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Title of Thesis: Humoral Immune Responses to a Malaria Vaccine Candidate: Towards a Correlate of Vaccine-Induced Protection

Eric Raphael Gottlieb, Master of Science, 2012

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Abstract:

Introduction: Identifying immune correlates of protection is a priority for malaria vaccine research. A successful pediatric Phase 2 clinical trial in Mali of FMP2.1/AS02$_A$, a recombinant apical membrane antigen 1 (AMA1)-based vaccine candidate, provided a source of serum samples from subjects who may have developed vaccine-induced, strain-specific protective immunity to clinical malaria illness. We studied IgG subclass and avidity patterns of antibodies to the malaria protein AMA1 in a subset of participants, with the objective of identifying immune responses that may be associated with protection against malaria. We hypothesized that the AMA1 vaccine candidate would induce production of cytophilic antibody subclasses IgG1 and IgG3, as well as overall IgG avidity maturation.

Methods: Titers of IgG1, IgG2, IgG3, and IgG4, as well as avidity of antibodies to AMA1, were determined by ELISA for ten AMA1 vaccine recipients and ten control subjects who had been randomized to receive a rabies vaccine in this double-blind trial at days 0, 90, and 150 after the first of three vaccinations. To identify statistically significant differences between the groups, responses in vaccine recipients were evaluated longitudinally and compared with responses in control subjects.

Results: IgG1, IgG2, IgG3, and IgG4 were induced more strongly in vaccine recipients than in control subjects. Additionally, vaccine recipients had higher ratios of cytophilic to non-cytophilic antibodies than control subjects. Avidity indices were not significantly different between the two groups at the three time points tested, and there were no significant differences in avidity between time points in either group.

Conclusion: Contrary to our hypothesis, both cytophilic and non-cytophilic antibodies were induced by the FMP2.1/AS02$_A$ vaccine candidate and immunization did not appear to stimulate avidity maturation. Therefore, IgG1, IgG2, IgG3, and IgG4 titers are candidate variables for a humoral immune correlate of vaccine-induced protection. These results are among the first reports of the subclasses and avidity of antibodies produced in response to a malaria vaccine candidate with allele-specific protective efficacy.

Humoral Immune Responses to a Malaria Vaccine Candidate: Towards a Correlate of Vaccine-Induced Protection

Eric Raphael Gottlieb

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AMA1: Apical Membrane Antigen 1, expressed by sporozoites and merozoites

$AS02_A$: Adjuvant System 2A, proprietary adjuvant provided by GlaxoSmithKline

ELISA: Enzyme-Linked Immunosorbent Assay

FMP1: Falciparum Malaria Protein 1, recombinant MSP1 protein

FMP2.1 Falciparum Malaria Protein 2.1, recombinant AMA1 protein

HLA: Human Leukocyte Antigen

IgG: Immunoglobulin G, includes subclasses IgG1-IgG4

IL-4: Interleukin 4

kDa: Kilodalton

MSP1/MSP2: Merozoite Surface Protein 1/2

PBS: Phosphate Buffered Saline

RON: Rhoptry Neck, family of merozoite proteins involved in erythrocyte invasion

ROS: Reactive Oxygen Species

WRAIR: Walter Reed Army Institute of Research

I. Introduction

More than 200 million people suffer from malaria each year and between 655,000 and 1.2 million people die, making malaria a leading cause of morbidity and mortality among children in developing nations (Murray et al., 2012; WHO, 2011). Development of an effective malaria vaccine is therefore a priority in global health, but this has been difficult because the *Plasmodium* parasites that cause the disease are genetically diverse, invade host cells through complex mechanisms, and are adept at evading the immune system. An additional challenge is that there are no defined immune correlates of protection. Identifying a biological measure of immunity would make it possible to design and evaluate vaccine candidates more efficiently. This study was undertaken to characterize the humoral immune response to an experimental malaria vaccine and identify candidate measures of protection.

Malaria vaccine candidates are designed to prevent or control the disease by inducing immune responses against antigens expressed during targeted stages of infection. In the liver stage, which occurs shortly after an individual is bitten by an infected mosquito, sporozoites invade hepatocytes, within which they multiply as much as 20,000-fold (Sturm et al., 2009), causing the cells to rupture. Large numbers of merozoites are released into the blood, where they invade erythrocytes and multiply 10 to 20-fold, again causing the cells to burst and releasing daughter merozoites to invade more cells. The severe pathology of *P. falciparum* malaria is largely a consequence of parasite proteins that are expressed on the surface of infected erythrocytes and aberrantly bind to the endothelium. This obstructs the vasculature, prevents clearance of infected erythrocytes by the spleen, and causes chronic inflammation, reduced tissue perfusion,

and a variety of effects on the surrounding tissue. It also causes the destruction of both infected and uninfected erythrocytes (Miller et al., 2002).

Several blood stage vaccine candidates have been developed with the objective of inducing an immune response that would inhibit erythrocyte invasion and merozoite replication to directly prevent severe disease. Two antigens to which antibody levels have been associated with natural immunity are Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Protein 1 (MSP1) (Dodoo et al., 2011). A vaccine including a recombinant fragment of the MSP1 protein derived from the 3D7 strain of *P. falciparum* and suspended in the adjuvant system $AS02_A$ (FMP1/$AS02_A$) was found to be safe and immunogenic in adults recruited at the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, MD (Ockenhouse et al., 2006) and in Bandiagara, Mali (M. Thera et al., 2006), but a subsequent clinical trial in children in Kenya showed that it was not protective (Ogutu et al., 2009).

The FMP2.1/$AS02_A$ vaccine candidate, which is composed of recombinant AMA1 from the same strain of parasite and is formulated with the same adjuvant, was also found to be safe and immunogenic in adults at WRAIR (Polhemus et al., 2007) and in Bandiagara (M. Thera et al., 2008). Although it did not significantly reduce the cumulative incidence of malaria in a subsequent study in children aged between one and six years in Bandiagara, the vaccine was found to effectively protect subjects from clinical episodes associated with infection with parasites that had an amino acid sequence matching that of the 3D7 vaccine strain at a highly polymorphic domain of AMA1, the C1 loop. Molecular epidemiological studies had previously found that the C1 loop is the portion of AMA1 that is the most significant determinant of allele-specific natural

protective immunity against clinical malaria (Takala et al., 2009). During the first 240 days after immunization, the vaccine candidate had more than 60% efficacy against episodes associated with infection by these strains (M. A. Thera et al., 2011). It is proposed that a vaccine combining this and other similarly effective antigens would confer broader protection (Heppner et al., 2005). However, the AMA1-Combination 1, which included a mixture of 3D7 and FVO-strain AMA1 antigen and was one of the first multivalent vaccine candidates, failed to confer either overall or allele-specific protection in a Phase 2 pediatric clinical trial conducted in Bancoumana, Mali (Ouattara et al., 2010; Sagara et al., 2009).

The success of FMP2.1/AS02$_A$ in conferring allele-specific protection provides a novel opportunity to study the humoral and cell-mediated immune responses that explain the efficacy of this vaccine candidate. This is a priority for vaccine research because it makes it possible to assess efficacy by immunizing healthy volunteers and testing for markers of immunity rather than conducting costly challenge experiments with live parasite infections. Only vaccine candidates that induce an effective immune response relative to a benchmark correlate of protection would graduate to expensive, large-scale field trials. There is also evidence that certain peptide epitopes can drive class switching to specific isotype subclasses (Tongren et al., 2006). Therefore it may be possible to design a more effective vaccine by identifying antibody subclasses that are associated with protection and incorporating these epitopes accordingly. Once a vaccine is licensed, a correlate provides a benchmark against which to measure protection after immunization. Correlates of protection have been established for vaccine-induced immunity to diseases including tetanus, diphtheria, and measles (Plotkin, 2010).

Most studies of naturally acquired immunity to malaria have observed a poor correlation between overall antibody titers to malaria antigens and clinical protection (Kusi et al., 2012; Olesen et al., 2010), although associations with antibodies to specific protein domains have been observed (Polley et al., 2004). However, in many cases where relationships with overall antibody titers are insignificant, IgG subclass titers have been shown to be associated with protection. The most common associations are with higher levels of the cytophilic subclasses IgG1 and IgG3. (Courtin et al., 2009; Ferreira et al., 1996; Leoratti et al., 2008; Mehrizi et al., 2011; Olesen et al., 2010; Richards et al., 2010; Stanisic et al., 2009). Cytophilic antibodies bridge the humoral and cell-mediated immune responses by interacting with receptors on neutrophils and monocytes through their constant (Fc) regions. IgG2 may also be cytophilic in individuals with a compatible receptor mutation, and titers of IgG2 have been shown to be associated with protection in these subjects. IgG4, which is not cytophilic, has been shown to be more highly expressed in subjects with complicated malaria, as compared with uncomplicated malaria and asymptomatic parasitemia. Subjects with fewer previous malaria attacks, who likely have a less mature immune response, have also been shown to have higher IgG4 titers than individuals with more previous exposure (Leoratti et al., 2008).

Studies of natural immunity have also shown that relative subclass titers are largely a function of the antigen, rather than the individual. In children in Papua New Guinea, the response to AMA1 and the 19 kDa fragment of MSP1 ($MSP1_{19}$) was IgG1-dominant, but IgG3 was more highly induced by MSP2 (Stanisic et al., 2009). Furthermore, children with high levels of IgG3 to AMA1 were not more likely to have higher levels of IgG3 than IgG1 to $MSP1_{19}$. Certain characteristics of the individual, such

as the sickle cell mutation (Ntoumi et al., 2002) and HLA alleles (Stirnadel et al., 2000), have been found to affect subclass patterns, but the impact is antigen-specific. From a functional standpoint, it should be noted that the subclass with the highest titer may or may not be the primary driver of protection. In the same cohort in Papua New Guinea, IgG3 titers were most strongly associated with protection, even for antigens that induced higher titers of IgG1 (Richards et al., 2010).

Avidity, or binding strength of antibodies to their targets, is another characteristic of the humoral immune response that may influence protection. This measure of intrinsic antibody function would distinguish effective responses from cases in which subjects have the same antibody titers but are not as well protected. Avidity has been shown to differ across malaria transmission settings and to be associated with different clinical presentations and history of malaria. In a study comparing clinically immune African subjects to acutely ill or recently recovered migrant workers in the Amazon, it was found that convalescent Amazonians and immune Africans had similarly high avidity antibodies, but acutely ill Amazonians had much lower avidity antibodies. This suggests that avidity maturation contributes to the development of immunity after exposure to malaria (Ferreira et al., 1996). Likewise, in the previously discussed study comparing subclass responses in subjects in Papua New Guinea with different clinical presentations and previous exposure to malaria, those with higher titers of IgG4, an indicator of an immature response, were also found to have lower avidity antibodies to the vaccine antigen than individuals who were better protected (Leoratti et al., 2008). Finally, low avidity has also been found to predict or otherwise be associated with the failure of licensed vaccines for other infectious diseases, such as respiratory syncytial virus (RSV)

(Delgado et al., 2009), *Haemophilus influenzae* type b (Lee et al., 2008), and meningococcal disease (Auckland et al., 2006).

The objective of the current study was to determine which IgG subclasses of AMA1-specific antibodies were preferentially induced by the FMP2.1/AS02$_A$ vaccine candidate as compared with natural exposure alone, whether there was a significant difference in avidity of anti-AMA1 antibodies between vaccine recipients and control subjects, and at which time point after immunization these differences were most significant. A subsequent study will test these candidate markers in a larger group of subjects in conjunction with data on clinical malaria episodes to detect any association between subclass titers or avidity and protection from malaria. Although a vaccine-induced immune response may not mirror natural immunity, we hypothesized that the cytophilic antibodies IgG1 and IgG3 would be induced by the vaccine candidate. We expected that IgG1 and IgG3 antibody titers would be equivalent in vaccine recipients and control subjects at the time of the first immunization (day 0, with subsequent immunizations on days 30 and 60), but that they would have increased significantly in vaccine recipients by day 90 and remain elevated or show a moderate decline by day 150. We also expected avidity of anti-AMA1 antibodies to be significantly higher in vaccine recipients at day 90 and day 150 than in control subjects.

II. Methods

Description of Serum Samples

Subjects were drawn from a Phase 2, double-blind, randomized, pediatric clinical trial of the FMP2.1/AS02$_A$ malaria vaccine candidate conducted in Bandiagara, Mali, West Africa between 2007 and 2008 (M. A. Thera et al., 2011). Children were randomized 1:1 to receive either the FMP2.1/AS02$_A$ malaria vaccine candidate or a rabies vaccine as a comparator vaccine on days 0, 30, and 60. Bandiagara is a rural town with a population of approximately 14,000. The peak malaria season in Bandiagara extends from July to December. *P. falciparum* is the dominant species of malaria-causing parasite and is transmitted by *Anopheles gambiae* mosquitoes (M. Thera et al., 2008). All participants in the clinical trial were between one and six years old and were healthy at the time of enrollment. Subjects included in this study had IgG titers to the vaccine protein, recombinant 3D7 strain-like Apical Membrane Antigen 1 (AMA1), between the 25th and 75th percentile of individuals with their respective vaccine assignment at day 90 after the first immunization, as measured by our collaborators at the Walter Reed Army Institute of Research (WRAIR).

Study Design

Ten subjects were randomly selected from the pools of recipients of the FMP2.1/AS02$_A$ vaccine and control subjects who received the RabAvert rabies vaccine (Chiron Vaccines, Emeryville, CA). The recombinant AMA1 vaccine antigen was provided by WRAIR (Silver Spring, MD) and the proprietary adjuvant system AS02$_A$ was provided by GlaxoSmithKline Biologicals (Rixensart, Belgium). The investigators

were blinded to vaccine assignments until all assays were complete. Serum samples from days 0, 90, and 150 after vaccination were tested by enzyme-linked immunosorbent assay (ELISA) for each subject. Positive control samples were drawn from a pool of sera collected from 15 adults on day 74 after vaccination in a Phase 1 clinical trial of a Merozoite Surface Protein 1 (MSP1) vaccine previously conducted in Bandiagara (M. Thera et al., 2006). All subjects contributing to the positive control pool had experienced a malaria episode between 12 and 46 days prior to the blood draw. This study was approved by the institutional review boards of the University of Maryland, Baltimore (IRB #HP00041298) and the University of Sciences, Techniques, and Technology, Mali (IRB #FWA00001769).

ELISA Protocol for IgG Subclasses

Ninety-six-well round-bottom Immulon 2 plates (Thermo Fisher Scientific, Waltham, MA) were coated with 100 µL per well of recombinant AMA1 diluted in PBS at a concentration of 0.5 µg/mL and incubated at 37 degrees Celsius for three hours. Plates were washed with PBS 0.05% Tween-20 (PBS Tween) six times by a Biotek ELx405 automated plate-washer (Biotek, Winooski, VT), blocked with 10% nonfat milk in PBS, and incubated at four degrees Celsius overnight.

Serum was diluted in PBS Tween with 10% nonfat milk (PBS milk Tween). Serum in diluent was added to duplicate wells and was serially diluted by a factor of two, such that 100 µL remained in each well. Wells with 100 µL of PBS milk Tween were used as negative controls. Dilutions for subject serum samples were determined by trial and error, such that a reliable titer could be obtained. A positive control sample was

included on each plate at a predetermined dilution. Plates were incubated for two hours at 37 degrees Celsius.

Plates were washed and 100 µL of subclass-specific horseradish peroxidase-conjugated (HRP) sheep-anti-human secondary antibodies (The Binding Site, Birmingham, UK) diluted in PBS milk Tween at concentrations of 1:400, 1:200, 1:250, and 1:100 for IgG1, IgG2, IgG3, and IgG4, respectively, was added to all wells. Plates were incubated at 37 degrees Celsius for one hour and then washed.

The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) was warmed to room temperature and 100 µL was added to each well. Plates were placed in the dark for fifteen minutes. The reaction was then stopped with 100 µL per well of 1M phosphoric acid, and optical densities (ODs) were read by a Spectramax M2 plate-reader (Molecular Devices, Sunnyvale, CA) at 450nm. Endpoint titer was defined as the dilution predicted to give an OD of 0.2. This was extrapolated from the dilution that would give an OD of 0.5, which is more reliably measured than a lower OD by our assay.

ELISA Protocol for Avidity and Overall IgG

Avidity was measured by an ELISA procedure similar to that used for subclass titers, except that duplicate assays were prepared and incubated with the primary serum. The plates were then washed, and 100 µL of 5M urea in PBS Tween was added to each well on one assay and 100 µL of PBS Tween was added to the other. The plates were placed at room temperature for 30 minutes. The urea was expected to detach a portion of the antibodies from the antigen according to their avidity: high avidity antibodies would be most resistant to urea. The plates were washed and 100 µL of HRP-conjugated goat

anti-human IgG was added at a dilution of 1:10,000 (Jackson ImmunoResearch Laboratories**,** West Grove, PA). Avidity index was calculated as the ratio of the endpoint titer of the urea assay to the endpoint titer of the PBS Tween assay. Reported titers for overall IgG were derived from the PBS Tween avidity assays.

Statistical Analysis

After all assays were complete, vaccine assignments were un-blinded. Titers and avidity indices for vaccine recipients were compared with those of negative controls for each subclass and time point using a one-tailed Mann-Whitney-Wilcoxon rank sum test. Cytophilic ratios were calculated as the sum of IgG1 and IgG3 titers divided by the sum of IgG2 and IgG4 titers. Cytophilic ratios for vaccine recipients and control subjects were compared using a one-tailed Mann-Whitney-Wilcoxon rank sum test. Differences in titers, avidity, and cytophilic ratios across time points were compared by using a one- or two-tailed Mann-Whitney-Wilcoxon rank sum test, as appropriate. Significance was defined as having a *P*-value of less than 0.05. All analyses were conducted in Microsoft Excel 2010 (Redmond, WA) or GraphPad Prism 5.0 (La Jolla, CA).

III. Results

IgG and Subclass Titers

Overall anti-AMA1 IgG and subclass titers for FMP2.1/AS02$_A$ recipients and control subjects, who received a rabies vaccine, are reported in **Table 1** and graphed in **Figure 1**. At both day 90 and day 150, anti-AMA1 IgG1, IgG2, IgG3, and IgG4 titers were significantly higher in FMP2.1/AS02$_A$ recipients than in negative control subjects ($P < 0.0001$ except IgG4 day 150 $P = 0.0001$). There were no significant differences between groups for any IgG subclass at day 0.

Differences in titers across time points are shown in **Table 2**. IgG and subclass titers in vaccine recipients were significantly higher at day 90 than at day 0 ($P < 0.0001$ except IgG2 $P = 0.0001$). The titers of IgG (day 90 vs. day 150 $P < 0.0001$, day 0 vs. day 150 $P < 0.0001$) and subclasses IgG1 (day 90 vs. day 150 $P = 0.001$, day 0 vs. day 150 $P < 0.0001$), IgG2 (day 90 vs. day 150 $P = 0.0051$, day 0 vs. day 150 $P = 0.0002$), and IgG3 (day 90 vs. day 150 $P < 0.0001$, day 0 vs. day 150 $P = 0.0001$) all declined from day 90 to day 150, but remained higher than at day 0. IgG4 titers did not significantly decrease from day 90 to day 150 (day 90 vs. day 150 $P = 0.1577$, day 0 vs. day 150 $P = 0.0001$).

Overall IgG titers were not significantly different between time points for control subjects (day 0 vs. day 90 $P = 0.5787$, day 0 vs. day 150 $P = 0.1431$, day 90 vs. day 150 $P = 0.0753$). However, titers of IgG2 ($P = 0.0471$) and IgG3 ($P = 0.0254$) increased from day 0 to day 150 in control subjects, and IgG3 titers increased from day 90 to day 150 ($P = 0.0147$).

Avidity

Anti-AMA1 IgG avidity for FMP2.1/AS02$_A$ recipients and control subjects are reported in **Table 1** and graphed in **Figure 1**. Differences in avidity across time points are shown in **Table 2**. Differences in avidity were not statistically significant at any of the time points tested, and avidity indices did not change significantly in either vaccine recipients or control subjects.

Table 1: Titers and avidity indices of antibodies to AMA1. Titers of IgG and all four subclasses were higher in FMP2.1/AS02$_A$ malaria vaccine recipients than in control subjects, but there was no significant difference in avidity. Geometric mean, minimum, and maximum titers are shown for FMP2.1/AS02$_A$ recipients and control subjects. P-values were determined by a Mann-Whitney-Wilcoxon rank sum test. Asterisks indicate significance ($P < 0.05$).

		Day 0				Day 90				Day 150			
		Geo. Mean	Min	Max	P-value	Geo. Mean	Min	Max	P-value	Geo. Mean	Min	Max	P-value
IgG	FMP 2.1/AS02$_A$	1,745	213	192,926	0.4524	958,344	674,591	1,505,372	*<0.0001	392,192	161,347	666,108	*<0.0001
	Rabies	1,845	195	6,558		1,281	106	11,695		3,943	129	14,820	
IgG1	FMP 2.1/AS02$_A$	423	13	37,211	0.4853	231,127	147,636	283,115	*<0.0001	94,377	47,536	453,823	*<0.0001
	Rabies	351	25	3,018		320	58	2,379		977	105	4,098	
IgG2	FMP 2.1/AS02$_A$	19	13	112	0.1726	5,450	3,337	12,429	*<0.0001	2,205	467	6,188	*<0.0001
	Rabies	22	13	59		24	13	84		39	13	83	
IgG3	FMP 2.1/AS02$_A$	19	13	62	0.4470	6,163	3,253	16,407	*<0.0001	1,350	586	3,636	*<0.0001
	Rabies	22	13	190		22	13	709		63	13	518	
IgG4	FMP 2.1/AS02$_A$	17	13	92	0.3683	2,237	290	16,566	*<0.0001	1,285	467	3,838	*0.0001
	Rabies	18	13	65		18	13	25		48	13	1,161	
		Mean	Min	Max	P-value	Mean	Min	Max	P-value	Mean	Min	Max	P-value
Avid.	FMP 2.1/AS02$_A$	0.35	0.09	0.89	0.1678	0.31	0.15	0.56	0.1965	0.30	0.15	0.52	0.0782
	Rabies	0.21	0.09	0.47		0.26	0.05	0.52		0.23	0.03	0.48	

Figure 1: Titers and avidity indices of antibodies to AMA1. IgG and subclass geometric mean titers and avidity indices at days 0, 90, and 150 are shown with 95% confidence intervals for FMP2.1/AS02$_A$ malaria vaccine recipients (●) and control subjects (■). Titers and avidity indices were measured by ELISA. Asterisks indicate significant differences ($P < 0.05$) in median titers and avidity indices between vaccine recipients and control subjects, as determined by a Mann-Whitney-Wilcoxon rank sum test.

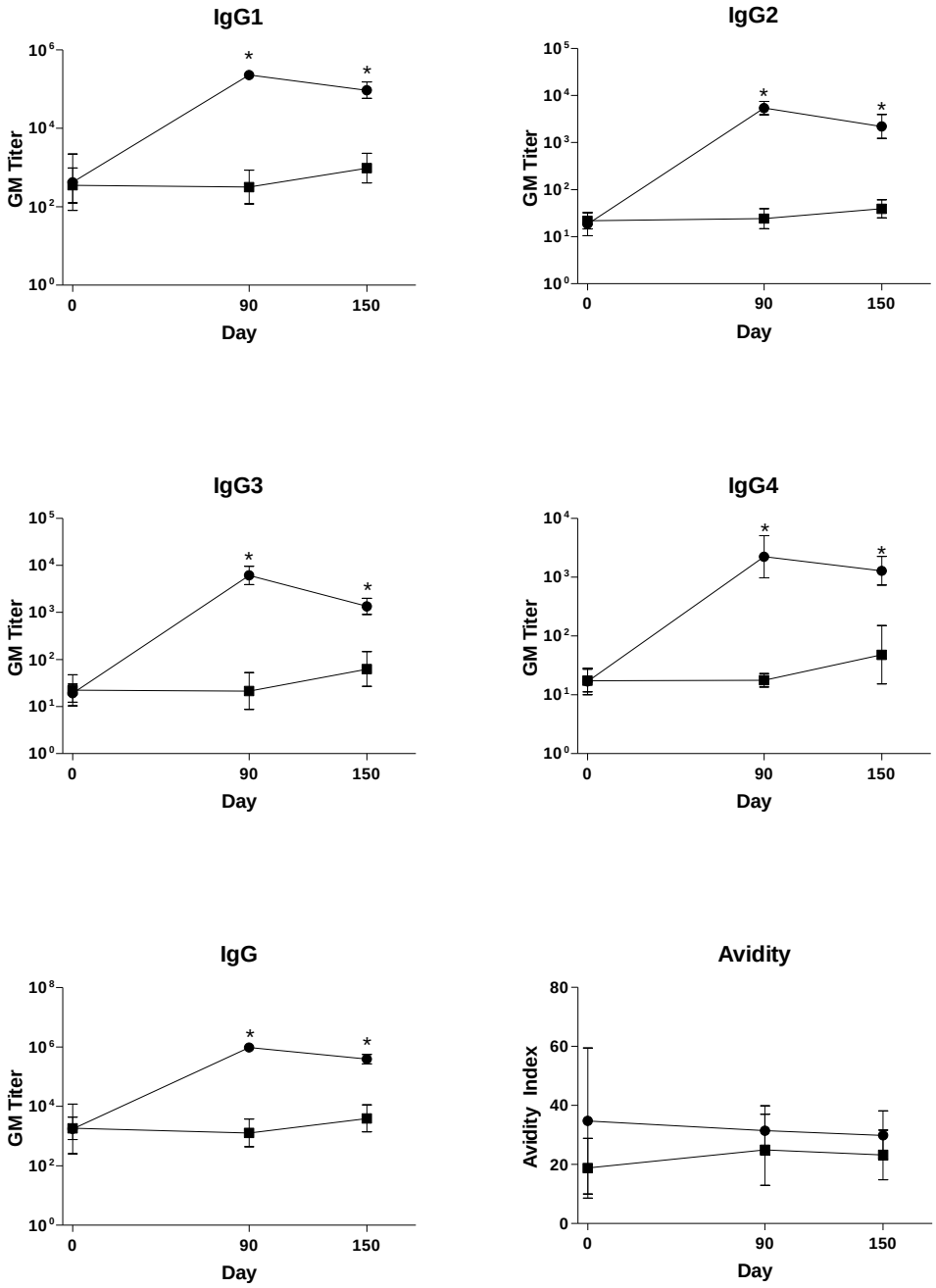


Table 2: Changes in titers and avidity indices between time points. Titers of overall IgG and all four subclasses were higher at days 90 and 150 than at day 0 in FMP2.1/AS02$_A$ malaria vaccine recipients. *P*-values were determined by a Mann-Whitney-Wilcoxon rank sum test. Asterisks indicate significance ($P < 0.05$).

		Day 0 vs. Day 90 P-value	**Day 0 vs. Day 150 P-value**	**Day 90 vs. Day 150 P-value**
IgG	**FMP 2.1/AS02$_A$**	*<0.0001	*<0.0001	*<0.0001
	Rabies	0.5787	0.1431	0.0753
IgG1	**FMP 2.1/AS02$_A$**	*<0.0001	*<0.0001	*0.001
	Rabies	0.8534	0.1655	0.0630
IgG2	**FMP 2.1/AS02$_A$**	*0.0001	*0.0002	*0.0051
	Rabies	0.7797	*0.0471	0.1226
IgG3	**FMP 2.1/AS02$_A$**	*<0.0001	*0.0001	*<0.0001
	Rabies	1.0	*0.0254	*0.0147
IgG4	**FMP 2.1/AS02$_A$**	*<0.0001	*0.0001	0.1577
	Rabies	0.6730	0.1431	0.2393
Avidity	**FMP 2.1/AS02$_A$**	0.5726	0.5414	0.6038
	Rabies	0.4002	0.4470	0.6842

Cytophilic Ratios

The ratios of cytophilic antibodies (IgG1 and IgG3) to non-cytophilic antibodies (IgG2 and IgG4) were higher at day 90 ($P = 0.0144$) and day 150 ($P = 0.001$) in vaccine recipients than in control subjects (**Table 3**, **Figure 2**). There was not a statistically significant change in cytophilic ratios in vaccine recipients between days 90 and 150 ($P = 0.5490$, **Table 4**).

Table 3: Differences in cytophilic ratios by vaccine assignment. Cytophilic ratios [(IgG1 + IgG3)/(IgG2+IgG4)] were higher in FMP2.1/AS02$_A$ malaria vaccine recipients than in control subjects at days 90 and 150. *P*-values were determined by a Mann-Whitney-Wilcoxon rank sum test. Asterisks indicate significance ($P < 0.05$).

	Day 0		Day 90		Day 150	
	Cytophilic Ratio	P-value	Cytophilic Ratio	P-value	Cytophilic Ratio	P-value
FMP 2.1/AS02$_A$	14.71	0.4657	30.66	*0.0144	41.28	*0.001
Rabies	12.79		13.83		14.01	

Figure 2: Cytophilic Ratios. Mean cytophilic ratios are shown with 95% confidence intervals for FMP2.1/AS02$_A$ malaria vaccine recipients (●) and control subjects (■) at days 0, 90, and 150. Cytophilic antibodies were more dominant in vaccine recipients than in control subjects. Asterisks indicate significant differences ($P < 0.05$) between FMP2.1/AS02$_A$ recipients and control subjects or differences between time points for vaccine recipients, as determined by a Mann-Whitney-Wilcoxon rank sum test.

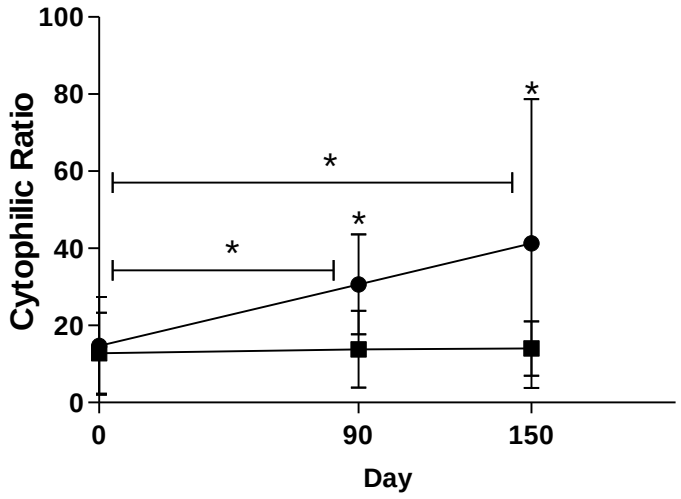


Table 4: Changes in cytophilic ratios between time points. Cytophilic ratios [(IgG1 + IgG3)/(IgG2+IgG4)] were higher at days 90 and 150 than at day 0 in FMP2.1/AS02$_A$ malaria vaccine recipients. *P*-values were determined by a Mann-Whitney-Wilcoxon rank sum test. Asterisks indicate significance ($P < 0.05$).

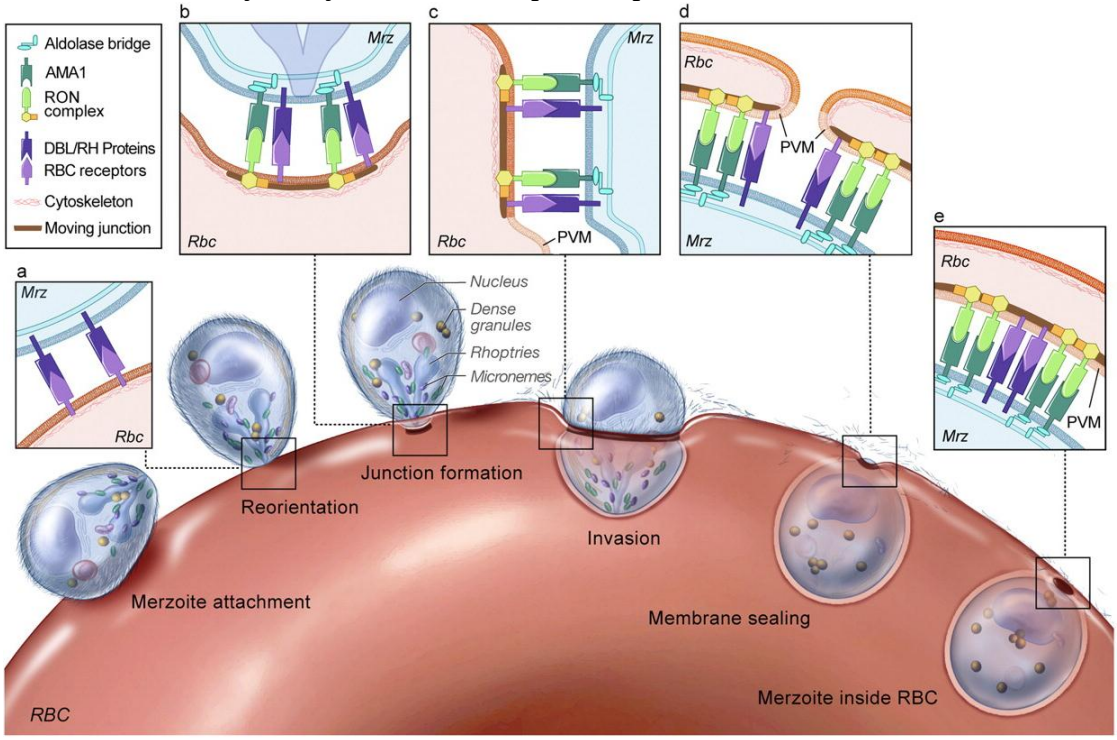
	Day 0 vs. Day 90	**Day 0 vs. Day 150**	**Day 90 vs. Day 150**
	P-value	**P-value**	**P-value**
FMP 2.1/AS02$_A$	*0.0140	*0.0157	0.5490
Rabies	0.4841	0.3019	0.3421

IV. Discussion

In this study, we showed that IgG1, IgG2, IgG3, and IgG4 antibodies to a recombinant *P. falciparum* 3D7 strain-like AMA1 protein were all induced by malaria vaccine candidate FMP2.1/AS02$_A$. The ratios of cytophilic to non-cytophilic antibodies were significantly higher in vaccine recipients than in control subjects. We did not detect avidity maturation, but this may have been because we did not select for subjects for whom the vaccine conferred protection. Other limitations to this study include that there were 10 assays out of 300 that could not be completed because the serum was depleted or was judged to be expired after repeated failed experiments. In a few cases, the judgment of the principal investigator was used to evaluate the validity of results, such as when to disregard aberrant ODs from individual wells on an ELISA plate.

A strong antibody response is a logical mechanism of protection because surface proteins, such as AMA1 and the MSP family, are accessible to antibodies and are critical to erythrocyte invasion (Dodoo et al., 2011). The invasion process begins with the merozoite attaching to the erythrocyte and reorienting itself to come into closer contact. The merozoite then secretes proteins into the erythrocyte from the neck of the rhoptry secretory organ. At this point a junction forms, allowing additional rhoptry secretion, invasion driven by merozoite motor proteins, and formation of a parasitophorous vacuole. Junction formation depends on AMA1 binding to a complex of rhoptry neck (RON) proteins, and this interaction has been blocked *in vitro* with monoclonal antibodies targeting specific epitopes on AMA1 (Srinivasan et al., 2011). The sequence of AMA1-mediated erythrocyte invasion is illustrated in **Figure 3.**

Figure 3: Sequence of AMA1-mediated merozoite invasion of erythrocytes. In the blood stage of infection, the merozoite attaches to the erythrocyte, reorients itself, and secretes rhoptry proteins into the erythrocyte. AMA1 binds to rhoptry neck (RON) proteins to form a junction, allowing further rhoptry protein secretion. The merozoite then invades the erythrocyte and forms a parasitophorous vacuole around itself.



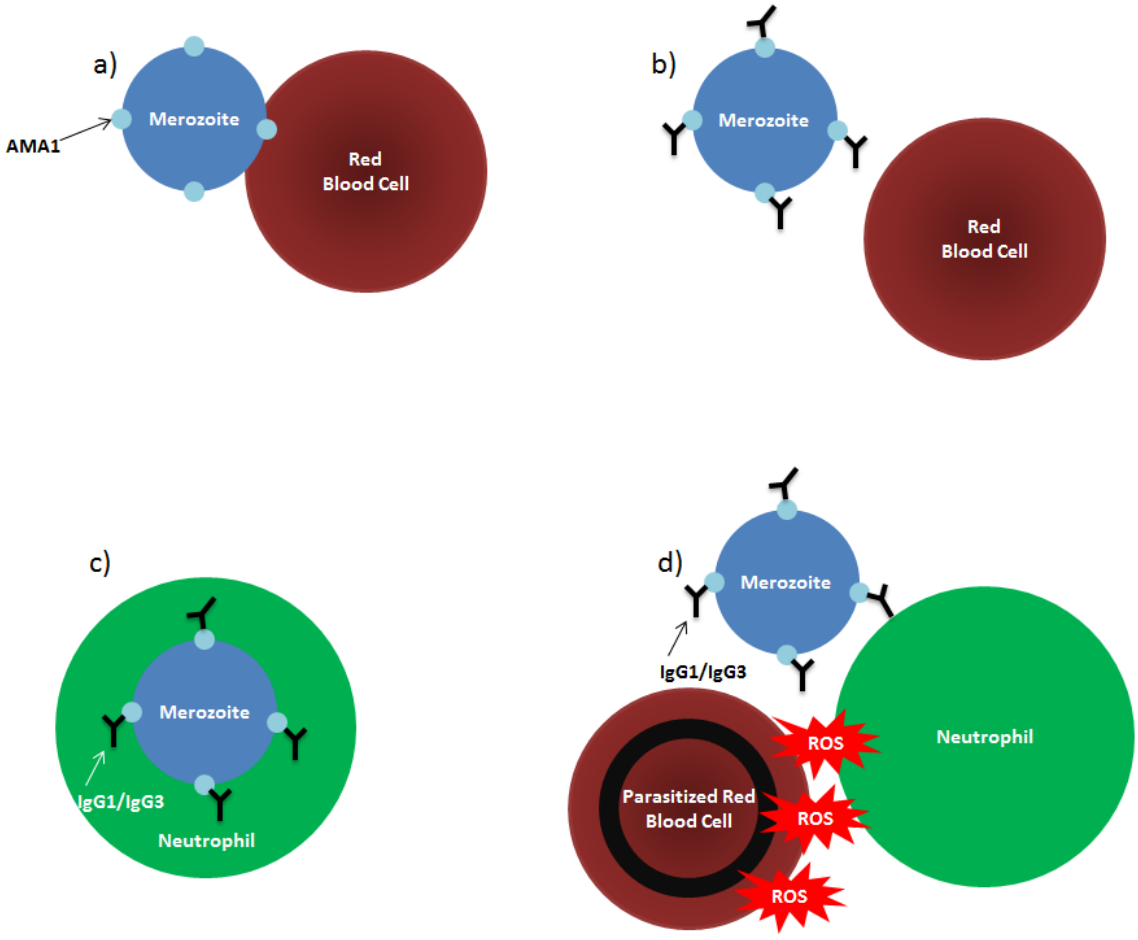
Reproduced from Srinivasan et al. (2011) with permission of the authors

Anti-AMA1 antibodies can prevent invasion by inhibiting critical processing steps and protein-protein interactions (Dutta et al., 2005). However, the relationship between overall antibody titers, invasion inhibitory activity, and protection remains unclear. For example, a study in mice showed that antibody levels were inversely associated with peak parasitemia after challenge, but were not associated with invasion inhibitory activity *in vitro* (Murhandarwati et al., 2010). Several mice in this study were completely protected without any antibody-mediated invasion inhibitory activity being detected. In humans, it has been shown that individuals with different levels of immunity may have similar total IgG levels (Olesen et al., 2010), but differences in subclass

profiles are associated with differences in protection (Courtin et al., 2009; Ferreira et al., 1996; Leoratti et al., 2008; Mehrizi et al., 2011; Olesen et al., 2010; Richards et al., 2010; Stanisic et al., 2009). These findings together imply that cellular immune responses mediated by specific antibody subclasses are critical for protection.

It was first shown in the 1980s that parasitized erythrocytes can stimulate neutrophils to undergo a metabolic burst in the presence of immune serum with active complement (Salmon et al., 1986). More recent studies have confirmed that an important function of antibodies is to opsonize merozoites and interact with neutrophils to stimulate both phagocytosis and production of reactive oxygen species (ROS), which are harmful to intra-erythrocytic parasites. Furthermore, IgG1 and IgG3 levels have been shown to correlate with ROS production, and lower ROS production is associated with a higher risk of clinical malaria illness (Joos et al., 2010). The function of cytophilic antibodies in mediating interactions between merozoites and immune cells is further supported by studies in which antibody fragments that recognize MSP1 and the FcγRI receptor, respectively, were connected by a flexible linker. This molecule stimulated a respiratory burst three times more efficiently than anti-MSP1 IgG1 (Pleass et al., 2003). The various mechanisms of antibody-mediated protection are illustrated in **Figure 4.**

Figure 4: Mechanisms of red blood cell (erythrocyte) invasion and antibody-mediated protection from blood stage disease. a) AMA1 mediates invasion of red blood cells by merozoites. **b)** Anti-AMA1 antibodies prevent merozoites from invading red blood cells. **c)** Cytophilic anti-AMA1 antibodies opsonize merozoites and stimulate phagocytosis by neutrophils. **d)** Cytophilic antibodies opsonize merozoites and stimulate neutrophils to produce reactive oxygen species (ROS), which are toxic to intra-erythrocytic parasites. This prevents merozoites from replicating and invading more red blood cells.



As expected, the cytophilic subclasses IgG1 and IgG3 were induced by the vaccine, but titers of the non-cytophilic subclasses IgG2 and IgG4 increased as well. The heightened levels of non-cytophilic antibodies might be undesirable for protection because these antibodies would likely compete with their cytophilic counterparts and limit the cell-mediated response (Ferreira et al., 1996; Leoratti et al., 2008; Ntoumi et al., 2002). Nonetheless, the higher ratio of cytophilic to non-cytophilic antibodies in vaccine recipients than in control subjects at days 90 and 150 indicates that the vaccine response is weighted towards the subclasses capable of inducing a cellular response. While not statistically significant in this study, the mean cytophilic ratio appeared to increase between day 90 and day 150 in vaccine recipients, and this may suggest a continued process of enhancement of the antibody repertoire.

Despite the importance of cytophilic antibodies, there is evidence that contradicts the assumption of a clear relationship between titers of these subclasses and protection. One case is a mouse study of the effects of knocking out IL-4, a key driver of class switch recombination. Compared with control mice, the levels of IgG1 were reduced 15 to 100-fold after immunization, as expected, and the levels of IgG2a/c and IgG2b were increased to compensate, but there was no significant difference in protection after challenge with *P. chabaudi* (Burns et al., 2004). In another study, IgG1 antibodies to AMA1 and MSP1 in mice immunized with these antigens were shown to be inversely associated with peak parasitemia after challenge. These relationships were used to prospectively predict peak parasitemia after subsequent challenges. The predictions were strongly correlated with observed parasitemia, except in mice with the highest pre-challenge antibody titers to MSP1. These four mice all had higher peak parasitemia than

was predicted. The authors proposed that excessive antibody titers to a single antigen might inhibit the development of a diverse repertoire that is needed for effective protection (Lynch et al., 2009).

Additionally, a moderate immune response that maintains a certain level of parasitemia might be beneficial for boosting cell-mediated immunity to the parasite**.** Experiments in mice have shown that robust T-cell (Stephens et al., 2010) and B-cell (Achtman et al., 2007) memory develops in response to chronic infection. These mice are more resistant to reinfection and high density parasitemia than those without chronic parasitemia, such as mice whose infections were cleared by chloroquine. In humans, initial parasitemia, as assessed by slide positivity, is associated with faster clearance of subsequent infections (Polley et al., 2004). Therefore, if excessive antibody titers do not permit low-level parasitemia to be maintained, they may be detrimental to long-term protection.

Contrary to what we had anticipated, we did not detect avidity maturation in either vaccine recipients or control subjects over this period. Avidity maturation has previously been reported in subjects with natural exposure to malaria, but antibodies to some antigens – even different variants of the same protein – more readily show evidence of avidity maturation than others. For example, in subjects in Uganda and Tanzania, antibodies to AMA1 had higher avidity than antibodies to MSP2 and antibodies to the 3D7 variant of MSP2 bound more strongly than antibodies against the FC27 variant (Reddy et al., 2012). This is likely due to properties of the protein antigen, such as hydrophilicity and stability of conformation. However, only avidity of antibodies to MSP2-3D7 was associated with protection. In studies of *P. chabaudi* infection in mice,

avidity maturation was detected over the course of several months and was accelerated by reinfection (Achtman et al., 2007).

The subjects in this study may not have shown avidity maturation for a number of reasons. First, since the vaccine was not completely protective, even against clinical malaria due to infection with parasites with 3D7-like AMA1, it is possible that avidity maturation only occurred in a subset of subjects for whom the vaccine conferred protection and was not apparent in our random sample. A follow-up study is planned to measure avidity maturation specifically in subjects who were protected by the vaccine. Alternatively, the lack of observed avidity maturation might be related to the kinetics of exposure to the vaccine antigen or the short time over which samples were collected. By comparison, Reddy et al. (2012) reported that avidity of antibodies to AMA1 gradually increased up to the age of 16, and therefore a change may not have been apparent over the five-month period of this study. Finally, evidence of avidity maturation in a small compartment of the antibody repertoire may have been masked by the avidity of the overall sample. It has been proposed that a small number of high avidity antibodies may rapidly bind to the merozoite and slow the kinetics of erythrocyte invasion, giving the bulk population of lower avidity antibodies more time to bind (Mehrizi et al., 2011; Reddy et al., 2012). However, these antibodies may be too sparse to be detected in unfractionated serum.

Overall, these results are significant because they are among the first descriptions of IgG subclass and avidity responses to a malaria vaccine candidate with allele-specific efficacy. Although there have been many studies of the association between naturally developed antibodies and protection against malaria, vaccine-induced immunity might

differ from the response to natural infection because of characteristics unique to vaccines. First, vaccine candidates such as FMP2.1/AS02$_A$ produce high concentrations of antibodies against one or a few antigens, as compared with the diverse repertoire induced by the whole parasite (Epstein et al., 2011). This alters the frequency of specific antigen-antibody interactions relative to others and may affect the mechanism of protection (Miura et al., 2011). In addition, vaccines are suspended in an artificial adjuvant (Heppner et al., 2005) and the mode of delivery is different from that of natural infection in that the vaccine is delivered by needle injection rather than a mosquito bite (M. A. Thera et al., 2011). Finally, vaccine candidates such as FMP2.1/AS02$_A$ contain recombinant proteins that may differ slightly from their natural counterparts in conformation and post-translational modifications (Olesen et al., 2010). Consequently, it is important that a correlate of vaccine-induced protection be determined through vaccine trials rather than studies of natural immunity.

Differences in population characteristics may also affect the response to the vaccine, and it is therefore important to study vaccine-induced immune responses in the population in which the vaccine candidate is intended to be provided. For example, there is evidence that previous exposure to malaria might interfere with vaccine-induced immunity, such as the *in vitro* finding that the addition of AMA1-depleted immune sera can blunt the invasion inhibitory activity of anti-AMA1 IgG antibodies from non-immune vaccine recipients (Miura et al., 2011). Immune function and protection against malaria are also influenced by co-infection with other pathogens as diverse as HIV (Ataíde et al., 2011; Laufer et al., 2007) and parasitic worms (K. Lyke et al., 2006; Su et al., 2006), which may have a different prevalence in malaria endemic countries. In addition,

differences in genetics, such as an HLA allele that is associated with higher antibodies to AMA1 (Johnson et al., 2004; K. E. Lyke et al., 2011), may cause variation between different populations. Finally, age is an important determinant of an immune response (Courtin et al., 2009; Dodoo et al., 2011), and the results of adult Phase 1 trials might differ from results of studies in children, who would be the most significant beneficiary of a malaria vaccine (Miller et al., 2002).

V. Conclusions

The FMP2.1/AS02$_A$ malaria vaccine candidate induced significant production of anti-AMA1 IgG1, IgG2, IgG3, and IgG4 antibodies. The high ratio of cytophilic to non-cytophilic antibodies in vaccine recipients, as compared with control subjects, is consistent with studies of natural immunity and cell-mediated protection. We did not detect avidity maturation in either group.

This is one of the first reports to describe the subclass composition and avidity of antibodies induced by a malaria vaccine candidate with allele-specific protective efficacy. Based on these findings, a future study is planned to measure titers of the four subclasses in 90 additional vaccine recipients and 30 control subjects, as well as any other subjects who had clinical malaria episodes associated with infection with parasites that had 3D7-like sequences in the C1 loop of AMA1. We will compare the results in this larger sample with a measure of outcomes to identify any associations with protection. These results will also be stratified by age and will exclude clinical episodes that occurred before day 74, at which point subjects would have been able to respond to all three immunizations in the study protocol.

A second pilot study will be conducted to detect differences in avidity in protected subjects that are not visible in the overall population. This is warranted because although the current study showed that there was not a significant difference in avidity between vaccine recipients and control subjects, it did not have the specificity to observe avidity maturation in a small subset of subjects who were protected by the vaccine candidate. It will also be important to combine this work with studies of vaccine-induced cellular immunity to fully characterize an effective response to the vaccine candidate.

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