

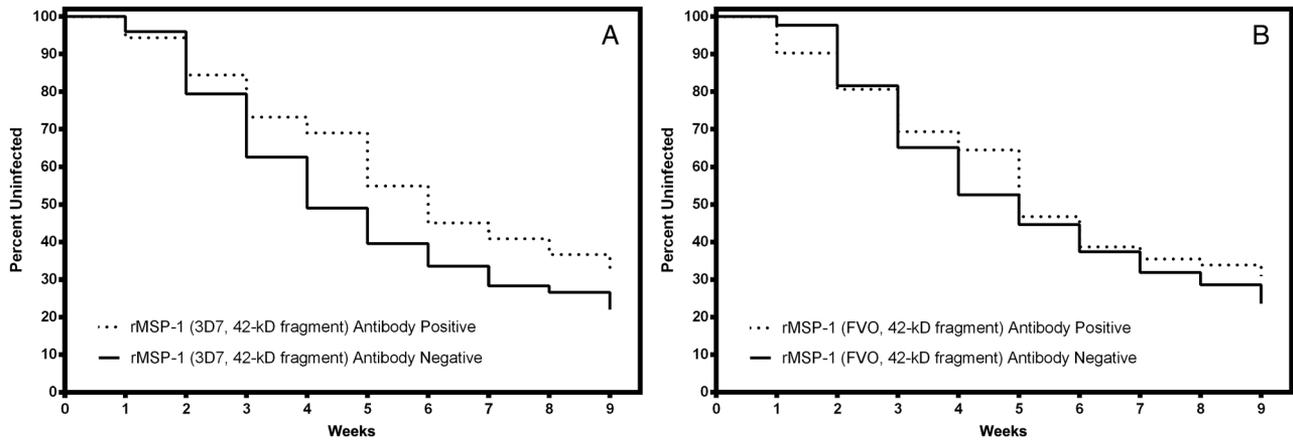
A greater proportion of adults than children had high-titer IgG antibodies to MSP1<sub>42</sub> 3D7 (49.5% vs 25.0%;  $P = .003$ ), whereas there was no difference for anti-MSP1<sub>42</sub> FVO antibodies (36.6% and 28.0%, respectively; [Table 2](#)). Antibody levels to MSP1<sub>42</sub> 3D7 and FVO did not differ significantly by age group among the children ([Table 2](#)).

### MSP1<sub>42</sub> Allele-Specific Immunity and Risk of (Re)infection

Kaplan-Meier survival analyses were performed to determine which variables were independently associated with risk of (re)infection. M2 3D7 peptide IFN- $\gamma$  ELISPOT responses ([Figure 1A](#)) and MSP1<sub>42</sub> 3D7 IFN- $\gamma$  ELISA responses ([Figure 1B](#)) were significantly associated with a decreased risk of (re)infection (hazard ratio [HR], 0.35 [95% confidence interval {CI}, .13–.98];  $P = .046$ ) and a delayed time to (re)infection (HR, 0.65 [95% CI, .44–.94];  $P = .023$ ). IFN- $\gamma$  recall to MSP1<sub>42</sub> FVO was present ([Figure 2C](#)) but not associated with a decreased risk of (re)infection (HR, 0.91 [95% CI, .062–1.34];  $P = .663$ ). IL-10 ELISA responses were not



**Figure 1.** Survival analyses for interferon  $\gamma$  (IFN- $\gamma$ ) responses to the *Plasmodium falciparum* merozoite surface protein 1 (MSP1). The solid black line represents nonresponders and the dashed line represents responders. A) Significant delay in the time to (re)infection was observed for those with IFN- $\gamma$  ELISPOT responses to the M2 3D7 peptide (hazard ratio [HR], 0.35 [95% confidence interval {CI}, .13–.98];  $P = .046$ ; and B) IFN- $\gamma$  enzyme-linked immunosorbent assay (ELISA) responses to recombinant MSP1<sub>42</sub> 3D7 (HR, 0.65 [95% CI, .44–.94];  $P = .023$ ; but not for those with C) IFN- $\gamma$  ELISA responses to recombinant MSP1<sub>42</sub> FVO (HR, 0.91 [95% CI, .62–1.34];  $P = .663$ ).



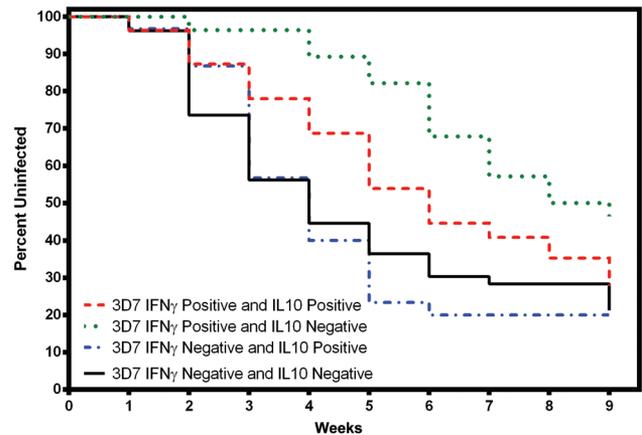
**Figure 2.** Survival analyses for immunoglobulin G (IgG) antibody responses to the 42-kD C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (MSP<sub>142</sub>). The solid black line represents nonresponders, and the dashed line represents responders. Study participants were considered negative for IgG antibody responses if levels were in the lower 2 quartiles, whereas those with responses in the upper 2 quartiles were considered antibody positive. *A*, A slight delay in the time to (re)infection was observed for those with high IgG antibody responses to MSP<sub>142</sub> 3D7, but this did not achieve statistical significance (hazard ratio, 1.02 [95% confidence interval {CI}, .71–1.49]; *P* = .897). *B*, Antibody responses to MSP<sub>142</sub> FVO were also not associated with a risk of (re)infection (HR, 1.0 [95% CI, .70–1.16]; *P* = .969).

an independent predictor of the risk of (re)infection (data not shown). Individuals with high-titer IgG antibody responses to MSP<sub>142</sub> 3D7 and FVO were not associated with a delayed time to (re)infection relative to that for individuals with responses in the lower 2 quartiles (Figure 2), even after adjustment for age.

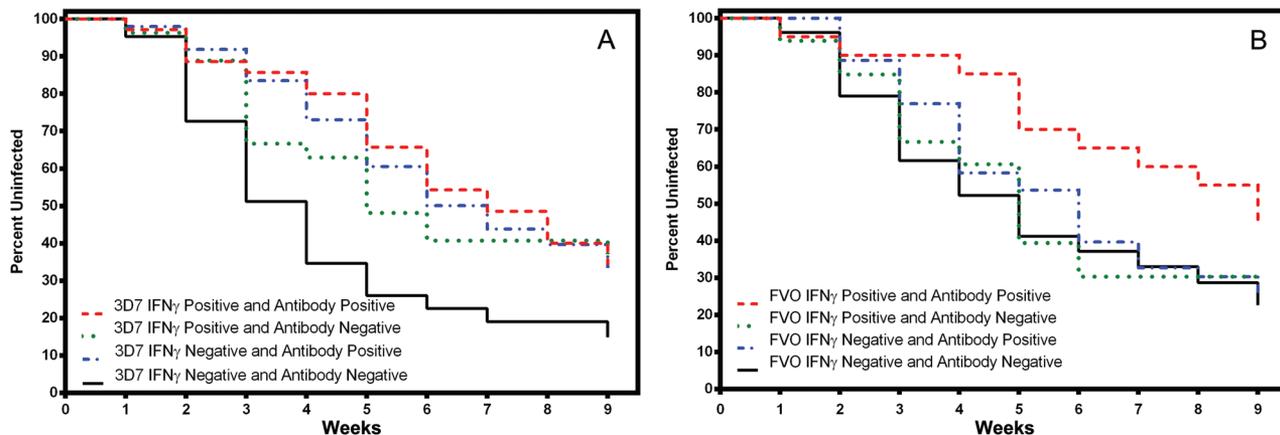
We next evaluated combined measurements of immunity to determine whether this was a better fit for the model. IFN- $\gamma$  and IL-10 responses were evaluated first because IL-10 has been associated with protection from malaria but may have an antagonistic effect on IFN- $\gamma$  responses [32]. Cytokine responses to MSP<sub>142</sub> 3D7 were categorized into 4 groups: IFN- $\gamma$  positive and IL-10 negative, IFN- $\gamma$  positive and IL-10 positive, IFN- $\gamma$  negative and IL-10 positive, IFN- $\gamma$  negative and IL-10 negative. Figure 3 demonstrates that individuals who produced only IFN- $\gamma$  had a significantly reduced risk of (re)infection as compared to IFN- $\gamma$  negative and IL-10 negative (HR, 0.65 [95% CI, .49–.88]) or the other 3 groups combined (HR, 0.48 [95% CI, .28–.82]; *P* = .008). Similar analyses were done for MSP<sub>142</sub> FVO, confirming that cytokine responses to this allele were not associated with a reduced risk of (re)infection (data not shown).

Combined MSP<sub>142</sub> 3D7 and FVO IFN- $\gamma$  and antibody responses were evaluated next to determine whether there was an additive effect for individuals with both responses. Responses were classified into 4 groups, similar to the description above: IFN- $\gamma$  positive and antibody positive, IFN- $\gamma$  positive and antibody negative, etc. For MSP<sub>142</sub> 3D7, any positive response was associated with significantly reduced risk of (re)infection, compared with the double-negative group (Figure 4A). Antibody responses to MSP<sub>142</sub> 3D7 and FVO were highly correlated (Spearman *r* = 0.78; *P* < .0001). Yet improved survival estimates were observed for MSP<sub>142</sub> FVO only when IFN- $\gamma$  and antibody

responses were included in the analysis (HR, 0.80 [95% CI, .64–.98]; *P* = .042; Figure 4B).



**Figure 3.** Survival analyses for interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10) responses to 42-kD C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (MSP<sub>142</sub>) 3D7. Cytokine responses to MSP<sub>142</sub> 3D7 were categorized into 4 groups: IFN- $\gamma$  positive and IL-10 positive (red), IFN- $\gamma$  positive and IL-10 negative (green), IFN- $\gamma$  negative and IL-10 positive (blue), IFN- $\gamma$  negative and IL-10 negative (black). Study participants with IFN- $\gamma$  positive and IL-10 negative responses (*n* = 55) had a significantly reduced risk of (re)infection as compared to those with IFN- $\gamma$  negative and IL-10 negative responses (hazard ratio [HR], 0.65 [95% confidence interval {CI}, .49–.88]) or the other 3 groups combined (HR, 0.48 [95% CI, .28–.82]; *P* = .008). By using study participants with no cytokine responses (ie, IFN- $\gamma$  negative and IL-10 negative) as the referent group (*n* = 53), those who were double positive for IFN- $\gamma$  and IL-10 (*n* = 28; HR, 0.82 [95% CI, .53–1.27]; *P* = .382) or IFN- $\gamma$  negative and IL-10 positive (*n* = 31; HR, 1.03 [95% CI, .62–1.72]; *P* = .897) did not have a significantly delayed time to (re)infection. Cytokine responses to MSP<sub>142</sub> FVO were too infrequent for meaningful analysis (data not shown).



**Figure 4.** Survival analyses for combined interferon  $\gamma$  (IFN- $\gamma$ ) and immunoglobulin G (IgG) antibody responses to 42-kD C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (MSP1<sub>42</sub>) 3D7 and FVO. Responses were divided into 4 categories: IFN- $\gamma$  positive and IL-10 positive (red), IFN- $\gamma$  positive and IL-10 negative (green), IFN- $\gamma$  negative and IL-10 positive (blue), IFN- $\gamma$  negative and IL-10 negative (black). *A*, For MSP1<sub>42</sub> 3D7, IFN- $\gamma$  positive and antibody positive (hazard ratio [HR], 0.78 [95% confidence interval {CI}, .66–.92];  $P = .0037$ ), IFN- $\gamma$  positive and antibody negative (HR, 0.50 [95% CI, .32–.78];  $P = .0022$ ), and IFN- $\gamma$  negative and antibody positive (HR, 0.74 [95% CI, .56–.98];  $P = .036$ ) responses were associated with a reduced risk of (re)infection, compared with those who were negative for both measurements. The combined effect for any positive IFN- $\gamma$  or antibody response relative to IFN- $\gamma$  negative or antibody negative response was an HR of 0.79 (95% CI, .7–.89;  $P = .0001$ ). *B*, For MSP1<sub>42</sub> FVO, those who were IFN- $\gamma$  positive and antibody positive were the only group who experienced a significant delay in the time to (re)infection (HR, 0.80 [95% CI, .64–.98];  $P = .042$ ), compared with those who were IFN- $\gamma$  negative and antibody negative. IFN- $\gamma$  positive and antibody positive (HR, 0.93 [95% CI, .73–1.19];  $P = .5672$ ) and IFN- $\gamma$  negative and antibody positive (HR, 0.85 [95% CI, .55–1.32];  $P = .472$ ) had nonsignificant delays to reinfection, relative to IFN- $\gamma$  negative and antibody negative individuals.

Finally, Cox proportional hazard models were created for all MSP1<sub>42</sub> 3D7 and FVO immunologic measurements (ie, IFN- $\gamma$ , IL-10, and antibodies), with adjustment for HbAS genotype, parasitemia status at baseline, and age (continuous). Table 3 demonstrates that the 3 immune responses to MSP1<sub>42</sub> FVO, the interactions among them, and HbAS genotype were not significant or informative. Colinearity was evaluated and found not to significantly influence the parameter estimates. However, age (continuous), and malaria status at baseline (ie, asymptomatic parasitemia and parasite density; [Supplementary Materials](#)) were significantly associated with a risk for (re)infection. The full model confirmed that IL-10 responders to MSP1<sub>42</sub> 3D7 had a 62% increase in the risk of (re)infection, compared with IL-10 nonresponders ( $P = .019$ ). In contrast, IFN- $\gamma$  responders to MSP1<sub>42</sub> 3D7 had a 46% decrease in the risk of (re)infection, compared with IFN- $\gamma$  nonresponders ( $P = .002$ ). In the full model, MSP1<sub>42</sub> 3D7 IgG antibody responses were not significantly associated with the risk of malaria but increasing age was associated with a lower risk of infection ( $P < .0001$ ). Finally, there were no significant differences in antibody and cytokine responses revealed by a comparison of the 23 children with febrile (re)infections to the 72 children who remained uninfected or asymptomatic when (re)infected.

## DISCUSSION

We sought to more clearly define cellular and humoral immune correlates of protection from *P. falciparum* blood-

stage infection in a malaria-holoendemic setting. We focused on immunity to MSP1<sub>42</sub>, the most abundant of the merozoite proteins, a ligand essential to erythrocyte invasion, and a malaria vaccine candidate. Taking into account various polymorphic MSP1<sub>42</sub> variants circulating in western Kenya [33], we

**Table 3. Hazard Ratio Estimates for Cox Regression Survival Analyses**

Antigen	Predictor Variable <sup>a</sup>	HR (95% CI)	<i>P</i>
3D7	IFN- $\gamma$ ELISA	<b>0.54</b> (.36–.80)	<b>.002</b>
3D7	IgG Ab ELISA	1.00 (.66–1.53)	.988
3D7	IL-10 ELISA	<b>1.62</b> (1.08–2.43)	<b>.019</b>
3D7	Age (continuous)	<b>0.97</b> (.95–.98)	<b>&lt;.0001</b>
3D7	Baseline Pf infection	<b>1.75</b> (1.13–2.74)	<b>.013</b>
3D7	HbAS genotype	0.95 (.61–1.47)	.820
FVO	IFN- $\gamma$ ELISA	0.93 (.62–1.38)	.703
FVO	IgG Ab ELISA	0.86 (.56–1.32)	.495
FVO	IL-10 ELISA	1.44 (.91–2.28)	.116
FVO	Age (continuous)	<b>0.97</b> (.95–.98)	<b>&lt;.0001</b>
FVO	Baseline Pf infection	<b>1.56</b> (1.01–2.41)	<b>.044</b>
FVO	HbAS genotype	0.81 (.53–1.24)	.335

Survival analysis for the full model, including both cellular and humoral immunity to MSP1<sub>42</sub>. Bold values denote significant results.

Abbreviations: Ab, antibody; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; HR, hazard ratio; IFN- $\gamma$ , interferon  $\gamma$ ; IgG, immunoglobulin G; IL-10, interleukin 10; MSP1<sub>42</sub>, 42-kD C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1; NS, not significant; Pf, *Plasmodium falciparum*.

<sup>a</sup> Adjusted for HbAS genotype, parasitemia status at baseline, and age.

also evaluated whether allele-specific immunity is relevant to these immune correlates. IgG antibodies to MSP1<sub>42</sub> 3D7 and FVO were abundant but not predictive of protection. In contrast, MSP1<sub>42</sub> 3D7-driven IFN- $\gamma$  responses measured by ELISA and ELISPOT correlated, respectively, with a 41% and 73% lower risk of (re)infection during the 3-month follow-up period, whereas IFN- $\gamma$  responses to the FVO variant were infrequent and not independently predictive of protection, suggesting that immunity to polymorphic T-cell epitopes within MSP1<sub>33</sub> is important to protection from parasitemia. In addition, IL-10 responses did not correlate with time to (re)infection; however, when IL-10 responses were observed to coexist with IFN- $\gamma$ , they were associated with abrogation of protection. This finding is consistent with the counterregulatory influence of IL-10 on IFN- $\gamma$  [32] and indicates that the cytokine milieu induced by malaria antigens should be considered during vaccine design and evaluation. Covariate analyses of the risk for (re)infection that include IFN- $\gamma$ , IgG antibody, and human genetic and demographic factors showed that only IFN- $\gamma$  responses to MSP1<sub>42</sub> 3D7 were protective and not additive or synergistic, that HbAS genotype was not relevant to the risk of (re)infection (contrary to results of a study from an area with seasonal malaria transmission [34]), and that asymptomatic parasitemia at baseline was associated with increased risk of (re)infection. A limitation of this study was an insufficient sample size to make firm conclusions regarding the relevance of these immune end points to the susceptibility to clinical malaria.

Protective immunity to asexual-stage *P. falciparum* is an age-related process signified by antiparasite immunity marked by control of parasite density and clinical immunity marked by reduced susceptibility to fever with parasitemia [2]. Since both phenotypes appear by late childhood and are maintained during adulthood, it is intuitive that asymptomatic low-density parasitemia is a critical factor to the development and maintenance of this immunity. Age, independent of malaria exposure history, may also be an important variable in the development of immunologic memory [35]. With respect to antibody responses, higher antimerozoite antigen IgG titers are found in asymptomatic infected children, compared with noninfected children, and rapidly wane following clinical malaria [36], suggesting that serologic responses are not stable [37, 38] and that memory B cells are slow to develop and, when considered alone, are a poor correlate of protection from parasitemia. An earlier study we conducted in a region of Kenya with low malaria transmission reported that functional antibodies to MSP1<sub>19</sub>, as measured by invasion inhibitory activity, may be more informative than standard serologic analysis [39]. However, MSP1<sub>19</sub> invasion inhibitory activity did not correlate with protection from malaria in an area of considerably higher transmission [33]. It is clear that more research is needed to understand the significance of antimerozoite antibodies to the control of parasitemia and susceptibility to clinical malaria,

including the breadth of merozoite antigens to which memory B cells and antibodies are generated [37, 40].

Studies of cellular immunity in regulating malaria susceptibility are challenging given the low frequency of T-cell subsets in peripheral blood and the many cytokines produced by antigen-specific T-cells, B-cells, and monocytes. Although we did not address the cellular source of IFN- $\gamma$  or IL-10, recent observations show that IL-10 is primarily secreted by activated effector CD4<sup>+</sup> T-cells that also secrete IFN- $\gamma$  [32]. This regulatory IL-10 may diminish the protective effect of IFN- $\gamma$  during blood-stage malaria while limiting immunopathology. Nevertheless, enhancing long-lived T-cell memory is a desired end point for human malaria vaccines, including those that include merozoite antigens such as MSP1 [41, 42]. Inadequate T-cell immunity may also result from elicitation of short-lived, effector T cells [43] and active interference [44]. Longitudinal studies of naturally infected humans have demonstrated instability of cytokine responses over time [28, 45] and qualitative differences in IFN- $\gamma$ -secreting MSP1-specific T-cells associated with age [43]. Further studies are underway to determine which T-cell subsets are associated with fewer subsequent malaria episodes and to provide a more expansive picture for the role of IL-10, in addition to transforming growth factor  $\beta$ , in inhibiting protective immunity.

Natural malaria infections in endemic areas display significant genetic heterogeneity and antigenic diversity [46], which complicate the correlation of antigen-specific immunity with strain-specific protection from infection. Studies of strain-specific immunity to MSP1<sub>42</sub> are further challenged by the fact that major T-cell and B-cell epitopes are encoded in segments of *msp1* that have undergone recombination, leading to different haplotypes that are not easily sequenced from mixed-strain infections [29, 33]. Using disparate yet region-specific genotyping methods for *msp1*, we observed allele-specific immunologic correlations of protection for antibodies to MSP1<sub>19</sub> [33] but apparently only for children, not adults. This may be due to strain-specific immunity that clears individual infections until more general immunity (including T-cell help) has been acquired [47]. Our past studies in this population show higher temporal stability of IgG to the C-terminal MSP1 FVO as compared to 3D7 [48], even though Uganda-PA and FVO, which share downstream B-cell epitopes but differ upstream where the T-cell epitopes are located, tended to be the dominant allele [33]. Combined with results presented here, this suggests that antibodies to FVO do not confer protection from infection unless IFN- $\gamma$  is also present. Immunity to 3D7 MSP1<sub>42</sub> was dominated by IFN- $\gamma$  responses, which would also cover the Uganda-PA predominant genotype. Studies in animal models demonstrate that immunity to MSP1 is strain specific [49, 50]; however, naturally acquired immunity in humans who experience mixed-strain infections are more difficult to deconvolute.

Additional studies that monitor the cumulative effect of malaria on the development and maintenance of B-cell and T-cell immunity should increase our understanding of the key elements underlying protective immunity to malaria. This information is pertinent to vaccine development and to understanding how decreasing malaria transmission intensity impacts the strength and quality of protective immunity.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank the parents and guardians, for enrolling their children in this study; our team of community health workers, without whom this study would not have been possible; Garret S. Bailey; and the Malaria Vaccine Development Branch, National Institutes of Health and Infectious Diseases. This work is published with the permission of the Director of the Kenya Medical Research Institute.

**Financial support.** This work was supported by National Institutes of Health RO1 AI43906 (J. W. K.), RO1 CA134051 (A. M. M.), Fogarty International Center 1D43TW006576 (P. O. S., K. C.), Burroughs Wellcome Fund 1006818 (A. E. D.), UL1RR031982, National Center for Research Resources (H. J. F.) and the intramural research program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases (C. L.).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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