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Analysis Of The Malaria Vaccine Potential Of Plasmodium Falciparum Merozoite Surface Protein-3

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ANALYSIS OF THE MALARIA VACCINE POTENTIAL OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-3

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CELL BIOLOGY

ABSTRACT

Malaria causes an estimated 1-3 million deaths each year, with the majority of deaths being a result of infection with *Plasmodium falciparum*. No commercially available vaccine currently exists, but multiple subunit-based vaccines are in development including one based on the promising vaccine candidate *P. falciparum* Merozoite Surface Protein-3 (PfMSP3). PfMSP3 varies in sequence between *P. falciparum* isolates, but all variation is restricted to the N-terminal domain. This observation has lead to the development of a PfMSP3-based vaccine comprised solely of the highly conserved, yet poorly immunogenic, C-terminal domain. The rationale for supporting the C-terminal domain relies entirely on its conserved sequence, but so far no study has measured of the effect of N-terminal domain diversity on antibody responses in the endemic setting. To address this urgent question, we used both *P. falciparum* DNA and human sera samples from individuals infected with *P. falciparum* from a longitudinal cohort study near Iquitos, Peru. We demonstrate that, in this hypoendemic transmission setting, sequence polymorphisms in PfMSP3 were extremely limited with only 10 mutations occurring over a 4-year period, whereas allele class frequency variation was significant, suggesting a possible immunologically driven selection pressure. By studying antibody responses against PfMSP3 domains by ELISA, we determined that the N-terminal domain was significantly more immunogenic than the C-terminal domain. Additionally, N-terminal domain antibodies are capable of providing significant cross-reactivity across sequence variants within the

same allele class, and some level of cross-reactivity between allele classes. N-terminal domain antibodies also exhibited potent IgG1/IgG3 antibody responses, an IgG isotype profile well known to correlate with protection against *P. falciparum* malaria, compared to the C-terminal domain, which had higher IgM levels. These data suggests that the impact of PfMSP3 genetic diversity on antibody cross-protection may be more limited than was anticipated, and supports the development of a novel trivalent PfMSP3-based vaccine constituent comprised of both highly immunogenic N-terminal domain alleles in addition to the conserved C-terminal domain.

Keywords: malaria, vaccine, *Plasmodium falciparum*, Merozoite Surface Protein-3

DEDICATION

To my dear family and my dear Lord

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First, I would like to thank Dr Rayner for holding a spot in his lab for me while I finished my 2nd year of medical school. In my time in his lab, I have grown considerably in the way in which I approach science and owe a significant part of that development to his mentorship and input. Secondly, I am grateful to all the members of the Rayner lab, both past and present, for their help and their friendship over the years. Thirdly, I greatly appreciate the guidance that I have received from my thesis committee. Their input in every area of this thesis has been invaluable and I am grateful for the many hours they have invested. And most importantly, I am deeply indebted to my family for all their love and support: to my wonderful wife, Alyssa, who has been my constant companion and helpmate and to my dear family, who continues to actively invest in my life.

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INTRODUCTION

Burden and distribution of disease

Malaria poses a severe threat to global public health, with up to 500 million confirmed cases worldwide and an estimated 1-3 million deaths annually (1). Approximately 90% of these deaths occur in Africa, with an overwhelming majority occurring in children under the age of five. One recent study reported mortality rates in children as high as 26 percent in rural Western Kenya (2). The global burden of malaria extends beyond the morbidity and mortality public health concerns and has a considerable impact on both the economic and scholastic development of affected countries. For example, in Africa alone, treatment and preventative measures against malaria account for approximately 40% of the healthcare budget; a cost of US$12 billion annually (3). It has also been estimated that malaria alone is responsible for a loss of 1% in yearly *per capita* economic growth in affected countries (4-6). As most of these countries have economies that are considered "developing" by GDP measures, the loss of even 1% of GDP is considerably more stifling to economic growth than in more developed countries. Additionally, these countries continue to suffer from a loss of work force due to malaria and, in children, delayed educational progress due to health-related absences from school.

Although hyperendemic environments, environments where contacting malaria is a frequent occurrence, are largely localized to Sub-Saharan Africa and parts of Southeast Asia and India, the global distribution of disease is enormous with nearly 2.4 billion people living in areas of potential malaria transmission (7). In fact, recent reports demonstrate, in the last 10 years, a net overall increase in worldwide malaria incidence, despite

numerous global eradication campaigns and billions of dollars spent annually on anti-malarials (8) (see Figure 1). This increase is largely attributed to globalization, specifically increased travel capabilities, and the increased use of technologies that facilitate a spread of disease, such as increases in irrigation usage.

Clinical pathology and parasite life cycle

Malaria is caused by infection with an apicomplexan parasite of the *Plasmodium* genus, which is spread through the bite of an infected *Anopheles* mosquito. Although four different *Plasmodium* species routinely infect humans, *Plasmodium falciparum* is responsible for virtually all malaria-related mortality. Classical signs and symptoms of *P. falciparum* malaria include fever and/or night sweats, respiratory distress, metabolic acidosis, and hepatosplenomegaly (enlargement of the liver and spleen). Additionally, severe *P. falciparum* malaria can also result in cerebral complications due to sequestration of parasites in the microvasculature, leading to ischemia of downstream tissue and, unless immediate treatment is administered, coma and/or death of the host.

Treatment consists exclusively of anti-parasitic drug administration, and some of the drugs still used for severe malaria such as quinine have been in use for more than 300 years. The emergence of drug-resistant parasites in the past decades has plagued effective treatment strategies, making some previously first-line anti-malarials such as chloroquine almost completely ineffective as a treatment regimen. Currently, the emergence of drug-resistant parasites is being slowed through the use of multi-drug regimens and efforts to increase compliance of full-course drug administration in patients with malaria. It is clear, however, that the ongoing emergence of drug-resistant parasites is unavoidable and

that future malaria treatment will rely on continual novel drug discovery efforts. However, as novel drug discovery efforts merely delay the inevitable emergence of drug-resistant parasites, there has been a major shift in resources toward development of effective preventative measures, which include the utilization of insecticide-treated bed nets (ITNs), vector-control campaigns, and development of an effective malaria vaccine.

ITNs have been widely studied as they provide immediate protection against *P. falciparum* infection by preventing *Anopheles* mosquitoes from biting the host. However, protection is only afforded if the ITNs are frequently used. Unfortunately, as most areas where the utilization of ITNs is encouraged are tropical climates, the resulting decreased air circulation caused by ITNs has made widespread adoption and frequent usage challenging problems. Vector-control programs, which are dependent on regular applications to be effective, also face challenging obstacles in today's environment. A leading concern that hinders vector-control efforts in the increased use of irrigation, which increases the number of mosquito breeding sites, making control difficult. Additionally, insecticide-resistance in mosquitoes is a growing concern, necessitating the continued development of novel insecticides. Consequently, there is little optimism that vector control campaigns alone will cause a major reduction in disease burden.

By far the most theoretically effective preventative measure against *P. falciparum* malaria would be the development of an effective malaria vaccine. With the recent increases in global malaria incidences, more resources are being devoted to vaccine research. The most well known has been the "Roll Back Malaria" partnership, largely supported through the Bill and Melinda Gates Foundation and The Global Fund, which hopes to ablate malaria-related mortality by 2015 through the development of an effective

vaccine. Unfortunately, progress has been slow as the size of the *P. falciparum* genome and the complexity of the parasite's life cycle makes the identification of optimal vaccine candidates difficult and sometimes controversial.

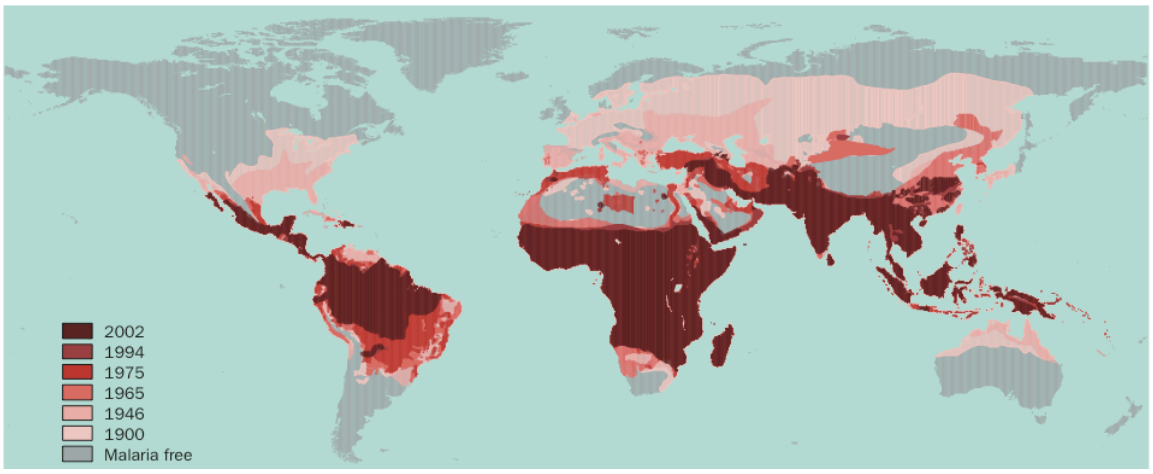

Figure 1. Malaria endemic regions of the world over the past 100 years. Between 1900-2002, there has been a reduction in the overall distribution of malaria. However, there has been an overall increase in the incidence of disease. Adapted from: Hay, S.I. *et al.* (2004) "The global distribution and population at risk of malaria: past, present, and future." The Lancet Infectious Diseases; *4*: 327-336.

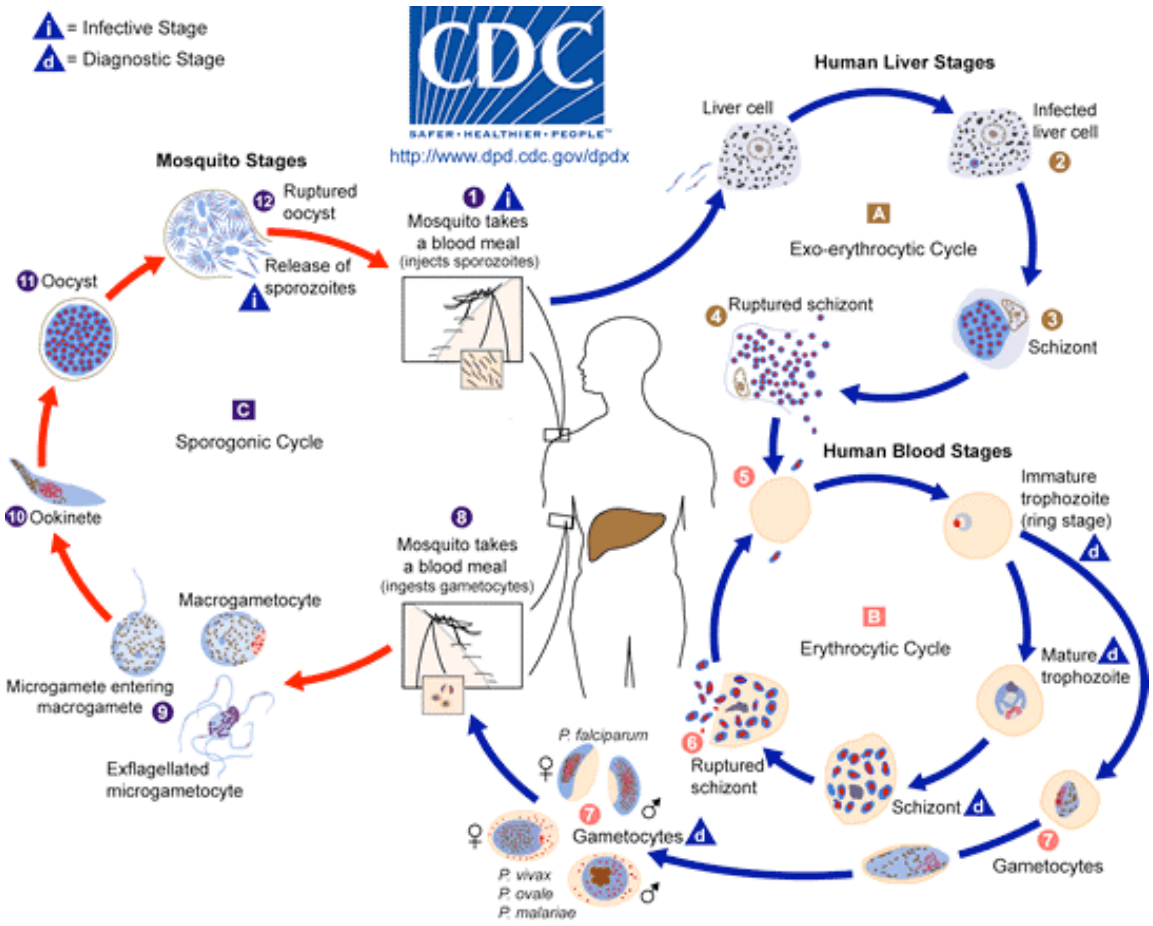

Figure 2. The Life cycle of *Plasmodium falciparum*. Image taken from www.dpd.cdc.gov/dpdx/HTML/Malaria.htm

The *Plasmodium falciparum* genome is 23Mb, encoding approximately 5,200 genes that together allow for a complex life cycle involving multiple stages in both mosquito and human hosts. Human infection occurs initially through the inoculation of sporozoite parasites into the host by the bite of an infected *Anopheles* mosquito. The sporozoites will enter the venous system and travel to the liver where they will invade hepatocytes, undergo extensive replication and ultimately emerge as infectious merozoites that will target and invade host erythrocytes. Importantly, until the emergence of merozoite from the hepatocyte, the host exhibits no signs or symptoms of disease as the parasites are effectively concealed from the host immune system within the hepatocytes. However, the emergence of merozoites from the liver elicits a sudden onset of disease

manifestations as the immune system immediately encounters foreign antigens. Within the invaded erythrocyte, the merozoite will metabolize host hemoglobin within an enclosed vacuole called the parasitophorous vacuole and progress through multiple stages of development defined as a ring stage, a trophozoite stage, and a schizont stage. As development progresses, the parasite undergoes asexual replication and each fully mature schizont will form 8-32 merozoite progeny that will erupt both the parasitophorous membrane and erythrocyte membrane and be released into the host circulatory system to invade neighboring erythrocytes. A small fraction of blood-stage parasites will differentiate into gametocytes that will initiate the mosquito-stage infection upon ingestion by a feeding *Anopheles* mosquito. As mentioned, the merozoite-mediated blood-stage infection is responsible for all malaria-related pathology and establishes chronic infection in the host, with the final outcome being either recovery, via immune- or drug-mediated control of the infection, or death of the host. Interestingly, merozoites exploit a weakness in the host immune response: the lack of Major Histocompatibility Class I molecules on erythrocytes (9). This results in the infected erythrocytes being incapable of signaling to host B- and T-cells of the presence of their intracellular infection. However, the emergence of merozoites from ruptured erythrocytes, an event that occurs every 48 hours, and the expression of parasite-derived antigens on the erythrocyte surface during development provide targets for immunological attack (10). Consequently, as these proteins will be encountered by the host immune system, they might be able to confer host protection against *P. falciparum* if immunologically-primed by an effective vaccine.

Evidence of Plasmodium falciparum immunity and whole-parasite vaccines

The gradual development of natural immunity to *P. falciparum* in the hyper-endemic setting has been widely accepted for over 50 years, and is most evident by the fact that the overwhelming majority of malaria mortality occurs in children under the age of five, before effective immunity has developed (11). The mechanism of that protection is believed to be antibody-mediated as the passive transfer of purified immunoglobulin from hyper-immune individuals to naïve individuals is able to recapitulate protection against *P. falciparum* challenge (12). The case for an effective vaccine was further strengthened by pioneering experiments performed in the late 1950's that demonstrated that inoculation with γ-irradiated attenuated *P. falciparum* sporozoites conferred protection against parasite challenge for up to 3 months (13), (14). This seminal study convinced the scientific community that, indeed, an effective vaccine was not only attainable, but also likely close to being produced. However, the whole parasite vaccination strategy faces clear challenges if it is to be widely used. The most important is that of scalability, since to be effective, thousands of attenuated *P. falciparum* parasites need to be inoculated into patients with each vaccine. Currently, the technology isn't available to facilitate the extraction of intact sporozoites from *Anopheles* mosquitoes for distribution to hundreds of millions of patients in the developing world. Secondly, the effectiveness of sporozoite attenuation is a clear host safely concern. As these parasites are fully infectious, any decrease in γ-irradiated effectiveness could render them fully viable and, thus, capable of instigating a potentially deadly infection in vaccinated patients. The obvious solution- to subject the parasites to higher levels of irradiation- isn't a valid option as this would significantly decrease infectivity and, thus, decrease vaccine efficacy. Although the risks of such an event occurring are considerably low, widespread adoption of whole-

parasite vaccines would only magnify the number of vaccine-induced malaria infections by incomplete parasite attenuation. Obviously, the potential of contacting malaria from a vaccine would make it difficult to garner patient acceptance and a few isolated reports of vaccine-instigated disease would be devastating to ongoing vaccine efforts. It has therefore been generally accepted that whole-parasite vaccines will not be scaleable, and their development has advanced little over the past few decades. One potential breakthrough, however, is the use of genetically attenuated sporozoites. By generating knock-out mutants of the genes uis3 or uis4, Kuma *et al* were able to create sporozoites that demonstrated wild type-level infectivity, yet would developmentally-arrest in the hepatocyte (15). The obvious benefit of such an attenuation strategy is that it alleviates concerns about contacting malaria due to ineffective parasite attenuation. Unfortunately, however, these genetically attenuated parasites are still subject to the same problems concerning scalability as the γ-irradiated parasites, and the initial studies have all been done in the rodent model of malaria *P. berghei*, and it is not yet known whether they will be transferable to human malaria parasites. Given these challenges, the vast majority of vaccine efforts have shifted towards subunit-based malaria vaccines.

Liver stage subunit vaccines

Subunit-based vaccines are advantageous over whole-parasite vaccines for multiple reasons. Firstly, massive production of a subunit vaccine candidate is readily achievable through the use of high-yield expression methods, such as *E. coli*- or yeast-based systems. Such techniques are capable of producing milligram quantities of proteins very rapidly. Secondly, subunit-based vaccines are highly stable and, thus, ideal for commer-

cial use, especially in a developing world environment where access to electricity, needed to preserve whole-parasite vaccines at 4°C, is sparse and unpredictable. Additionally, the high stability of subunit-based vaccines allows for the co-administration of adjuvants. Adjuvants, which are added to enhance vaccine efficacy, are incompatible with live vaccines as they often contain charged particles (*e.g.* aluminum) or non-polar solvents (*e.g.* oil-immersion solvents) that greatly decrease parasite viability. Thirdly, subunit-based vaccines allow for the administration of multiple vaccine constituents in a single multivalent vaccine. Such advantage could allow for the development of a malaria vaccine that could induce protection against all stages through eliciting responses against multiple stage-specific targets. The use of subunit-based vaccines, therefore, effectively resolves the concerns that plague whole-parasite vaccine development and has consequently become the vaccine method of choice for the vast majority of upcoming malaria vaccines.

As a result of the scalability and safety concerns that have plagued development of γ-irradiated sporozoite vaccines, efforts began to try and identify the key antigens that mediated that protection in the hope that it could be recapitulated in a subunit-based vaccine. Within a few years, the target was identified: the Circumsporozoite protein (13) (CSP). CSP is the dominant antigen on the sporozoite surface and the major target of antibodies that mediated protection through γ-irradiated sporozoite vaccination. Further studies demonstrated that the target of those antibodies was a CSP-specific motif, the NANP repeat, and that CSP also has several potent T-cell epitopes. It therefore became the major constituent of the most advanced malaria vaccine, to date, the RTS,S vaccine. RTS,S is comprised of four main constituents: the CSP Repeat NANP region, the C-terminal portion of CSP that contains the T-cell epitopes and fused to the Surface antigen

of Hepatitis B, and, as an adjuvant, the addition of Hepatitis B Surface antigen, alone. Currently, RTS,S is progressing towards a Phase 3 clinical trial and is the only malaria vaccine to reach Phase 3, despite somewhat discouraging performance in Phase 2 clinical trials, with an efficacy rate of only 35.3% against subsequent clinical episodes of malaria (16). This level of protection suggests that RTS,S still has immunogenicity concerns, despite two decades of development. It also has the major problem that because it targets a sporozoite protein, CSP, it attempts to provide protection through the inhibition of *P. falciparum* development before it reaches the hepatocyte. Importantly, as the liver stage of the *P. falciparum* life cycle is merely a developmental stage leading up to the release of thousands of merozoites into the blood stream, if even a single sporozoite is able to escape the RTS,S vaccine-induced immune response, it will result in the release of thousands of merozoites into the blood stream and the development of a blood stage infection. As it is the blood-stage that is responsible for all malaria symptoms, the decrease in severe malaria symptoms seen in RTS,S trials is likely the result of the ability of RTS,S to decrease the number of merozoite released from the liver, thereby allowing the immune system a few more days to mount an effective response before the *P. falciparum* parasite burden reaches severe levels. While this approach may reduce severe symptoms, the trial results suggest that RTS,S is only partially effective at doing so, and is therefore not likely to be a long-term solution to the malaria vaccine problem.

The decision to target the sporozoite antigen CSP was made in part on the initial γ-irradiated sporozoite trials and in part on the belief that the optimal stage to target for vaccination might be the stage with the fewest viable parasites (approximately 8-12 sporozoites are injected per *Anopheles* bite compared to thousands of released merozoi-

tes). However, this approach ignores the desired end outcome of *P. falciparum* vaccine development and an understanding of *P. falciparum* parasite/host relationship. Firstly, it can be strongly argued that the ultimate goal of an effective *P. falciparum* vaccine is not to induce sterile immunity in vaccinated individuals (*i.e.* total parasite clearance) but to control the *P. falciparum* parasite load, especially in children under the age of five, until they can develop their own effective natural immunity. In fact, the strongest argument for this assertion is the inability of hyper-immune individuals to develop sterile immunity, despite decades of continual parasite exposure– it seem highly unlikely that a malaria vaccine could instigate protection at a level that the human immune system is unable to achieve, despite repeated exposure over decades. Secondly, the use of liver stage vaccines attempts to completely ablate the host/parasite relationship by obstructing *P. falciparum* development. This is in sharp contrast to the goal of blood-stage vaccines, which attempt only to reduce host symptoms through a reduction in the total parasite burden. This difference is critically important, as natural selection will ensure that *P. falciparum* parasites adapt to liver stage vaccines. However, since blood-stage vaccines share the same goal as *P. falciparum* parasites- protecting the host while allowing parasite development- it can be argued that this approach will apply significantly less pressure driving natural selection to adapt. Given these significant concerns, sporozoites and liver-stages may not be the best choice for vaccine development.

Blood-stage subunit vaccines

The development of blood-stage *P. falciparum* vaccines began to emerge in the late 1980's with the understanding of the problems plaguing liver-stage-specific vaccines

and the clear advantages of targeting the stage directly responsible for host morbidity and mortality. As mentioned previously, erythrocytes do not contain the major histocompatibility complex I, and therefore are unable to display *P. falciparum* antigens to the immune system. As a result, the immune response to systemic infection is limited to those antigens either expressed on the surface of the infected erythrocyte or found on the surface of merozoites after their release from the erythrocyte. Of the vaccine candidates expressed on the erythrocyte surface, *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) has been the most widely studied. Unfortunately, there are 60 homologs of PfEMP1, encoded by var genes, in the *P. falciparum* genome, and the specific var gene being expressed at any one time can switch under a poorly understood antigenic variation system (17). As antibody responses to a single allele of PfEMP1 demonstrate limited cross protection against other homologs (18), complications with PfEMP1 variation have effectively ruled it out of general vaccine development. One important exception is correlation of expression of a single var gene, var2csa, with pregnancy-associated malaria (19). Thus, while there is hope that a var2csa-specific vaccine may be able to protect against malaria-induced fetal mortality (20), little work is otherwise being done on PfEMP1-based malaria vaccines.

There are currently at least 11 antigens that have been identified on the merozoite surface, termed Merozoite Surface Proteins (MSPs), and all are being considered as potential vaccine candidates. EM studies performed in the early 1990's revealed that the merozoite surface coat is highly organized and comprised of multiple proteinaceous complexes (21). By far the most widely studied merozoite surface vaccine candidate is *P. falciparum* Merozoite Surface Protein-1 (PfMSP1), which constitutes approximately 30%

of the merozoite surface (22). PfMSP1 undergoes proteolytic cleavage, a phenomenon characteristic of all MSP's (23). However, unique to PfMSP1 is a GPI-anchored C-terminal 19kD (termed $PfMSP1_{19}$) fragment that remains attached to the merozoite after PfMSP1 proteolysis and is retained on the parasite surface even after invasion (24). Importantly, antibodies against $PfMSP1_{19}$ have been shown to inhibit *P. falciparum* invasion both *in vitro* (24) and *in vivo* (25) and the presence of $PfMSP1_{19}$ antibodies in African children has been shown to correlate with protection against *P. falciparum* malaria (26). Thus, PfMSP1 is considered one of the most promising vaccine candidates to date and is currently undergoing multiple phase II trials, either as a mono- or a multi-valent vaccine constituent. Unfortunately, however, recent data has raised concerns about PfMSP1's future as an effective vaccine candidate. $PfMSP1_{19}$ demonstrates little sequence variation, and it was thought that structural constraints were responsible for its sequence stability. However, O'Donnell *et al* showed that it was possible to replace the C-terminal 19kD region of PfMSP1 with the homologous C-terminus of the rodent *Plasmodium* species *Plasmodium chabaudi*, which shares only 34% homology with $PfMSP1_{19}$, and the chimeric protein could recapitulate native PfMSP1 function (27). The 19kDa region is therefore not as sequence constrained as had been though, raising the possibility that a vaccine against the $PfMSP1_{19}$ region might result in the rise of escape mutants. Another PfMSP1 vaccine, FMP-1, has recently undergone a phase IIb clinical trial looking at protection in 374 children in Western Kenya. The vaccine constituent was $PfMSP1_{42}$, the 42kD C-terminal portion of PfMSP1, and includes $PfMSP1_{19}$. Unfortunately, no protective efficacy was detected by $PfMSP1_{42}$ compared to the control group, leading the investigators to recommend that subsequent PfMSP1-constituted vaccines

focus on other formulations and antigenic constructs (28). PfMSP1 based vaccines have therefore some way to go to fulfill their promise.

A malaria vaccine roadmap

Over 60% of current *P. falciparum* vaccines in development target one of only four different proteins (29), PfMSP1 being one of the four. With a genome of over 5,200 genes, obviously the pool of candidates being studied needs to be widened. However, with new antigens being identified yearly, the number of vaccine candidates undergoing clinical trials will quickly outpace the available funds unless measures are taken to ensure only the most promising candidates are being studied. Instead of applying strict criteria to upcoming candidates early in their development that either rejects them from further study or supports their development, no entering antigen, to date, has been ruled out of ongoing vaccine studies at the onset. A failure to implement this critical step in the vaccine development pipeline has the potential to be very costly in the future, both financially and scientifically, as more extensive studies are demanded. A devastating example is the failure of the SPf66 vaccine, a vaccine comprised of PfMSP1 and other merozoite-associated proteins, which endured 9 clinical trials before being abandoned (30). Importantly, only a few small-scale studies were performed prior to the decision to rapidly advance SPf66 to clinical trials (31). Fortunately, the importance of immediate evaluation of entering candidates has been recognized and a plan has been developed by a worldwide collaboration that addresses the need to narrow down the list of candidates progressing through the vaccine development pipeline (www.malariavaccineroadmap.net). Importantly, this article lists key "focus areas" that each candidate should be subjected to with

the ultimate development infrastructure being a large number of candidates undergoing basic research at the entry level, but only a few progressing to the expensive Phase IIb and Phase III clinical trials. Thus, in order for such a development pipeline to occur, critical go/no go criteria must be applied to candidates early in the developmental pipeline.

The first criterion that an effective candidate must demonstrate is that it must be relatively conserved throughout the population or else generate antibodies that demonstrate cross-protection between variants. Vaccination with an antigen that is polymorphic will only result in the rise of "escape mutants" rendering the vaccine ineffective, unless effective cross-reactive antibody responses are generated. Secondly, a candidate antigen must be able to elicit a strong and enduring immune response. With the focus of an effective malaria vaccine being sub-Saharan Africa, an area with sparse access to healthcare, frequent vaccinations are not financially or demographically feasible. Thirdly, a vaccine candidate must be supported by data demonstrating that it correlates with protection against malaria. Without such data, a legitimate concern is that subsequent clinical trials will demonstrate a candidate ineffective at conferring protection, as was the case for the SPf66 vaccine. Thus, critical data is needed at the outset that assigns a go/no-go designation to novel vaccine candidates, either removing them from ongoing vaccine research or supporting their continuing development through the vaccine pipeline.

Hypoendemic transmission environments: an opportunity to study vaccine candidates

If data is to be gathered at the outset, as recommended by the Malaria Vaccine Roadmap, that allows a go/no-go designation to be assigned to novel vaccine candidates both hyperendemic and hypoendemic environments must be utilized. Hyperendemic en-

vironments are characterized by high *P. falciparum* frequency-of-infection (FOI) rates and loosely define environments that exhibit high malaria transmission, with FOI rates as high as multiple infectious bites/person/night. In contrast, hypoendemic environments describe malaria transmission settings that exhibit much lower FOI rates, with individuals receiving up to a few infectious bites per year. Obviously, the hyperendemic environment provides the ideal setting for studying protection of novel vaccine candidates, as frequent infections allow studies to be performed that can correlate an increase in time-to-next-infection and/or a decrease in symptoms with antibody levels against the novel candidate. Such settings are routinely used in phase II studies to test vaccines and prove instrumental in assessing vaccine efficacy. Importantly, however, the frequent inoculation rate characteristic of hyperendemic environments makes them poor settings for the initial characterization of antibody responses against novel vaccine candidates. The major reason for this is that frequent inoculation usually results in simultaneous infection with multiple *P. falciparum* genotypes, which makes assessing cross-reactivity between genotypes (*i.e.* vaccine candidates) impossible. Consequently, the investigator is unable to distinguish whether antibodies that bind a given *P. falciparum* strain antigen do so as a result of infection with that strain or due to cross-reaction from infection with a heterologous strain. Additionally, hyperendemic environments are not conducive for studying antigenic variation and antibody responses over time. This is due to the high level of genetic diversity in these regions, which makes it difficult to distinguish whether variation was spontaneous or due to inoculation with a mutant genotype. Importantly, as almost all *P. falciparum* vaccine candidates demonstrate variation, either sequence polymorphisms or the presence of multiple isoforms, it makes sense to abandon hyperendemic settings for

environments that exhibit low within-host diversity and high population-level diversity for these initial characterization studies. Such attributes are characteristic of hypoendemic transmission environments.

A considerable advantage of a hypoendemic transmission setting is that genetic analysis of individual infections is possible on both a macro scale, between villages or transmission seasons, and a micro scale, between subsequent infections within an individual, because such infections are likely to be spaced rather than continuous and overlapping, as is common in hyperendemic environments. It is with these advantages in mind that a longitudinal cohort study, termed Molecular and Immunological Genetics In the Amazon (MIGIA), was initiated in the Peruvian Amazon outside Iquitos in 2003. In this region, *P. falciparum* was introduced only 10 years previously and has maintained a stable hypoendemic environment with an inoculation rate of less than one infection per person per year (32,33). To accomplish the recommendations set forth by the Malaria Vaccine Roadmap, this thesis involves collaboration with the MIGIA cohort study and describes the analysis of samples from individuals in this hypoendemic setting. A detailed description of the MIGIA cohort study is provided in Chapter one of this thesis.

Briefly, the MIGIA cohort study has focused on a small cluster of villages within Zungarococha, a site of residual hypoendemic malaria transmission since an epidemic in 1995 (Figure 3). The villages are located about 1km apart and the residents have homogenous income levels, housing construction, and 24-hour access to medical care by the physicians involved in the study. In addition to passive case detection (*i.e.* a symptomatic individual presenting to the clinic), the study employs comprehensive active case detection through the instigation of weekly active longitudinal sampling during the 7-month

malaria season. In January, a community-wide survey is conducted, followed by random weekly sampling of 300 individuals for 1 month each month during the 7-month transmission season. At the completion of the season another community-wide survey is conducted. Throughout the season, sick individuals are directed to the local health outpost in Zungarococha to receive appropriate treatment. All treated individuals are followed for compliance and control of infection. This level of centralized healthcare has been provided through collaboration with the Peru National Ministry of Health (MINSA), which maintains the Zungarococha health outpost and has kept and made available all patient records from 1998 to the present. It is important to note that the detection of *P. falciparum* infection occurs in both symptomatic individuals, by passive case detection, as well as asymptomatic individuals, by active case detection. As classical immunity against *P. falciparum* is characterized by persistent low-level infection in the absence of symptoms, the presence of parasites in asymptomatic individuals by active case detection suggests malaria protection exists in these individuals. In contrast, passive case detection is defined by the presentation of symptomatic individuals to the health outpost and therefore defines a malaria susceptible (non-protected) population. Making this distinction has considerable implications for the evaluation of vaccine candidates as stratification of infections into these two groups allows for the study of determinants (i.e. specific characteristics of antigens/antibodies, population demographics etc.) that correlate with protection against malaria; defined by a statistically significant correlation with protected individuals in the absence of any correlation in the non-protected group.

Importantly, a recent study of PfMSP1 block 2 variation demonstrated that extensive diversity exists within the Zungarococha community. By amplifying block 2 of

PfMSP1 with allele-specific primers by nested PCR, Branch *et al* established that block 2 diversity is considerable despite the recent introduction of *P. falciparum* into the community (personal communication). In fact, all 3 alleles of PfMSP1 Block 2 (K1, Mad20, and RO33) are present in Zungarococha and define 13 different alleles. Additionally, comparison with Block 2 variation in the hyperendemic setting of Western Africa reveals considerable population-level diversity in Zungarococha despite a significantly lower frequency of infection. In fact, only 21% of Zungarococha infections had multiple PfMSP1 Block 2 alleles compared with 89% in Africa. This stark difference can be explained by the low frequency of infection present in Zungarococha (0.04 infections/person/month versus 7.3 infections/person/month). Thus, the combination of low within-host diversity and high population-level diversity makes the MIGIA cohort study an ideal environment for the characterization of novel vaccine candidate evolution and the study of vaccine-specific antibody dynamics.

Figure 3. Schema of the Zungarococha Community. Located approximately 1Km apart, the community is a cluster of 4 villages: Zungarococha village (ZG, n= 805), Puerto Almendra (PA, n= 272), Ninarumi (NR, n = 590), and Llanchama (LC, n = 203). The community health post is located in Zungarococha village. Adapted from Branch, OH *et al.* (2005). "Clustered local transmission and asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* malaria infections in a recently emerged, hypoendemic Peruvian Amazon community." Malaria Journal; *4*: 27-43.

P. falciparum Merozoite Surface Protein-3: a promising vaccine candidate

The identification of *P. falciparum* Merozoite Surface Protein-3 (PfMSP3) is unique in that it remains the only vaccine candidate to be identified based on its role in conferring protection against malaria in the endemic setting (34), (35). It was identified by two different groups simultaneously; both using pooled sera from malaria-immune African patients. Importantly, PfMSP3 is a major target of cytophilic antibodies, IgG1 and IgG3 (34), (36); isotypes whose predominance in malaria-infected individuals strongly correlates with protection against malaria (37),(38), (39), (40), (41).

PfMSP3 is a 48kD secreted protein that associates with the merozoite surface through an unknown mechanism. Although no crystal structure has yet been solved, biochemical studies of PfMSP3 suggest it exists on the merozoite surface in a head-to-head homotetramer and forms a highly elongated 500Å structure postulated to bind unknown erythrocyte surface ligands (42), (43). In support of this putative role, electromicroscopy scanning of the merozoite surface corroborates the presence of 300Å spikes extending from the merozoite surface (21).

PfMSP3 consists of two domains, an N-terminal domain characterized by three heptad repeat regions believed to form coil/coil structures and a C-terminal domain, comprised of a glutamic acid-rich domain and a putative leucine zipper motif (42) (Figure 4). Interestingly, the heptad repeats of PfMSP3 are formed by alanine residues

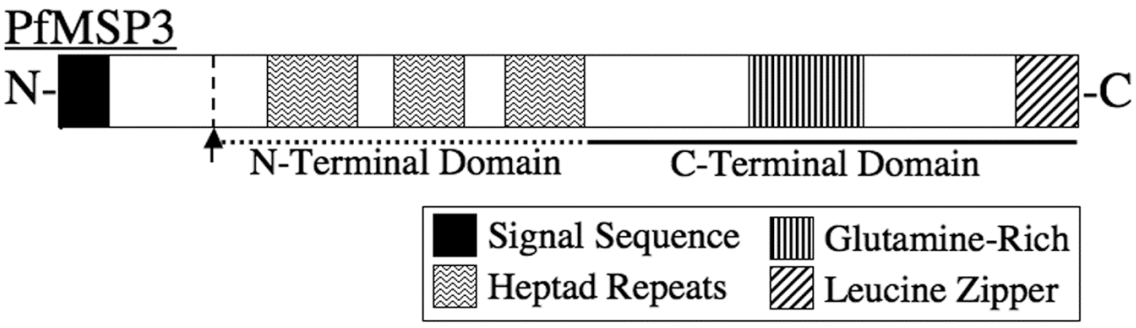

Figure 4. Domains of PfMSP3. PfMSP3 contains 2 domains: a polymorphic N-terminal domain, characterized by 3 heptad repeat regions, and a C-terminal domain comprised of a glutamine-rich region and a putative leucine zipper motif.

in an AxxAxxx configuration (35),(44); an unusual structural occurrence only described in 3 other organisms (45), (46), (47). Importantly, genetic variants of PfMSP3 are known to exist, but virtually all polymorphisms are limited to the N-terminal domain (48). Two allele classes have been identified, termed K1 and 3D7, with the 3D7 allele being slightly smaller due to the presence of two indel mutations (48). It is important to note that al-

though sequence variation exists within the heptad repeat regions of the N-terminal domain, the alanine residues and hydrophobic residues believed to stabilize alpha helical structures are almost exclusively conserved, suggesting that structural conservation is maintained (49). In stark contrast, the C-terminal domain is almost entirely conserved among strains (49).

The presence of an N-terminal signal sequence targets PfMSP3 for export where, upon secretion into the parasitophorous vacuole, the extreme N-terminus is cleaved by an unknown protease (50) and the remaining mature processed protein associates with the merozoite surface. As it lacks either a transmembrane domain or a GPI anchor, the most likely mechanism for PfMSP3's surface localization is via an undetermined protein-protein interaction possibly with the acidic-basic rich antigen (51). It is important to note that the mature processed form of PfMSP3 contains the intact N-terminal domain, preserving the two allele classes and all sequence polymorphisms (50).

As mentioned, PfMSP3 was initially identified using pooled purified immunoglobulin from malaria-immune individuals. Subsequent studies revealed it induces a potent IgG1 and IgG3 isotype response and has significant inhibitory potential in an assay that correlates *in vivo* protection, the Antibody-Dependent Cellular Inhibition (ADCI) assay (52). The ability of PfMSP3 alone to confer protection has been demonstrated by several animal models. Using SCID mice grafted with human erythrocytes, Druilhe *et al* demonstrated that human monocytes and anti-PfMSP3 antibodies were sufficient to protect SCID mice against *P. falciparum* infection (53). Additionally, vaccination of *Aotus nancymai* monkeys, the closest available animal model for *P. falciparum* malaria, with full length PfMSP3 exhibited 86% protection against *P. falciparum* challenge (54). This

protection was greater than that induced by the leading vaccine candidate PfMSP1, the positive control used in this study and remains the highest protection ever achieved for a malaria vaccine in an animal model. Interestingly, after challenge with *P. falciparum* parasites, sera from both PfMSP1-vaccinated and PvS25-vaccinated (negative control) groups demonstrated the presence of anti-PfMSP3 antibodies that correlated with protection against *P. falciparum*, despite having no previous exposure to PfMSP3 other than *P. falciparum* challenge, arguing that anti-PfMSP3 antibody responses are an important component of the natural response to *P. falciparum* infection.

The most compelling evidence supporting PfMSP3 as a promising vaccine candidate is that it is the target of antibodies that inhibit parasite growth in cooperation with human monocytes in an *in vitro* assay termed the Antibody-Dependent Cellular Inhibition (ADCI) assay (52). ADCI activity seems to strongly parallel *in vivo* protection against *P. falciparum,* and this correlation is supported by multiple observations. Firstly, the ADCI assay relies on the potential of antibodies to cross-link human monocytes (55); an IgG-dependent phenomenon similarly seen *in vivo*, as protection against *P. falciparum* in the endemic setting has been shown to be an antibody-mediated process (12). This was demonstrated by early immunoglobulin transfer studies, which showed recapitulation of protection in malaria-naïve individuals simply through the transfer of purified immunoglobulin from malaria-immune patients (12). Secondly, inhibition by ADCI is known to occur through the cross-linking of Fcγ receptors, which is mediated by cytophilic IgG isotypes. It is significant, therefore, that purified immunoglobulin from malaria-immune individuals demonstrates a strong cytophilic profile, comprised of isotypes IgG1 and IgG3, which suggests they confer protection through cooperation with immune effector (56). Thirdly,

the ADCI assay relies on the ability of cytophilic IgG isotypes to cross-link monocytes. It has been demonstrated that, by testing purified immunoglobulin from malaria-immune adults in combination with different effector cells to identify the cells responsible for conferring protection, only monocytes are capable of inhibiting *P. falciparum* growth *in vitro* (57), (58), (56). Thus, the reliance of ADCI on monocytes and cooperation with IgG of mainly cytophilic classes strongly supports it as an effective assay of protection against *P. falciparum*.

Mechanistically, the ADCI assay facilitates the cross-linking of monocyte receptors Fcγ RIIa and RIIIa, which release an unknown soluble factor that inhibits parasitic growth *in vitro* (59). Although the factor responsible for parasite inhibition has yet to be identified, it is known to act in cooperation with TNF-α and causes *P. falciparum* growth arrest at the junction between the trophozoite/schizont stages, possibly through a cyclin-dependent kinase mechanism (59), (60). Recently, a novel ADCI assay has been developed using the immortal cell line THP-1 in lieu of purified patient monocytes as this removes any concerns about ADCI accuracy due to variant Fcγ RIIa polymorphisms, which are a common occurrence in donor monocytes(55).

Although PfMSP3 is strongly supported as an effective candidate by both *in vitro* and *in vivo* data that demonstrates its ability to confer protection against *P. falciparum*, the optimal domain for inclusion in a PfMSP3-based vaccine has yet to be determined. Due to the ability of the C-terminus of PfMSP3 to induce ADCI, compounded by its highly conserved sequence, it has been chosen as the sole constituent of a monovalent PfMSP3-based vaccine (61), which has been the subject of both Phase 1a (62), (63) and 1b (64) clinical trials. Importantly, the rationale given for excluding the N-terminal do-

main is exclusively based on concerns regarding sequence variation, but such variation is only a concern if it limits cross-reaction of antibodies across different alleles. To date there has been no study to address the occurrence of cross-protective antibodies, and until such data is gathered, the decision to rule-out the N-terminal domain from vaccine development is premature and unfounded.

Recent data does however provide some support for the inclusion of the N-terminal domain in a PfMSP3-based vaccine. In the PfMSP3 *Aotus* monkey vaccine trial described above, the immune response against PfMSP3, which conferred 85% protection against *P. falciparum* challenge, was strongly allele specific, indicating the N-terminal domain was the target of those protective antibodies rather than the C-terminal domain which is conserved between alleles (54). Additionally, those antibodies were also found to cross-react with $PfMSP1_{19}$, another leading vaccine candidate and the target of invasion-inhibiting antibodies. Unfortunately, the anti-$PfMSP1_{19}$ antibodies were not characterized further but their presence suggests an N-terminal PfMSP3 vaccine might have collateral mechanisms of mediating protection against *P. falciparum*. Another study looking at the role of PfMSP3 in 319 children from The Gambia found that increasing antibody levels against the N-terminus of PfMSP3 resulted in a greater reduced risk of clinical malaria (65). Although antibodies against the C-terminus were also associated with protection, there was no change in the reduced risk of malaria by increasing levels of C-terminal domain reactivity. Additionally, the N-terminal domains seem to be the target of positive natural selection (66), (65). This led the authors to suggest including both N-terminal alleles in addition to the C-terminus in a PfMSP3-based vaccine.

With several studies demonstrating that the N-terminal domain of PfMSP3 confers protection against *P. falciparum* infection *in vivo*, clearly its role as a potential vaccine candidate needs to be further analyzed and until such data is gathered, the decision to proceed with a monovalent C-terminal domain-based subunit vaccine is premature and unsupported. In this pursuit, we decided to conduct an extensive evaluation of PfMSP3 domains, both N-terminal and C-terminal domains, to identify the optimal vaccine candidate based on the guidelines recommended by the Malaria Vaccine Roadmap. Through collaboration with the MIGIA cohort study, we assessed *P. falciparum* genetic variation in a hypoendemic transmission setting, and tested sera from individuals who were infected with *P. falciparum* to identify antibody characteristics indicative of effective vaccine-induced immune responses. In Chapter one, recently published in the *American Journal of Tropical Medicine and Hygiene,* we characterized the sequence variation within the N-terminal domain of PfMSP3 and demonstrated that genetic diversity is relatively stable in the hypoendemic setting. Although allelic variation exists, we detected a significant shift in allele frequencies between subsequent transmission seasons, suggesting a possible immunologically-mediated selection pressure. In Chapter two we conducted a systematic analysis of the ability of the PfMSP3 N-terminal and C-terminal domains to induce antibody responses and subsequently characterized those antibodies by assessing their ability to cross-react between PfMSP3 alleles classes and between sequence variants within a given allele class. Finally, we characterized the isotype profile of anti-PfMSP3 domain antibodies to identify the domain that induces the most potent cytophilic isotype responses. These studies reveal that antibodies against the PfMSP3 N-terminal domain cross-react strongly within an allele class, and also to some extent be-

tween allele classes, reducing concerns about N-terminal domain sequence variability. This data argues strongly for the inclusion of the N-terminal domain in any PfMSP3 vaccine, and lays the foundation for the future development of a novel trivalent PfMSP3 subunit-vaccine, discussed in the Summary Discussion below.

GENETIC DIVERSITY OF THE MALARIA VACCINE CANDIDATE *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-3 IN A HYPOENDEMIC TRANSMISSION ENVIRONMENT

by

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ABSTRACT

The N-terminal domain of *Plasmodium falciparum* Merozoite Surface Protein-3 (PfMSP3) has been excluded from malaria vaccine development largely due to genetic diversity concerns. However no study to date has followed N-terminal diversity over time. This study describes PfMSP3 variation in a hypoendemic longitudinal cohort in the Peruvian Amazon over the 2003-2006 transmission seasons. PCR was used to amplify the N-terminal domain in 630 distinct *P. falciparum* infections, which were allele-typed by size and also screened for sequence variation using a new high-throughput technique, denaturing High Performance Liquid Chromatography (dHPLC). PfMSP3 allele frequencies fluctuated significantly over the four-year period, but sequence variation was very limited with only 10 mutations being identified out of 630 infections screened. The sequence of the PfMSP3 N-terminal domain is relatively stable over time in this setting, and further studies of its status as a vaccine candidate are therefore warranted.

INTRODUCTION

Plasmodium falciparum is responsible for between 300--500 million clinical episodes of malaria and between 1 and 3 million deaths annually, with approximately 90% of mortalities occurring in young children.[1,2] With the continual threat of the emergence of drug-resistant *P. falciparum* strains, the need for a safe and effective malaria vaccine is more urgent than ever. Although a handful of *P. falciparum* vaccine candidates, such as MSP1,[3-5] AMA1,[6,7] and CSP[8-10] have been extensively studied for many years, there are many other promising vaccine candidates which have received much less attention. For many of these candidates, a pool which has radically increased with the recent rapid increase in genomic data,[11] there is little, if any, supporting data, particularly regarding the candidate's sequence diversity or role in the generation of protective immunity *in vivo.* Given the problems of sequence diversity and poor antigenicity that can afflict even the most well studied vaccine candidates, there is critical need for the rapid and early characterization of novel vaccine candidate potential and the adoption of high throughput techniques to facilitate this type of analysis. It is with such concerns in mind that a collaborative malaria vaccine roadmap was recently released, which identifies key issues that need to be addressed in order for a vaccine candidate to effectively progress through the development pipeline (www.malariavaccineroadmap.net). With the increasing cost of vaccine clinical trials, this roadmap proposes that strict go/no-go criteria be enforced on candidate antigens--- *i.e.*, studies be conducted early that characterize the candidate's vaccine po-

tential and either lay the groundwork for subsequent studies or remove it from the pool of viable candidates.

P. falciparum Merozoite Surface Protein--3 (PfMSP3) is a major vaccine candidate that has not yet advanced to Phase II field trials. PfMSP3 is a highly immunogenic non-integral protein expressed on the surface of merozoites and has been suggested to be involved in erythrocyte binding, although its function remains unknown.[12,13] Structurally, it possesses three blocks of heptad repeats that are proposed to form alpha-helical coil/coil domains, a hydrophobic glutamine-rich domain, and a putative leucine zipper domain at the extreme C-terminus.[14,15] Importantly, it is a major target of antibodies from malaria-immune African individuals and immunization studies using full-length PfMSP3 elicited significant protection in a non-human primate model.[16-18] PfMSP3 exists as two allele classes, termed K1 and 3D7 after the *P. falciparum* strains in which they were first identified. The majority of both intra- and inter-allele differences are localized to the heptad repeat region, which defines the N-terminal domain. Variation in the PfMSP3 N-terminal domain occurs largely between the blocks of heptad repeats and consists of both indels and SNPs. Conversely, the C-terminal domain, comprised of the glutamine-rich region and the leucine zipper motif, is almost entirely conserved.[19] Although the presence of variation in the PfMSP3 N-terminus has been established for some time using lab-adapted *P. falciparum* isolates,[19] only one study has previously looked at natural variation in *PfMSP3* sequences, with 48 and 50 *PfMSP3* genotypes sequenced from samples in Nigeria and Thailand respectively.[20] PfMSP3 is, therefore, a strong vaccine candidate with limited epidemiologic data; data that is needed to support its continued development along the proposed malaria vaccine roadmap.

This study investigates genetic variation in the PfMSP3 N-terminal domain in samples collected from the Malaria Immunology and Genetics in the Amazon (MIGIA) cohort study, a longitudinal study of *P. falciparum* transmission in Zungarococha; a cluster of four villages located in the Peruvian Amazon. In this community, *P. falciparum* transmission is hypoendemic, with inoculation rates of < 0.04 infections per person per month during the 7--month transmission season, with most infections consisting of a single genetic type.[21] Although transmission rates are low, there is still a significant level of overall genetic diversity, with at least 5 different genetic types defined for PfMSP1 Block 2 (P. Sutton and OH Branch, manuscript in preparation).

To date, no study has yet looked at the extent of PfMSP3 variation in hypoendemic settings such as in South America. Such data is important because the increased frequency of simple infections in such a setting enables us to look at changes in allele frequency over time, which might provide evidence for or against the presence of allele- and variant-specific immune responses. In order to characterize genetic variation within PfMSP3 in a hypoendemic setting, we initiated a retrospective study using blood samples obtained from *P. falciparum*-infected individuals enrolled in the MIGIA cohort study. To significantly increase our power and enable us to screen hundreds of samples, we adapted the recently developed high throughput Denaturing High Performance Liquid Chromatography (dHPLC) genotyping technology to screen for PfMSP3 variation.[22-25] The existence of extensive clinical data generated by the MIGIA study also allowed us to test for correlates of protection with specific PfMSP3 genotypes. This manuscript is therefore a comprehensive analysis of PfMSP3 genotypes across multiple transmission seasons, and

is an important window into the genetic diversity dynamics of this important vaccine candidate in a hypoendemic setting.

MATERIALS AND METHODS

Study location and malaria transmission. A detailed description of the four villages that comprise the longitudinal cohort study has been previously published.[21] Briefly, the study enrolls residents from four villages of the Zungarococha community: Zungarococha village, Puerto Almendra, Ninarumi and Llanchama. The community is located South of Iquitos in the Peruvian Amazon and is supported by the Nanay River, a tributary of the Amazon River. The village of Ninarumi contains a port that brings in boats from outside the community. The village residents have homogenous housing construction, income levels and access to healthcare, provided by the MIGIA cohort physicians. The women generally work in or near their home and the majority of men work as either community fishermen or in local agriculture. Importantly, travel outside the community is rare with the most frequent travel being to the city of Iquitos, where malaria transmission is non-existent.

The Zungarococha community was chosen as the focus of the MIGIA cohort due to the presence of continuing stable hypoendemic transmission of both *P. vivax* and *P. falciparum,* introduced during an epidemic in 1992. The annual malaria season lasts for seven months and closely follows the rainy season, which occurs between January and July. The major vector of malaria transmission is *Anopheles darlingi*, an anthropophilic species that has proliferated in the area since the early 1990's.[26]

Sample collection and extraction. All individuals enrolled in the MIGIA study have access to healthcare provided at a local health post located in Zungarococha village. Upon presentation to the health post, symptomatic individuals were tested for malaria parasites by Geimsa-stained microscopy. If positive, and consent was given, patients submitted a 0.5ml blood sample by finger venipuncture and underwent a comprehensive medical evaluation. PCR verification of the microscopy result was subsequently performed using species-specific primers. *Plasmodium* infection was immediately treated with a combination of Mefloquine (0.5 mg/kg for 7 days) and Artesunate (4 mg/kg daily for 3 days).

P. falciparum infections were considered asymptomatic if the medical history and physical exam revealed no malaria-related signs or symptoms and the patient had not presented to the clinic. Clinical presentation or symptomatic infections identified in the community were labeled symptomatic infections.

The submitted blood sample was separated into a serum and packed RBC fraction by centrifugation and each was cataloged and stored at -80°C. *P. falciparum* DNA was extracted using a commercially available kit (Qiagen) and stored at -80°C until needed.

Only *P. falciparum* infections were included in this study, which spanned 4 years, from 2003--2006. Samples were chosen at random but any infection occurring within 60 days of the initial infection date was excluded to minimize the risk of parasite recrudescence due to incomplete drug clearance.

Nested PCR and allele-typing. Nested PCR was used to amplify the N-terminal region of PfMSP3 using the external PCR primers 5'--ATAATGTTGCTAGTAAAGAAATTG--3' and 5'--

AATACATCATCATTTTCCTTAG--3' and the internal primers 5'--ATAATCTTAACTTAAGAAATGC--3' and 5'--**CGGCGGGGGC**GATAAGCATTTTTTGCC--3'. The bold annotation identifies the CG clamp, which was added to the 5' end of the internal reverse primer to facilitate the subsequent analysis by dHPLC. 1.5µl of *P. falciparum*-infected patient extracted DNA was amplified using ChoiceTaq (Denville) and underwent 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 65°C for 1 minute, followed by a final elongation step of 5 minutes at 65°C. The nested PCR reaction involved the same conditions except that 1µl of the external PCR reaction was used as the template. Classification of the PfMSP3 allele was performed by size migration using 2% agarose gel electrophoresis supplemented with ethidium bromide.

Denaturing high performance liquid chromatography and sequencing. Denaturing High Performance Liquid Chromatography (dHPLC) analysis was performed using a WAVE column (Transgenomics) and standard protocols. Prior to screening, optimal WAVE temperatures for each PfMSP3 allele were determined using Wavemaker software (Transgenomics). 2µl of nested PCR mixture was diluted 1:10 in ddH_20 and submitted for dHPLC analysis. The submitted sample was mixed with a control sequence from the homologous allele and heterologous strand formation was performed by denaturation and subsequent reannealing of the mixture. The sample was then bound to the WAVE column and eluted using a linear acetonitrile gradient. The presence of a second elution peak on the elution chromatograph was indicative of a mutation and all detected mutants were immediately sequenced to identify the mutation. As positive controls, 3D7 and Borneo were complexed with the Peruvian alleles HB3 and K1, respectively. As an addi-

tional quality control step and to define the sensitivity and specificity of the technique, 10% of all samples that exhibited no mutations by dHPLC were randomly sequenced.

Ethics committee approval. This study was approved by review boards of the University of Alabama at Birmingham, Universidad Peruana Cayetano Heredia, and the Peruvian Ministerio de Salud, Instituto Naccional de Salud. Written consent was obtained from all participants prior to study enrollment.

Statistical analysis. Descriptive statistics, such as frequencies, percentages, means, and standard deviations, were used to summarize all study variables of interest. Comparisons between proportions of alleles (K1 and 3D7) for communities, mutant, gender, symptom status, subsequent allele, subsequent *P. falciparum* infections, year of infection, age group ($< 15, \geq 15$), time group to next *P. falciparum* infection (0--6 months, ≥ 6 months), and comparisons between all other proportions of interest were performed using the two-group chi-square test or Fisher's exact test when the assumptions on the chi-square were not tenable. Comparisons between means of actual age and days to next *P. falciparum* infection were performed using the usual two-group t-test, or the two-group t-test for unequal variances when needed. All statistical tests were two-sided and were performed using a 5% significance level (i.e. alpha = 0.05). SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC) was used to perform all statistical analyses.

RESULTS

PfMSP3 allele frequency varies significantly between subsequent years and between villages. The Zungarococha community consists of 4 villages each approximately 2--3 km apart and supported by tributaries of the Amazon River: Zungarococha village

(ZG, N = 805), Puerto Almendra (PA, N = 272), Ninarumi (NR, N = 590), and Llanchama (LL, N = 203) (Fig. 1A). *P. falciparum* transmission is hypoendemic at this study site, transmitted largely by the anthropophilic vector *Anopheles darlingi. P. falciparum* transmission is seasonal, occurring largely between January and August of each year with entomological bite rates described as high as 10--24 bites/person/hour between April--July. This corresponds to an EIR of 0.13 infections/person/month during the 7--month transmission season.[21]

630 *P. falciparum* infections were randomly chosen for inclusion in this study: 101 from 2003, 174 from 2004, 250 from 2005, and 105 from 2006 (Fig. 1B). Samples were chosen to give the widest possible distribution across all 4 villages, although numbers for each village were not equal because the villages do not carry an equal burden of infections: although Zungarococha village is the largest village in the community, it had the least number of infections included in this study. The highest number of infections sampled was from Ninarumi, the second largest village.

Nested PCR was used to amplify and genotype *PfMSP3* from *P. falciparum* infected individuals. The primers amplified the PfMSP3 N-terminal domain, where the majority of genetic diversity has been shown to occur[19] (nucleotides 117--507 in the 3D7 strain, excluding primer sequence) and the allele class identified using agarose gel electrophoresis. Size differences confirmed that both 3D7 and K1 allele classes of PfMSP3 are present in the Zungarococha community (Fig. 2A) and eight samples from each allele classes were sequenced to identify the strain. The sequence of the smaller, 3D7 allele class samples were all identical to the published *PfMSP3* HB3 reference strain (GenBank Acc. No.

AF001137), while the larger, K1 allele class samples were all identical to *PfMSP3* from the K1 reference strain (GenBank Acc. No. AF001151).

Genotyping all 630 samples based on size established that between 2003--2006, a significant majority, 570 (90.3%), of infections were of the 3D7 allele class and only 57 infections were of the K1 allele class. Only 3 infections were complex infections, containing both allele classes, in keeping with the generally "simple" infections observed in hypoendemic transmission areas. Interestingly, the allele frequency distribution fluctuated significantly between subsequent years with the K1 allele class frequency exhibited a decreasing trend; K1 class frequencies ranged from 19.1% in 2004 to 1.0% in 2006 (Fig. 2B). The change in allele class frequency between 2004 and 2005 was statistically significant by chi-square analysis ($p < 0.001$). Additionally, in comparison with the 2003 allele frequency, the decrease in K1 class frequency in both 2005 and 2006 was also statistically significant ($p < 0.001$).

To assess if any correlation might be identified that could explain the significant allele class frequency variation, we looked at the village level for any correlation that exists between *PfMSP3* allele and age, sex, community, patient symptom status, and subsequent infection. As shown in Fig. 2C, only community was significantly associated with *PfMSP3* allele class status. Interestingly, Puerto Almendra demonstrated a significant increase in K1 class infections (> 3 fold, $p < 0.0001$) compared to the other villages. Additionally, Llanchama, the smallest of the villages, was associated with a significant decrease (> 16 fold, $p = 0.002$) in K1 class infections compared to the other villages, with only a single K1 class infection being detected across all four transmission season.

dHPLC can detect single nucleotide differences in PfMSP3 sequence. Due to the large number of samples allele-typed in this study, we adopted a high-throughput approach to screen for SNPs and indels within each amplicon: denaturing High Performance Liquid Chromatography (dHPLC). To our knowledge, this is the first time that dHPLC has been used to screen for SNPs in P. falciparum genes. As described in the methods section, the technique detects mutations through the formation of heteroduplex bubbles. Briefly, a test amplicon is denatured and reannealed in the presence of a reference amplicon of known sequence. If the test and reference sequences are identical, only homoduplexes form, but if there are SNPs or indels in the test sequence that are not found in the reference sequence, then heteroduplexes will form. The reannealed fragments are then bound to a WAVE column (Transgenomics), and eluted with a linear acetonitrile gradient. Homoduplexes elute from the column as a single peak. However, if a heteroduplex bubble is present, it will elute at a different acetonitrile concentration, and therefore form a second peak distinct from the homoduplex peak. Any samples that elute as two peaks from the WAVE column would then be sequenced to confirm the presence and identify of the SNP(s) and/or indels. dHPLC is therefore a rapid method to screen for deviations from a reference sequence, and because the cost is significantly lower (10% or less) than sequencing, dHPLC is of particular use when the expected SNP frequency is low--- it allows the user to rapidly screen large numbers of samples and only sequence those that are likely to contain SNPs.

We initially tested the sensitivity of dHPLC using PfMSP3-specific primers containing a 3'-CG clamp by mixing the HB3 allele either with itself or the same fragment amplified from genomic DNA from the 3D7 strain. Both HB3 and 3D7 are of the same al-

lele class, but 3D7 has previously been shown to contain a single SNP relative to HB3. dHPLC analysis showed a single peak when HB3 was reannealed only to itself, but two elution peaks when 3D7 and HB3 alleles were reannealed (Figure 3A). Sequencing the 3D7 amplicon confirmed the single SNP, showing that dHPLC has the sensitivity to detect single nucleotide changes in *PfMSP3.* Mixing K1 sequences with those from a different K1-class allele, genomic DNA from the Borneo *P. falciparum* strain (a kind gift from John Barnwell) also revealed the presence of a heteroduplex. Sequencing the Borneo fragment showed that K1 and Borneo differ by 37 nucleotides, establishing a range of detection for the PfMSP3-specific dHPLC assay of 1--37 nucleotide differences. The sensitivity of dHPLC withstood wide changes in either reference or unknown amplicon concentrations (data not shown) as has been previously noted, increasing its utility with field *P. falciparum* samples where DNA concentrations can vary.

PfMSP3 coil/coil domain sequence variation is limited. To characterize the level of sequence variation within the N-terminal domain of PfMSP3, all 630 samples that had been allele-typed were subjected to dHPLC analysis. All 3D7 allele class samples were mixed with HB3 sequences as a reference, since as mentioned above, initial sequencing of eight 3D7 class alleles showed all were identical in sequence to the HB3 allele. K1 allele class samples were mixed with K1 sequences as a reference, again because our initial sequencing had identified this as the predominant K1 class allele. Of the 630 samples screened, dHPLC detected heteroduplexes in 14 samples, all in the 3D7 allele class. All 14 samples were sequenced: 3 were false positives with no detectable mutation, while 11 samples had mutations that were different to the HB3 reference sequence: 10 true mutations and one mutation that had double overlapping peaks at one position on the sequenc-

ing chromatogram and therefore presumably resulted from Taq polymerase error. 86 samples that were mutation-negative by dHPLC were also submitted to direct sequencing to test for sensitivity, and all 86 samples were found to be identical to the reference sequence, as predicted by dHPLC. We therefore estimate that using dHPLC to screen for PfMSP3 variation has a sensitivity of 100% and a specificity of 96.6%. Sensitivity is calculated by the equation A/(A+C), where "A" is the number of true positives in the dHPLC-mutant positive group (11) and "C" is the number of true positives in the dHPLC-mutant negative group (0). Specificity is calculated by the equation D/(B+D), where "D" is the number of true negatives in dHPLC-mutant negative group (86) and "B" is the number of true negatives in the dHPLC-mutant positive group (3).

In 630 samples screened, we therefore found SNPs in only 10 samples (Figure 3B), all of which were 3D7-class alleles. Direct sequencing of these ten mutants established that all were identical and contained the same SNP: a single C→T nucleotide substitution at position 203, which resulted in a non-synonymous mutation L68S. Interestingly, this is the SNP that differentiates the HB3 and 3D7 PfMSP3 sequences. All 10 samples were collected during the 2003 transmission season, but they were distributed widely across the community: 3 occurred in Zungarococha village, 5 in Ninarumi, and 2 in Puerto Almendra. No significant correlation was detected between these samples and age, sex, symptom status or village (data not shown). To rule out the possibility that the detected mutations were due to the introduction of a completely new strain into the Zungarococha community, another polymorphic loci, *PfMSP2,* was amplified and sequenced from 6 of these 10 samples. Three of the *PfMSP2* genes were identical to the HB3 *PfMSP2*, but 3

PfMSP2 genes were significantly different (data not shown), suggesting that not all *PfMSP3* mutant strains shared the same origin.

Assessing for evidence of protection for PfMSP3 alleles. The longitudinal MIGIA cohort study employs active case detection in addition to passive case detection. This allows for the identification of not only symptomatic infections, but also asymptomatic infections that remain undiagnosed in the absence of active detection. As premunition is characterized by the absence of malaria symptoms in combination with an inability to clear *P. falciparum* parasites, asymptomatic infections in Peruvian individuals might indicate protection against *P. falciparum*. We therefore compared our *PfMSP3* dHPLC genotyping data, which identified all infections as either HB3, 3D7 or K1 alleles, with the available epidemiologic data to analyze whether there was any propensity for a specific allele type to be associated with a specific clinical outcome.

Because of the hypoendemic transmission and low rate of mixed infections at this study site, it is possible to follow subsequent infections in single individuals that are separated by time. We therefore assessed whether the *PfMSP3* allele frequency in these subsequent infections in the same individual was any different to the population level allele frequency. If there is strong allele-specific immunity to the PfMSP3 N-terminal domain, one would expect that allele frequencies would change in subsequent infections – that is, an HB3 infection would be more likely to be followed by a K1 infection, or vice-versa. There were 106 successive infection pairs (individuals with 2 *P. falciparum* infections in a <500 day period) in the 630 infections for which we had genotyped *PfMSP3*. Comparing the genotypes for the first and second infections in these individuals, we found no statistically significant deviation from whole population allele frequencies– that

is, there was no trend for an infection of one allele to be followed by an infection of another allele, as would be expected if protection was largely allele-specific (Table 1A, $p = 0.93$). Additionally, we tested for the possibility that infection with either allele class would lead to a decrease in subsequent *P. falciparum* infections but this relationship was not significant ($p = 0.17$).

The propensity of individuals to experience another *P. falciparum* infection within a 6-month period was also used to assess for possible protection as well as assessing for an increase in the "time to next infection". As the infection rate has remained relatively steady at 0.13 infections/person/month during the 7-month transmission season,[21] any significant decrease in infection rates in individuals infected with either allele would be suggestive of immune-mediated protection. As shown in Table 1A, no significant delay in time-to-next-infection was detected for either homologous or heterologous allele infections. There was, however, a non-significant trend that K1-infected individuals had a decreased "time to next infection" value (on average 26 days earlier, $p > 0.05$). However, as we showed above that the K1 allele class frequency is significantly associated with community, any difference is most likely due to transmission differences between the villages.

We also tested for any association that might exist between the presence of either *PfMSP3* allele and the rate of asymptomatic infections in subsequent infections. If the asymptomatic rate is higher in subsequent infections with the homologous antigen--- *i.e.* subsequent HB3 infection after a primary HB3 infection--- then immune selection might be a significant factor in mediating this phenomenon. The rate of asymptomatic infections in subsequent heterologous infection is also immensely informative as, if rates are

similar to homologous infections, it suggests some degree of cross-protection. Conversely, a significant increase in asymptomatic infections found in homologous infections, but absent in asymptomatic heterologous infections would suggest a significant strain-specific immune response. Compared to primary infections, no statistical significant difference in HB3 or K1 asymptomatic rates was detected for either homologous or heterologous subsequent infections (Table 1B). Even after pooling the infections into homologous/heterologous subsequent infections, we were unable to detect a significant increase in asymptomatic infections for either group.

DISCUSSION

In this manuscript, we describe the utilization of dHPLC to screen for variation within the malaria vaccine candidate PfMSP3 in a hypoendemic setting. As dHPLC has been demonstrated to exhibit a sensitivity and specificity of up to 100% in some tests, possesses a high throughput capacity, and is considerably more cost-efficient compared with direct sequencing, we decided to adapt it to screen for PfMSP3 N-terminal domain variation. Even with our resequencing of both mutation positive and negative samples for specificity and sensitivity analysis, our overall costs were only approximately 25% of the cost of direct sequencing all 630 isolates. We therefore believe that dHPLC is an effective high throughput approach for *P. falciparum* genotyping, particularly in hypoendemic transmission regions where genetic variation is suspected to be low. Although we applied dHPLC here to study a vaccine candidate antigen, another obvious application would be for drug resistance screening, where SNPs associated with drug resistance in PfCRT, PfDHFR, and PfDHPS are well established.

Using nested PCR we allele typed 630 individual *P. falciparum* infections and found that both 3D7 and K1 alleles classes of PfMSP3 were present in the Zungarococha community. Direct sequencing of those samples identified 3D7 class alleles as identical to the previously published sequences for HB3, and K1 class alleles as identical to the K1 sequence itself. Interestingly, 3D7 class alleles were dominant throughout the community and, over time, became increasingly more dominant. Between 2003 and 2006, the allele frequency shifted significantly, with 3D7 class percent increasing from 80.9% to 99% of all infections. Although the possibility exists that the observed changes in allelic frequency are due to extraneous factors such as genetic drift, which is a major contributor to allele variation in small populations, we believe our study population is sufficiently large that the effects of genetic drift are minimal. Such large scale and directional changes in allele frequencies over such a short time frame are unlikely to be caused by genetic drift, but this remains a formal possibility. Thus, it seems most likely that an as of yet unidentified selection pressure is responsible for driving the observed allele frequency variation. Importantly, if this selection pressure is immunologically-mediated, the target of that response is most likely against the N-terminal domain as the C-terminal domain is almost entirely conserved between both alleles.

Screening for sequence variation identified only 10 mutations in *PfMSP3* in 630 infections. All mutations occurred within the 2003 transmission season and were identical, a C→T non-synonymous substitution at position 203. It is interesting to note that this is the same SNP that was most commonly observed in other global studies, being present in 48% (20 out of 42 total) of 3D7 allele class variants in Nigeria and Thailand.[20] The frequency and global distribution of this SNP suggests a globally conserved function, per-

haps in immune evasion. Although the possibility exists that the lack of PfMSP3 variation that we observed in the Zungarococha community is due to an absence of genetic diversity in this setting, work by Sutton *et al.* (manuscript in preparation) has demonstrated that MSP1-block 2 variation in the Zungarococha community is significant and is similar in spectrum, though not in scale, to diversity seen in a hyperendemic environment (P. Sutton, OH Branch, manuscript in preparation). Additionally, microsatellite studies by Branch *et al.* suggest extensive diversity within the community (OH Branch, personal communication). This implies that the lack of variation in PfMSP3 is likely due not to a lack of genome-wide diversity in the community, but to a combination of the relative stability of the N-terminal domain antigen and the relatively recent introduction of *P. falciparum* in the Zungarococha community. Subsequent studies of PfMSP3 mutants by sequencing the highly polymorphic PfMSP2 revealed that 3 of those infections contained PfMSP2 sequences that were significantly different than HB3, suggesting the introduction of new 3D7 class strains into the community can explain some, but perhaps not all, PfMSP3 mutations. It is likely that the introduction of new *P. falciparum* strains occurred through the naval port located in Ninarumi. In support of this, all 3 PfMSP2-variant infections occurred in Ninarumi (data not shown).

A significant strength of the MIGIA cohort study is the use of active case detection in addition to passive detection of *P. falciparum* infections. Such infections, marked by a lack of malaria-related symptoms in the presence of active parasitemia, mimic protection in malaria-immune individuals, a phenomenon termed premunition, and might predict malaria-immune status in these individuals. Thus, the use of asymptomatic individuals,

detected through active case detection, allows us to assess for correlates of protection for *P. falciparum* alleles.

To identify correlates of protection for PfMSP3 alleles, we combined our PfMSP3 allele data with the available epidemiologic data. Evidence of protection was assessed by testing whether the presence of either PfMSP3 allele correlated with an increased frequency of subsequent allele infections, the rate of subsequent infections, or an increase in the rate of subsequent asymptomatic infections. Although we were unable to find significant correlations of protection for either PfMSP3 allele, this outcome does not rule out the potential for allele-specific protection. Such correlates of protection for PfMSP3 alleles, although important to assess for, are indirect outcomes of host protection and infrequent *P. falciparum* infections, which define the hypoendemic environment, make the identification of significant correlations unfavorable due to a lack of statistical power. More direct tests, such as directly testing antibody levels, will be critical to clearly identifying the role that PfMSP3 plays in mediating immunity in the hypoendemic setting.

In terms of PfMSP3 vaccine development, our central finding of genetic stability in the PfMSP3 N-terminal domain supports a reassessment of the inclusion of this domain in a PfMSP3-based *P. falciparum* vaccine, especially since genetic variation has been the major factor excluding N-terminal domain-based vaccine development. As a large population of the world lives in hypoendemic environments, the observed stable nature of the N-terminal domain of PfMSP3 in our study is significant. Previous reports have demonstrated more extensive PfMSP3 N-terminal domain variation in the hyperendemic setting, but this variation may simply represent a much larger pool of population-level genetic diversity, and does not necessarily mean that the N-terminal domain should be considered

hyper-variable. In such hyperendemic environments the high inoculation rate makes the differentiation of new polymorphisms from infections with heterologous strains difficult to distinguish. In contrast, the hypoendemic environment removes the confounding of heterologous/variant strain inoculation, allowing the dynamics of candidate polymorphisms to be easily studied.

Recent studies have suggested the N-terminal domain of PfMSP3 is significantly more immunogenic than the C-terminal domain,[20] supporting our recommendation that the N-terminal domain be reassessed for future vaccine development. Importantly, as PfMSP3 N-terminal domain variation occurs largely between the heptad repeat motifs, which are almost entirely conserved, concerns about N-terminal domain variation can be largely alleviated if antibody responses against the N-terminal domain are not allele-specific, but instead provide cross-protection between variants. Our genotyping data certainly shows no evidence for allele-specific responses, although this clearly needs to be followed up with immunologic data. The potential of antibodies to mediate cross-protection between N-terminal sequence variants therefore needs to be studied to either support the current C-terminal vaccine constituent or alternatively to provide evidence that supports a novel PfMSP3 N-terminal domain-based subunit vaccine. Such studies are ongoing in our lab.

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REFERENCES

1. Breman JG, 2001. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg 64:* 1--11.

2. Marsh K, 1992. Malaria---a neglected disease? *Parasitology 104 Suppl:* S53--69.

3. Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA, 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med 172:* 379--382.

4. Malkin E, Long CA, Stowers AW, Zou L, Singh S, Macdonald NJ, Narum DL, Miles AP, Orcutt AC, Muratova O, Moretz SE, Zhou H, Diouf A, Fay M, Tierney E, Leese P, Mahanty S, Miller LH, Saul A, Martin LB, 2007. Phase 1 Study of Two Merozoite Surface Protein 1 (MSP1(42)) Vaccines for Plasmodium falciparum Malaria. *PLoS Clin Trials 2:* e12.

5. Yuen D, Leung WH, Cheung R, Hashimoto C, Ng SF, Ho W, Hui G, 2006. Antigenicity and immunogenicity of the N-terminal 33-kDa processing fragment of the Plasmodium falciparum merozoite surface protein 1, MSP1: Implications for vaccine development. *Vaccine 25:* 490--499.

6. Malkin EM, Diemert DJ, McArthur JH, Perreault JR, Miles AP, Giersing BK, Mullen GE, Orcutt A, Muratova O, Awkal M, Zhou H, Wang J, Stowers A, Long CA, Mahanty S, Miller LH, Saul A, Durbin AP, 2005. Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for Plasmodium falciparum ma-

laria. *Infect Immun 73:* 3677--3685.

7. Remarque EJ, Faber BW, Kocken CH, Thomas AW, 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol 24:* 74--84.

8. Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B, Nussenzweig V, 1993. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med 177:* 1287--1298.

9. Doherty JF, Pinder M, Tornieporth N, Carton C, Vigneron L, Milligan P, Ballou WR, Holland CA, Kester KE, Voss G, Momin P, Greenwood BM, McAdam KP, Cohen J, 1999. A phase I safety and immunogenicity trial with the candidate malaria vaccine RTS,S/SBAS2 in semi-immune adults in The Gambia. *Am J Trop Med Hyg 61:* 865--868.

10. Heppner DGJ, Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, Stewart VA, Dubois P, Lanar DE, Krzych U, Moris P, Angov E, Cummings JF, Leach A, Hall BT, Dutta S, Schwenk R, Hillier C, Barbosa A, Ware LA, Nair L, Darko CA, Withers MR, Ogutu B, Polhemus ME, Fukuda M, Pichyangkul S, Gettyacamin M, Diggs C, Soisson L, Milman J, Dubois MC, Garcon N, Tucker K, Wittes J, Plowe CV, Thera MA, Duombo OK, Pau MG, Goudsmit J, Ballou WR, Cohen J, 2005. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine 23:* 2243--2250.

11. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg

SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B, 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature 419:* 498--511.

12. Mills KE, Pearce JA, Crabb BS, Cowman AF, 2002. Truncation of merozoite surface protein 3 disrupts its trafficking and that of acidic-basic repeat protein to the surface of Plasmodium falciparum merozoites. *Mol Microbiol 43:* 1401--1411.

13. Rodriguez LE, Curtidor H, Ocampo M, Garcia J, Puentes A, Valbuena J, Vera R, Lopez R, Patarroyo ME, 2005. Identifying Plasmodium falciparum merozoite surface antigen 3 (MSP3) protein peptides that bind specifically to erythrocytes and inhibit merozoite invasion. *Protein Sci 14:* 1778--1786.

14. Mulhern TD, Howlett GJ, Reid GE, Simpson RJ, McColl DJ, Anders RF, Norton RS, 1995. Solution structure of a polypeptide containing four heptad repeat units from a merozoite surface antigen of Plasmodium falciparum. *Biochemistry 34:* 3479--3491.

15. Burgess BR, Schuck P, Garboczi DN, 2005. Dissection of merozoite surface protein 3, a representative of a family of plasmodium falciparum surface proteins, reveals an oligomeric and highly elongated molecule. *J Biol Chem 280:* 37236--37245.

16. Oeuvray C, Bouharoun-Tayoun H, Grass-Masse H, Lepers JP, Ralamboranto L, Tartar A, Druilhe P, 1994. A novel merozoite surface antigen of Plasmodium falci-

parum (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. *Mem Inst Oswaldo Cruz 89 Suppl 2:* 77--80.

17. McColl DJ, Silva A, Foley M, Kun JF, Favaloro JM, Thompson JK, Marshall VM, Coppel RL, Kemp DJ, Anders RF, 1994. Molecular variation in a novel polymorphic antigen associated with Plasmodium falciparum merozoites. *Mol Biochem Parasitol 68:* 53--67.

18. Oeuvray C, Bouharoun-Tayoun H, Gras-Masse H, Bottius E, Kaidoh T, Aikawa M, Filgueira MC, Tartar A, Druilhe P, 1994. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. *Blood 84:* 1594--1602.

19. Huber W, Felger I, Matile H, Lipps HJ, Steiger S, Beck HP, 1997. Limited sequence polymorphism in the Plasmodium falciparum merozoite surface protein 3. *Mol Biochem Parasitol 87:* 231--234.

20. Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, Bojang KA, Conway DJ, 2007. Plasmodium falciparum Merozoite Surface Protein 3 Is a Target of Allele-Specific Immunity and Alleles Are Maintained by Natural Selection. *J Infect Dis 195:* 279--287.

21. Branch O, Casapia WM, Gamboa DV, Hernandez JN, Alava FF, Roncal N, Alvarez E, Perez EJ, Gotuzzo E, 2005. Clustered local transmission and asymptomatic Plasmodium falciparum and Plasmodium vivax malaria infections in a recently emerged, hypoendemic Peruvian Amazon community. *Malar J 4:* 27.

22. O'Donovan MC, Oefner PJ, Roberts SC, Austin J, Hoogendoorn B, Guy C, Speight

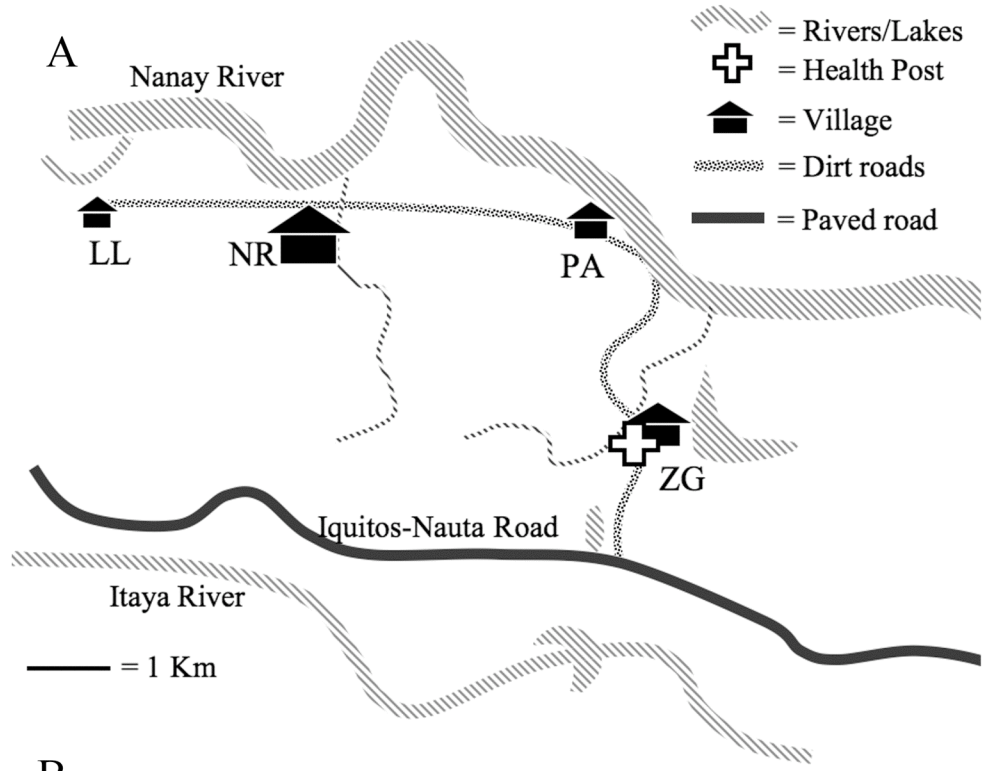
G, Upadhyaya M, Sommer SS, McGuffin P, 1998. Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. *Genomics 52:* 44--49.

23. Balogh K, Patocs A, Majnik J, Racz K, Hunyady L, 2004. Genetic screening methods for the detection of mutations responsible for multiple endocrine neoplasia type 1. *Mol Genet Metab 83:* 74--81.

24. Keller G, Hartmann A, Mueller J, Hofler H, 2001. Denaturing high pressure liquid chromatography (DHPLC) for the analysis of somatic p53 mutations. *Lab Invest 81:* 1735--1737.

25. Wolford JK, Blunt D, Ballecer C, Prochazka M, 2000. High-throughput SNP detection by using DNA pooling and denaturing high performance liquid chromatography (DHPLC). *Hum Genet 107:* 483--487.

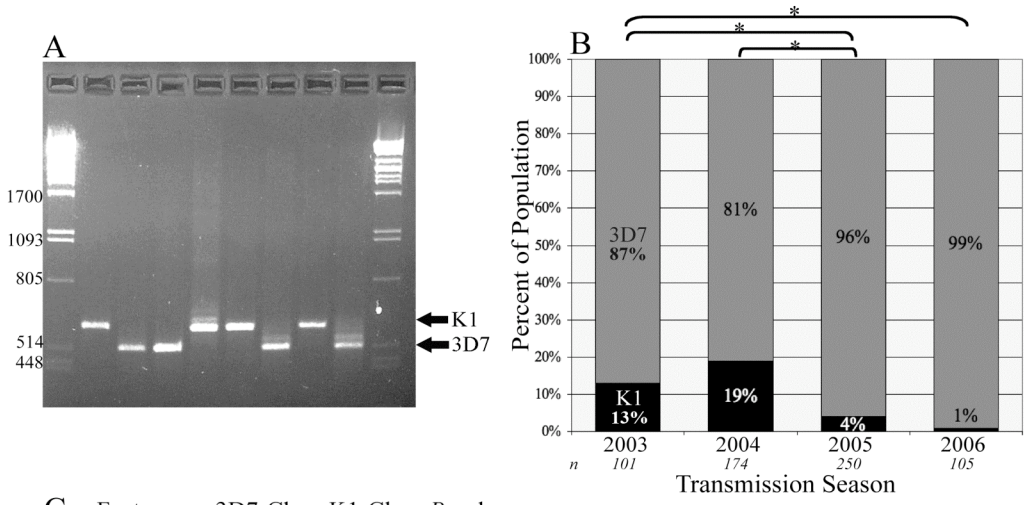
26. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, Pinedo-Cancino V, Patz JA, 2006. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of Falciparum malaria in the Peruvian Amazon. *Am J Trop Med Hyg 74:* 3--11.



B

Village	2003	2004	2005	2006	**Total**
Zungarococha	16	32	42	14	104
Puerto Almendra	41	33	53	14	141
Ninarumi	44	88	91	33	256
Llanchama	0	21	64	44	129
Total:	101	174	250	105	

Figure 1. *P. falciparum* transmission in Zungarococha from 2003--2006. (A) Schema of Zungarococha, a small community of 4 villages fed by the Nanay and Itaya river systems: Zungarococha village (ZG, N = 805), Puerto Almendra (PA, N = 272), Ninarumi (NR, N = 590), and Llanchama (LL, N = 203). The community health post is located in Zungarococha village. (B) Zungarococha community *P. falciparum* infection demographics from 2003--2006. 630 *P. falciparum* infections, spanning all villages, were analyzed in this study.



C

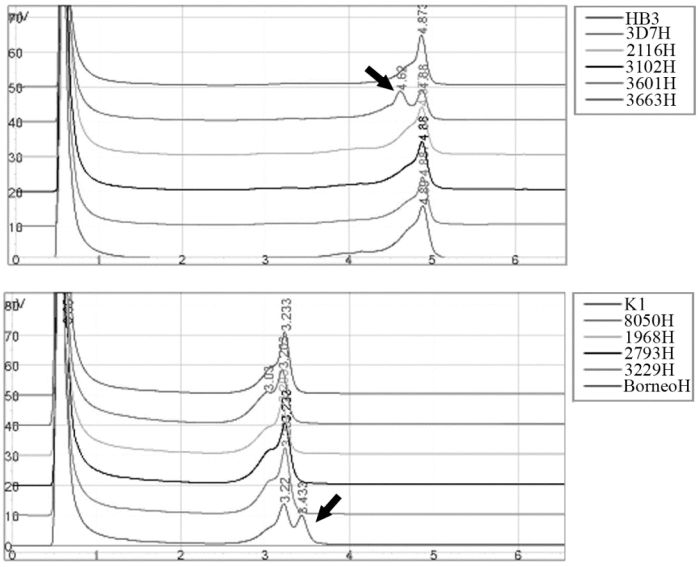
Factor	3D7-Class	K1-Class	*P*-value
Age Group			*0.5745*
< 15	189	21	
>=15	381	36	
Sex			*0.2860*
Male	309	35	
Female	263	22	
Community			*0.0158*
ZG	98	6	
PA	115	26	
NR	232	24	
LL	128	1	
Symptom Status			*0.9960*
None	167	15	
Sx	368	33	

D

Village	3D7-Class	K1-Class	*P*-value
Zungarococha			*0.2025*
ZG	98	6	
Others	475	51	
Puerto Almendra			*0.0001*
PA	115	26	
Others	458	31	
Ninarumi			*0.8128*
NR	232	24	
Others	341	33	
Llanchama			*0.0002*
LL	128	1	
Others	445	56	

Figure 2. PfMSP3 allelic diversity in the Zungarococha community. (A) Agarose gel of nested PCR amplicons from *P. falciparum* DNA extracted from 8 infected Peruvian individuals demonstrates both PfMSP3 K1 (upper arrow) and 3D7 (lower arrow) allele classes are present in the Zungarococha community. (B) PfMSP3 allele class frequency exhibits statistically significant fluctuations, by chi-square, between subsequent years. * = $p < 0.001$. PfMSP3 allele classes are significantly associated with community (C), with K1 infections being significantly elevated and decreased in Puerto Almendra and Llanchama, respectively (D).

A



B

	2003	2004	2005	2006	Total
HB3 Mutants	10	0	0	0	**10**
Identical to HB3	78	141	240	104	**563**
K1 Mutants	0	0	0	0	**0**
Identical to K1	13	33	10	1	**57**

Figure 3. PfMSP3 sequence variation in the Zungarococha community. (A) dHPLC elution chromatograms demonstrate that adapting dHPLC to screen for PfMSP3 variation can detect sequence differences of between 1 and 37 nucleotide differences, as evident by the secondary elution peak (arrows). (B) Screening 630 distinct *P. falciparum* infections yielded only 10 mutations in PfMSP3. 86 dHPLC-negative samples were sequenced to ensure adequate sensitivity.

A

	HB3	K1	*P*-value
Subsequent Allele			*0.9309*
HB3	86	13	
K1	6	1	
Subsequent Pf Infxn			*0.1651*
No	412	36	
Yes	161	21	
Next Infxn (Days)			*0.5179*
Mean	263	237	
n	73	12	

B

	Asym.	Sym.	%Asym	*P*-value
Homologous				*0.4335*
HB3:HB3	26	42	38.2%	
K1:K1	0	1	0.0%	
Heterologous				*0.9126*
HB3:K1	1	2	33.3%	
K1:HB3	3	7	30.0%	
Total Homol.	26	42	38.2%	*0.6095*
Total Hetero.	4	9	30.8%	

Table 1. Assessing for allele-specific protection in the Zungarococha community. (A) Assessing for an association between PfMSP3 allele and subsequent allele, the potential to experience a subsequent *P. falciparum* infection, and the time-to-next-infection failed to identify a significant association. (B) Comparison of PfMSP3 allele-typed infection and subsequent infection symptom status. No significant association was found that suggested any shift in the asymptomatic rate.

THE N-TERMINAL DOMAIN OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-3 IS TARGETED BY CROSS-REACTIVE ANTIBODIES: IMPLICATIONS FOR VACCINE DEVELOPMENT

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ABSTRACT

Infection with *Plasmodium falciparum* causes 300-500 million clinical cases of malaria and 1-3 million deaths each year. Although no commercially available vaccine currently exists, multiple subunit-based vaccines are in development including one based on the promising vaccine candidate *P. falciparum* Merozoite Surface Protein-3 (PfMSP3). PfMSP3 contains two domains: a N-terminal domain, which is known to contain sequence polymorphisms, and a more conserved C-terminal domain. Due largely to variation concerns, the N-terminal domain has been effectively ruled out of ongoing vaccine development. However, recent data showing that antibodies against the N-terminal domain correlate with protection against malaria suggest that this decision should be re-evaluated. Sequence variation is only a concern if antibodies generated by exposure to one variant are unable to cross-react with other variants. To test whether the PfMSP3 N-terminal domain can provide effective cross-reactivity between sequence and allele variants, we studied antibody responses against PfMSP3 domains by ELISA from *P. falciparum* infected individuals in a hypoendemic transmission environment near Iquitos, Peru. Hypoendemic settings are under-utilized for vaccine studies, but infrequent infections with generally single strains of *P. falciparum* provide an ideal environment to study the effect of genetic variation on immune responses, because the complexity of multiple overlapping infections is removed. We established that the PfMSP3 N-terminal domain is significantly more immunogenic than the C-terminal domain, and by comparing antibody responses to the *PfMSP3* genotype in each infection, found that N-terminal domain antibodies are capable of providing significant cross-reactivity across sequence variants

within the same allele class, and even some level of cross-reactivity between allele classes. N-terminal domain antibodies also exhibited potent IgG1/IgG3 antibody responses, an IgG isotype profile well known to correlate with protection against *P. falciparum* malaria, compared to the C-terminal domain, which had higher IgM levels. This data suggests that the impact of PfMSP3 genetic diversity on antibody cross-protection may be more limited than was anticipated, and supports the inclusion of the highly immunogenic N-terminal domain in any PfMSP3 based vaccine.

INTRODUCTION

Infection with *Plasmodium falciparum* causes an estimated 300-500 million clinical episodes of malaria and between 1 and 3 million deaths annually, with over 90% of mortality occurring in children under the age of five [1,2]. The global distribution of malaria is enormous with an estimated 2.4 billion people living in areas of potential malaria transmission [3]. Although effective treatment regimens exist, anti-malaria drug administration alone is not likely to manage the global burden of disease and the emergence of drug resistant parasites remain a serious concern, even with the latest generation of anti-malarials [4]. Although the development of effective vaccines remains a top priority, no vaccine is currently available and deployment is likely years away. Thus there is still an urgent need to not only identify promising potential malaria vaccine targets, but also to determine the optimal antigenic domain(s) of each target that should be tested for inclusion in a subunit-based vaccine.

P. falciparum Merozoite Surface Protein-3 (PfMSP3) was originally identified as a vaccine candidate based on its role in mediating protection in the hyperendemic setting. In these studies, PfMSP3, a 42kD protein expressed on the merozoite surface, was the major target of antibodies from individuals that were protected from severe *P. falciparum* malaria [5,6]. Structurally, PfMSP3 contains two domains, an N-terminal domain characterized by three heptad repeat regions believed to form alpha-helical coil/coil domains, and a C-terminal domain consisting of a low complexity glutamine-rich region and a putative leucine zipper motif [7,8]. PfMSP3 is proteolytically cleaved at the start of the N-terminal domain and has no transmembrane domain or GPI anchor and, therefore, is pre-

sumed to associate with the merozoite surface through interaction with an unknown surface protein(s). Several studies have shown that PfMSP3 is genetically variable and exists in two major allele classes, with almost all observed sequence variation restricted to the N-terminal domain [9-11]. The two allele classes are distinguished by the presence or absence of large indels in the N-terminal domain, but there have also been many observed single nucleotide polymorphisms and smaller indels within each allele class. This variation is largely, although not exclusively, localized to areas between the heptad repeat regions with the residues believed to form the coil/coil tertiary structure remaining almost entirely conserved [7,12]. Although no known function has been established for PfMSP3, it has been postulated to be involved in erythrocyte binding [13]. In support of this putative role, biochemical studies suggest that PfMSP3 likely exists as a long filament-like homotetramer that extends from the merozoite surface and is possibly analogous to the 500Å spiked structures described by merozoite surface EM studies [8,14].

PfMSP3 is an important vaccine target and the majority of such studies have focused on studying the C-terminal domain, as concerns about N-terminal domain genetic diversity have preemptively ruled it out of vaccine development. A long synthetic peptide consisting of 90 amino acids of the C-terminal domain has progressed through both Phase 1a and Phase 1b trials, and generated antibodies capable of killing *P. falciparum* parasites *in vitro* and in an *in vivo* mouse model [15-17]. Recently, however, data from several studies have called the decision to ignore the N-terminal domain into question. A study of 319 children from a hyperendemic environment in The Gambia detected a decreased risk for subsequent clinical malaria with increasing levels of antibodies targeting the N-terminal domain. Although antibodies against the C-terminal domain were also associ-

ated with protection, no change in risk was associated with increasing antibody levels [18]. Furthermore, a vaccine trial using full-length PfMSP3 in *Aotus* monkeys demonstrated significant protection against *P. falciparum* challenge [19], and while no direct comparison between PfMSP3 domains was conducted using immunized *Aotus* sera, the responses were highly allele-specific suggesting the target of those antibodies was the N-terminal domain. The primary concern with inclusion of the PfMSP3 N-terminal domain in a vaccine is therefore the presence of genetic diversity, rather than functional considerations. However, genetic diversity is only a concern if it limits antibody cross-reactivity between allele-classes or between variant sequences within an allele-class. If the PfMSP3 N-terminal domain is to be formally ruled in or out of the vaccine development process in a go/no-go decision as suggested by the malaria vaccine roadmap, there needs to be a systematic analysis of the ability of naturally generated antibodies to mediate cross-protection between PfMSP3 allele-classes and between polymorphic mutants within each class.

Most *P. falciparum* antibody studies are carried out in hyperendemic transmission environments, where individuals are repeatedly exposed to *P. falciparum* antigens and the immune responses are therefore usually stronger. However, such study sites are not suited to measure the impact of genetic diversity on immune responses, because individuals are exposed to multiple infections over the course of a year, with frequent and often overlapping infections that can containing multiple different *P. falciparum* genotypes simultaneously, making it difficult to tease apart the specific allele infection history of a given individual. By contrast hypoendemic transmission environments, where infections are spaced by up to a year apart and frequently consist of only a single detectable *P.*

falciparum genotype, are an under-utilized setting for vaccine development studies. The simple infection profiles, especially in the context of a longitudinal cohort where infection history is followed over a number of years, allows for a direct assessment of the impact of genetic diversity on immune responses by comparing genotype data with immune response data. We have previously followed PfMSP3 genetic diversity in over 600 individuals over four years in the ongoing MIGIA (Malaria Immunity and Genetics in the Amazon) longitudinal cohort study near Iquitos, Peru, where transmission is limited to less than a single infection per person per year [20]. In this study, we used serum samples from those same individuals to perform a comprehensive assessment of the impact of genetic diversity on the generation of cross-reactive anti-PfMSP3 antibodies, with important implications for PfMSP3-based vaccine development.

MATERIALS AND METHODS

Study Site, Subjects and Sample Collection

A detailed description of the four villages that comprise the MIGIA longitudinal cohort study has been previously described [20]. Briefly, the study enrolls residents from four villages in the Zungarococha community, which is located just south of Iquitos in the Peruvian Amazon: Zungarococha village, Puerto Almendra, Ninarumi and Llanchama. The residents have homogenous housing construction, income levels, and access to healthcare, which is provided by the MIGIA cohort physicians and acknowledge infrequent travel outside the community.

The annual malaria season lasts for seven months and closely follows the rainy season, which occurs between January and July. The major vector of malaria transmission in this region is *Anopheles darlingi*, an anthropophilic species that has proliferated in the area since the early 1990's [21]. In this environment, the frequency of infection (FOI) is 0.4 infections/person/month during the 7-month transmission season [20].

All individuals included in this study had confirmed *P. falciparum* infections, which had previously been genotyped for PfMSP3 allele and screened for sequence variation by our lab [11]. Sera samples were collected at the time of patient presentation and were used to study antibody responses by ELISA.

Antigen Construction and Purification

All PfMSP3 constructs were amplified, by PCR, using appropriate genomic DNA from the HB3 (for the HB3 N-terminal domain construct and the C-terminal domain construct), K1 (for the K1 N-terminal domain construct), or 3D7 strain (for the 3D7 and Nig80 N-terminal domain constructs). The forward and reverse primers used were 5'-CCGGCTCGAGGATTTTAGTGGTGGAGAATTTTCGTGGCC-3' and 5'-CGGGATCCTTATTCCCAACCTAAAATATAATC-3', 5'-CCGGCTCGAGTCTATGGAATTCGGAGGTTTTAC-3' and 5'-CGGGATCCTTATTCCCAACCTAAAATATAATC-3', and 5'-CCGGCTCGAGGATTATATTTTAGGTTGGGAATTTGGAGG-3' and 5'-CGGGATCCTTAATGATTTTTAAAATATTTGGATAATTC-3' for HB3 and 3D7 N-terminal domains, K1 N-terminal domain, and Conserved domain, respectively. The Nig80 N-terminal fragment was created using PCR splicing. The primers used were 5'-ATGCGGATCCGATTTTAGTGGTGGAGAATTTTTGTGGCCTGG-3'

and 5'- CTGCTTCTTTAGCAGCTTCTTCTGCCTCTTTAGAAGCATTTTCAG-CATCTTCGGAAGC -3' for the 5' fragment, 5'-GCTGCTAATGATGCTGAAAATGCTTCAAAAGAGGC-3' and 5'-AATTCTGCAGTTATTCCCAACCTAAAATATAATC-3'

for the 3' fragment, and 5'-ATGCGGATCCGATTTTAGTGGTGGAGAATTTTTGTGGCCTGG-3' and 5'-AATTCTGCAGTTATTCCCAACCTAAAATATAATC-3'

for the splicing reaction. The underlined nucleotides identify the Xho1, BamH1, and Pst1 restriction enzyme sequences. Construct inserts were ligated into the expression vector pET15b or pRSETA, which express the construct with the addition of a N-terminally located hexahistidine tag, and then transformed into the *E. coli* BL21(DE3)pLysS "Rosetta" strain for expression. All constructs were verified for sequence accuracy by direct sequencing.

One-liter cultures of the appropriate construct transformants were grown to an optical density of 0.6 at 600nm and construct expression was induced by the addition of 1mM IPTG. At 60-minutes post-incubation, the cells were pelleted and resuspended in 30ml of 50mM $NaPO_4$, pH 8.0 supplemented with protease inhibitors and 5mM β-mercaptoethanol. Lysis was induced by three freeze/thaw cycles, followed by DNAse treatment (10μg/ml) for 1hr at 25°C. The supernatant fraction containing soluble PfMSP3 antigen was obtained by centrifugation at 18,500rpm for 15 minutes and transferred to a 50ml conical tube for purification.

Each construct was subjected to an initial high-temperature purification step of 65°C for 25 minutes to induce denaturation of non-PfMSP3 construct proteins, which

were removed by centrifugation at 18,500rpm for 15 minutes. Subsequent purification was done by loading the sample onto a Ni-NTA agarose column (Qiagen) equilibrated in 50mM $NaPO_4$, pH 8.0, and eluted with a 0-500mM imidazole gradient. Fractions containing the PfMSP3 antigen were then pooled and loaded onto a Hi-Prep 16/10 Q XL anion-exchange column (Amersham Biosciences) equilibrated in 50mM $NaPO_4$, pH 8.0, and eluted with a 0-500mM NaCl gradient. Highly pure PfMSP3 construct antigen was dialyzed against PBS and then stored at -80°C until needed. Antigen purity was confirmed to be > 95% by Coomassie blue stained SDS-PAGE.

Circular Dichroism

Circular Dichroism (CD) spectra were obtained on an Aviv model 400 CD spectrometer equipped with a Peltier temperature controller (Lakewood, NJ). The instrument was standardized using (1S)-(+)-10-camphor sulfonic acid. Samples were placed in 1.0 mm cells (150 µl volume) and equilibrated in the instrument for 10 minutes at 20 EC. Data were collected from 260 nm to 185 nm in 0.5 nm increments with a 10 sec averaging time per point. Spectra were baseline corrected and smoothed, and converted to mean residue ellipticity for plotting and analysis. Secondary structural calculations were performed with PROSEC.

Enzyme-Linked Immunosorbent Assays (ELISA)

Enzyme-Linked Immunosorbent Assays (ELISA) were used to measure antibody titers against each PfMSP3 antigen. Purified PfMSP3 antigen was diluted to 1ng/µl in borate buffer solution (BBS) and 50ul was added to 96-well Maxisorp ELISA plates (Nunc) and incubated overnight at 4°C. The following day, the plates were washed 3x with PBS + 0.05% Tween-20 and blocked for 2 hours with 5% milk/PBS at room tem-

perature. The plates were subsequently washed 3x with PBS/Tween-20 to remove unbound blocking proteins and stored at -20°C.

Peruvian patient sera were diluted 1:100 in AB Wash solution (PBS, 0.05% Tween-20, 0.05% BSA) + 1.5% milk and added, in duplicate, to the appropriate antigen-coated plates. After a 90-minute incubation at room temperature, the plates were washed 4x with AB Wash solution to remove non-binding antibodies. Bound antibodies were detected by the addition of HRP-conjugated anti-IgG (Chemicon) at a 1:5,000 dilution in Ab Wash solution for 1 hour at room temperature. For the isotype studies, HRP-conjugated IgG-isotype-specific (Southern Biotech) and IgM-specific (Fisher Scientific) secondary antibodies were used at a dilution of 1:1000. ChromoPure human IgG (Jackson ImmunoResearch Laboratories) was used to standardize antibody responses, following dilutions in PBS of between 0-1.2μg/ml. All plates were read at 450nm using a Uniread 800 ELISA plate reader (GeneMate, Kaysville, UT).). Serially-diluted positive pools were run concurrently with each set of ELISAs to ensure all OD_{450} measurements remained within the linear range and to facilitate ELISA normalization. All ELISA data were normalized to a linear trend line that fit the mean serial dilution curve specific to each antigen. That curve was generated for each antigen by averaging all positive pool serial dilution curves that remained within the linear range.

Ethics committee approval.

This study was approved by review boards of the University of Alabama at Birmingham, Universidad Peruana Cayetano Heredia, and the Peruvian Ministerio de Salud, Instituto Naccional de Salud. Written consent was obtained from all participants prior to study enrollment.

Statistical Analysis

Descriptive statistics, such as frequencies, percentages, means, and standard deviations, were used to summarize all study variables of interest. Comparisons between proportions of ELISA antigens and allele infections (Figure 2A) were performed using the two-group chi-square test or Fisher's exact test when the assumptions on the chi-square were not tenable. Comparisons between mean IgG levels for ELISA antigens, separately for allele infections (Figure 2B), and for specific antibody responses (Figure 5) were performed using the Kruskal-Wallis test since IgG levels and antibody responses were determined to deviate greatly from a normal distribution. When a statistically significant overall result was obtained, the Dunn multiple comparisons procedure was used to determine which specific pairs of means were significantly different. Spearman correlation analyses were performed to examine the relationships between IgG levels of the ELISA antigens, separately for allele infections (Figure 3). All statistical tests were two-sided and were performed using a 5% significance level (*i.e.* alpha = 0.05). SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC) was used to perform all statistical analyses.

RESULTS

PfMSP3 Construct Antigens

To test for domain-specific anti-*PfMSP3* responses in serum samples from the MIGIA study site near Iguitos, Peru, three constructs were generated: two N-terminal domains, one from each allele class, and one C-Terminal domain (Figure 1A). Our previ-

ous genotyping study established that the 3D7-like and K1-like *PfMSP3* alleles circulating at the MIGIA study site were identical in sequence to the previously published HB3 and K1 *PfMSP3* sequences respectively; the N-terminal domain antigens were therefore constructed using HB3 and K1 *P. falciparum* gDNA so that the sequence exactly matched the PfMSP3 antigens to which infected individuals had been exposed (see Materials and Methods). Purification of these domains by high-temperature incubation followed by both affinity and anion-exchange chromatography yielded milligram quantities of highly pure protein (> 95%) as determined by Coomassie-stained SDS-PAGE (Figure 1B). The C-terminal domain appears as a homodimer even under reducing conditions, suggesting the presence of strong intermolecular attraction. This is consistent with previous reports that suggest a dimerization function for the C-terminal domain [8] and suggests correct folding for the C-terminal domain. To verify that all PfMSP3 construct antigens were folded correctly, we subjected each construct to far-UV Circular dichroism (CD) spectroscopy. As shown in Figure 1C, CD analysis reveals the presence of peaks at 190nm and 215nm for all three antigens indicating the domains to be highly enriched in alpha-helical domains. This is consistent with previous PfMSP3 CD results and suggests the integrity of each antigen's tertiary structure was not affected during expression or purification [8]. With this confirmation of both high PfMSP3 antigen purity and structural integrity, we proceeded to use these antigens to test for anti-PfMSP3 antibody responses in ELISA assays.

A majority of individuals generate anti-PfMSP3 that cross-react across allele classes

Sera samples from 369 distinct *P. falciparum* infections, all previously genotyped for the infecting PfMSP3 allele, were included in this study. Of these 369 infections, 335 contained the HB3 PfMSP3 allele and 34 infections contained the K1 allele, and none were co-infected with more than one allele. This is in keeping with the established allele distribution at this study site, where the HB3 *PfMSP3* allele was present in 90.3% of infections and only 3/630 infections contained more than one allele over a four year period [11]. A time-of-infection antiserum sample from all 369 infections was tested against all three antigens, and results normalized and converted into absolute concentration of anti-PfMSP3 IgG (see Materials and Methods). Positive responses, as measured by ELISA, were defined as responses greater than 3 standard deviations above the mean of our *P. falciparum* negative samples and cutoff values corresponded to 0.888 μg/ml, 1.026 μg/ml, and 0.969 μg/ml for the HB3 N-term, K1 N-Term, and C-Term antigens, respectively.

Overall, *P. falciparum*-infected patient sera demonstrated a high prevalence of anti-PfMSP3 antibody responses against all three antigens, despite the low transmission setting where the average infection rate is less than one infection per person per year [20]. Because all antiserum samples were collected from individuals who had had their infecting *PfMSP3* genotype established previously [11], we were able to compare infecting genotype with antibody responses to determine the prevalence of domain-specific antibody responses. A significantly higher percentage of HB3-infected individuals had positive responses against the HB3 N-terminal domain than either the K1 N-terminal domain or the C-terminal domain (see Fig 2A, $p < 0.0001$), suggesting a majority of responses

were against the infecting N-terminal domain. However, 57% of HB3-infected individuals also generated positive responses against the non-infecting K1 N-terminal domain. Although these could represent long-lasting antibody responses from a previous infection with a K1 PfMSP3 allele type, this is unlikely due to the low frequency of K1 infections at the study site (10% of infections over four years), and because the longitudinal epidemiologic data collected during the MIGIA study establishes that none of these individuals had been previously infected with a K1 *PfMSP3* allele within the last 5 months. Analysis of K1-infected individuals is more restricted because of the smaller numbers (34/369 infections, reflecting the low prevalence of the K1-allele at the study site), but there was a significant increase in anti-K1 responses in the K1-infected individuals relative to HB3-infected individuals (p value < 0.0001), again suggesting the majority of responses were infecting domain-specific.

To characterize the strength of the observed domain-specific responses, we analyzed the distribution of positive responses in IgG antibody levels [μg/ml] and compared the response to the infecting allele class (Figure 2B). For HB3-infected individuals it was clear that not only were positive responses to the infecting N-terminal domain more prevalent than responses to the C-terminal domain, they were also much stronger (Figure 2B). More than twice as many HB3-infected individuals had anti-HB3 antibody levels greater than 10μg/ml as had anti-C-terminal antibodies greater than 10μg/ml ($p < 0.0001$), and more than three times as many had anti-HB3 antibody levels above 20ug/ml ($p < 0.0001$). Comparison of the mean antibody responses demonstrates that HB3-infected individuals exhibited an overall 2.2-fold increase in antibody levels against the HB3 N-terminal domain compared to the C-terminal domain ($p < 0.05$) (Figure 2C). In

K1-infected individuals, we detected no significant difference in antibody levels between K1 N-terminal domain and C-terminal domain antibodies, but this is in part due to the lower numbers of K1-infections. Antibody responses against the N-terminal domains clearly do exhibit allele-specificity, as responses against the heterologous N-terminal domain (K1 N-term antigen in HB3-infected individuals, HB3 N-term antigen in K1-infected individuals) are 2.8-3.1-fold lower than the response against the homologous N-terminal domain (4.6 μg/ml versus 14.1 μg/ml for HB3-infected, $p < 0.05$). However, the mean antibody responses to the heterologous N-terminal domain, although statistically significant compared to the antibody responses to the conserved C-terminal domain, still approximated C-terminal domain antibody levels (in HB3 infected individuals, the mean response against the K1 N-terminal domain was 4.6 μg/ml versus 6.5μg/ml against the C-terminal domain). This suggests that inter-allele cross-reactivity with a non-infecting N-terminal domain is nearly as strong as the response to the conserved C-terminus, despite the fact that the C-terminal domain is part of the PfMSP3 antigen presented, immunologically, in all infections.

Inter-allele and Intra-allele cross-reactive anti-PfMSP3 responses

If anti-PfMSP3 N-terminal domain antibodies are truly cross-reactive between allele classes, the strength of the IgG response to one allele class should correlate with the response to the other. To test this, we generated scatter plots looking at pair-wise antibody responses against both PfMSP3 N-terminal domains. As evident in Figure 3A, although we observed a polarization toward the homologous N-terminal allele, there was a strong positive correlation between the strength of response against both the homologous

and heterologous domains (Spearman's $\rho = 0.5645$, $p < 0.0001$ for HB3-infected, Spearman's $\rho = 0.4780$, $p = 0.0043$ for K1-infected).

PfMSP3 is widely considered a polymorphic antigen, but there has been little study into whether these polymorphisms affect the strength or specificity of the anti-PfMSP3 immune responses, which is the key consideration for vaccine development. Given that the HB3 3D7-class allele was the predominant allele at our study site, we generated N-terminal domain antigens for two other 3D7-class alleles to test for the strength of intra-allele cross-reactive responses. One antigen was identical to the 3D7 allele, which differs from the HB3 sequence at only a single non-synonymous single nucleotide polymorphism (SNP). This is an important antigen to test because this SNP is the most prevalent polymorphism in the 3D7-class alleles and has been found in *P. falciparum* populations across the world [9], suggesting it may be functionally important, perhaps in immune evasion. 3D7 alleles have also been detected at the MIGIA study site, with 10/563 previously genotyped 3D7-class alleles containing this SNP [11]. None of these infections were included in this study, but, although unlikely, it is possible that some individuals had previously been exposed to this allele. As a more extreme difference, we chose a previously published 3D7-class PfMSP3 sequence from Nigeria, which possesses, in addition to the 3D7 SNP, a heptad indel mutation. No similar sequence has been reported in South America, and given the extent of our previous genotyping study, it is extremely unlikely that any of the individuals at the MIGIA study site have been previously exposed to this allele. For this analysis, 241 HB3-infected antiserum samples, all ELISA positive for the HB3 N-terminal domain antigen, were tested against both the 3D7 and Nig80 antigens, results normalized as above, and the responses compared to the re-

sponses against the infecting allele. HB3-infected individuals exhibited strong cross-reactivity against both 3D7 and Nig80 antigens (Figure 3B), with strong positive correlations between responses to the homologous and heterologous antigen (Spearman's $\rho = 0.8985$, $p < 0.0001$ for 3D7, Spearman's $\rho = 0.8685$, $p < 0.0001$ for Nig80). Intra-allele sequence diversity therefore appears to have only limited impact on the strength of responses to the PfMSP3 N-terminal domain.

IgG Isotypes profiles for anti-PfMSP3 antibody responses.

The correlation of cytophilic IgG isotype responses with protection against *P. falciparum* malaria has been widely described in both hypoendemic and hyperendemic environments. We were therefore interested to establish whether the observed N-terminal domain inter-allele cross-reactive antibodies were comprised of largely cytophilic antibody classes, IgG1 and IgG3. To test this, isotype-specific secondary antibodies were used to establish isotype class distribution in all antiserum samples that scored as positive by total IgG ELISA. As shown in Figure 5 and in keeping with previous data, PfMSP3 domains elicit dominantly IgG1 and IgG3 cytophilic antibodies. The C-terminal domain elicited a significantly higher IgM response compared to either N-terminal domain ($p < 0.05$). Mean IgG3 responses were slightly elevated compared to IgG1 levels when comparing N-terminal domain responses in HB3-infected individuals, whereas K1-infected individuals exhibited equal cytophilic responses against the K1 N-terminal domain. Compared to the C-terminal domain cytophilic responses, the HB3 N-terminal domain exhibited a significantly higher IgG1 and IgG3 isotype response ($p < 0.05$) while the K1 N-terminal domain did not.

DISCUSSION

In this manuscript, we conduct a systematic comparison of both N-terminal and C-terminal PfMSP3 domains to identify the optimal constituent for vaccine inclusion. As the N-terminal domain contains the majority of sequence variation and the indels that define the two PfMSP3 alleles, it has largely been excluded from vaccine development. However, recent studies have concluded that the optimal PfMSP3-based subunit vaccine include both domains as antibodies against the N-terminal domain correlated strongly with a reduced risk of clinical malaria. Importantly, however, no direct comparison of domain cross-reactivity can be conducted in hyperendemic environments as the frequent infection with multiple genotypes makes it impossible to characterize cross-protection between intra- and inter-allelic variants and between heterologous domains. In the hypoendemic environment, the widely spaced infections allows for single genotype infections to generate antibody responses that can then be tested against heterologous domains to assess cross-reactivity potential.

By comparing total IgG antibody responses against the N-terminal domains and C-terminal domain, the N-terminal domain was significantly more immunogenic compared to the C-terminal domain eliciting, in HB3 infected individuals, a 2.3-fold increase in total IgG antibody levels and a 3.6-fold increase in individuals who exhibit IgG antibody levels greater than 20μg/ml. This is significant, as *P. falciparum* antigens are generally poor immunogens and usually require adjuvant co-administration to boost antibody responses. Our results confirm results in The Gambia, which demonstrated significantly higher N-terminal domain antibody responses, especially in young children [18].

Based purely on the strength of the immune response, studies in both Africa and South America confirm that the PfMSP3 N-terminal domain is a powerful immunogen. However, to be useful as a vaccine candidate, the PfMSP3 N-terminal domain must generate antibody responses that cross-react either between allele classes, or between polymorphic variants within an allele class. We assessed the ability of N-terminal domain antibodies to mediate both intra- and inter-allelic cross-reactivity by comparing the responses of HB3 infected individuals to different alleles in the same class, 3D7 and Nig80, and also against K1, the circulating allele in the K1-allele class. In one respect, cross-reactivity between allele classes was limited, with responses to the heterologous non-infecting allele significantly lower in mean level than responses to the homologous, infecting allele. This is similar to previous studies in Western Africa which used full-length PfMSP3 constructs in competition ELISAs, rather than sub-domain antigens [18]. However, cross-reactivity between N-terminal domain allele classes was still substantial enough that cross-reactive antibody levels were comparable to the responses against the C-terminal domain – 68% of HB3 infected individuals were still able to elicit positive responses against the K1 N-terminal domain. While such numbers from a hyperendemic transmission setting could be attributed to antibodies generated during a recent K1-class infection, such an explanation is extremely unlikely in this transmission environment. At the MIGIA study site individuals are infected on average less than once per year, and given the very low frequency of K1-like alleles at the study site (10% over 2003-2006), the majority of HB3-infected individuals are unlikely to have experienced a K1-class infection within the last 12 months. Inter-allele class immune responses, while clearly lower than immune responses to the infecting allele, are therefore apparently just as

strong as the immune responses to the C-terminal domain, which contains the current lead PfMSP3 vaccine candidate.

Much more significant cross-reactivity was detected at the intra-allele level, with responses against 3D7 and Nig80 exhibiting significant pair-wise correlation with the homologous HB3 N-terminal domain ($p < 0.0001$). This result is highly significant, as a considerable majority of N-terminal domain sequence variation is intra-allelic, whether due to SNPs or the presence of heptad indel mutations. To our knowledge, this is the first study to measure intra-allele cross-reactivity, and the observed high cross-reactivity potential of N-terminal domain antibodies suggests they target conserved epitopes; perhaps the residues responsible for stabilizing coil/coil domains which are almost exclusively conserved. If such conserved epitopes are indeed the target of N-terminal domain antibodies, then concerns about intra-allelic sequence variation impacting vaccine efficacy can largely be alleviated.

Antibodies to PfMSP3 were predominantly cytophilic IgG1 and IgG3 isotypes, with IgG3 responses slightly elevated above IgG1 levels against all domains. No significant IgG2 or IgG4 responses were detected against any PfMSP3 domain, in concordance with non-cytophilic isotypes results for other malaria antigens. Interestingly, the C-terminal domain exhibited a significant increase in IgM antibodies compared to either N-terminal domain, suggesting responses against the C-terminal domain might precede N-terminal domain responses. Another possibility is that a decrease in C-terminal domain antigen presentation might be delaying immunoglobulin class-switching. The correlation of cytophilic responses with protection against *P. falciparum* has been widely confirmed in endemic environments. It is believed that cytophilic antibodies confer protection

through either phagocytosis or cross-linking Fcγ IIa receptors on blood monocytes [22]. In support of this putative protective role for cytophilic antibodies, the presence of a loss-of-function mutation in Fcγ IIa strongly correlates with an increase risk for clinical malaria [23-24].

Because the size of the *P. falciparum* genome presents a large number of potential vaccine candidates, it is critical that go/no-go decisions based on solid evidence are applied to limit the number of candidates entering expensive later stage vaccine trials. The PfMSP3 N-terminal domain had previously been eliminated from consideration based on concerns about sequence diversity, but such concerns are only valid if that diversity prevents the generation of cross-reactive antibodies that can recognize multiple alleles and sequence variants. This study establishes that the N-terminal domain is highly immunogenic, generates antibodies that strongly cross-react within a given allele-class, and cross-react between allele classes at a level comparable to the responses against the more conserved, but less immunogenic, C-terminal domain. We therefore believe that the PfMSP3 N-terminal domain deserves renewed attention for inclusion in a blood stage *P. falciparum* vaccine, and trials using a mix of two antigens, one representing each allele class, is the most logical formulation to induce strong and cross-protective immune responses.

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REFERENCES

1. Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg*. 2001;64:1-11.
2. Marsh K. Malaria--a neglected disease? *Parasitology*. 1992;104 Suppl:S53-69.
3. Guerra CA, Gikandi PW, Tatem AJ et al. The limits and intensity of Plasmodium falciparum transmission: implications for malaria control and elimination worldwide. *PLoS Med*. 2008;5:e38.
4. Abdulla S, Sagara I, Borrmann S et al. Efficacy and safety of artemether-lumefantrine dispersible tablets compared with crushed commercial tablets in African infants and children with uncomplicated malaria: a randomised, single-blind, multicentre trial. *Lancet*. 2008;372:1819-1827.
5. Oeuvray C, Bouharoun-Tayoun H, Gras-Masse H et al. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. *Blood*. 1994;84:1594-1602.
6. McColl DJ, Silva A, Foley M et al. Molecular variation in a novel polymorphic antigen associated with Plasmodium falciparum merozoites. *Mol Biochem Parasitol*. 1994;68:53-67.
7. Mulhern TD, Howlett GJ, Reid GE et al. Solution structure of a polypeptide containing four heptad repeat units from a merozoite surface antigen of Plasmodium falciparum. *Biochemistry*. 1995;34:3479-3491.
8. Burgess BR, Schuck P, Garboczi DN. Dissection of merozoite surface protein 3, a representative of a family of plasmodium falciparum surface proteins, reveals an oligomeric and highly elongated molecule. *J Biol Chem*. 2005;280:37236-37245.
9. Huber W, Felger I, Matile H, Lipps HJ, Steiger S, Beck HP. Limited sequence polymorphism in the Plasmodium falciparum merozoite surface protein 3. *Mol Biochem Parasitol*. 1997;87:231-234.

10. Escalante AA, Lal AA, Ayala FJ. Genetic polymorphism and natural selection in the malaria parasite Plasmodium falciparum. *Genetics*. 1998;149:189-202.
11. Jordan SJ, Branch OH, Castro JC, Oster RA, Rayner JC. Genetic diversity of the malaria vaccine candidate Plasmodium falciparum merozoite surface protein-3 in a hypoendemic transmission environment. *Am J Trop Med Hyg*. 2009;80:479-486.
12. McColl DJ, Anders RF. Conservation of structural motifs and antigenic diversity in the Plasmodium falciparum merozoite surface protein-3 (MSP-3). *Mol Biochem Parasitol*. 1997;90:21-31.
13. Rodriguez LE, Curtidor H, Ocampo M et al. Identifying Plasmodium falciparum merozoite surface antigen 3 (MSP3) protein peptides that bind specifically to erythrocytes and inhibit merozoite invasion. *Protein Sci*. 2005;14:1778-1786.
14. Bannister LH, Mitchell GH, Butcher GA, Dennis ED, Cohen S. Structure and development of the surface coat of erythrocytic merozoites of Plasmodium knowlesi. *Cell Tissue Res*. 1986;245:281-290.
15. Druilhe P, Spertini F, Soesoe D et al. A Malaria Vaccine That Elicits in Humans Antibodies Able to Kill Plasmodiumfalciparum. *PLoS Med*. 2005;2:e344.
16. Sirima SB, Nebie I, Ouedraogo A et al. Safety and immunogenicity of the Plasmodium falciparum merozoite surface protein-3 long synthethic peptide (MSP3-LSP) malaria vaccine in healthy, semi-immune adult males in Burkina Faso, West Africa. *Vaccine*. 2006
17. Badell E, Oeuvray C, Moreno A et al. Human malaria in immunocompromised mice: an in vivo model to study defense mechanisms against Plasmodium falciparum. *J Exp Med*. 2000;192:1653-1660.
18. Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, Marsh K. Naturally acquired antibodies to polymorphic and conserved epitopes of Plasmodium falciparum merozoite surface protein 3. *Parasite Immunol*. 2007;29:387-394.
19. Hisaeda H, Saul A, Reece JJ et al. Merozoite surface protein 3 and protection against

malaria in Aotus nancymai monkeys. *J Infect Dis*. 2002;185:657-664.

20. Branch O, Casapia WM, Gamboa DV et al. Clustered local transmission and asymptomatic Plasmodium falciparum and Plasmodium vivax malaria infections in a recently emerged, hypoendemic Peruvian Amazon community. *Malar J*. 2005;4:27.
21. Vittor AY, Gilman RH, Tielsch J et al. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of Falciparum malaria in the Peruvian Amazon. *Am J Trop Med Hyg*. 2006;74:3-11.
22. Jafarshad A, Dziegiel MH, Lundquist R, Nielsen LK, Singh S, Druilhe PL. A Novel Antibody-Dependent Cellular Cytotoxicity Mechanism Involved in Defense against Malaria Requires Costimulation of Monocytes Fc{gamma}RII and Fc{gamma}RIII. *J Immunol*. 2007;178:3099-3106.
23. Brouwer KC, Lal AA, Mirel LB et al. Polymorphism of Fc receptor IIa for immunoglobulin G is associated with placental malaria in HIV-1-positive women in western Kenya. *J Infect Dis*. 2004;190:1192-1198.
24. Nasr A, Iriemenam NC, Troye-Blomberg M et al. Fc gamma receptor IIa (CD32) polymorphism and antibody responses to asexual blood-stage antigens of Plasmodium falciparum malaria in Sudanese patients. *Scand J Immunol*. 2007;66:87-96.

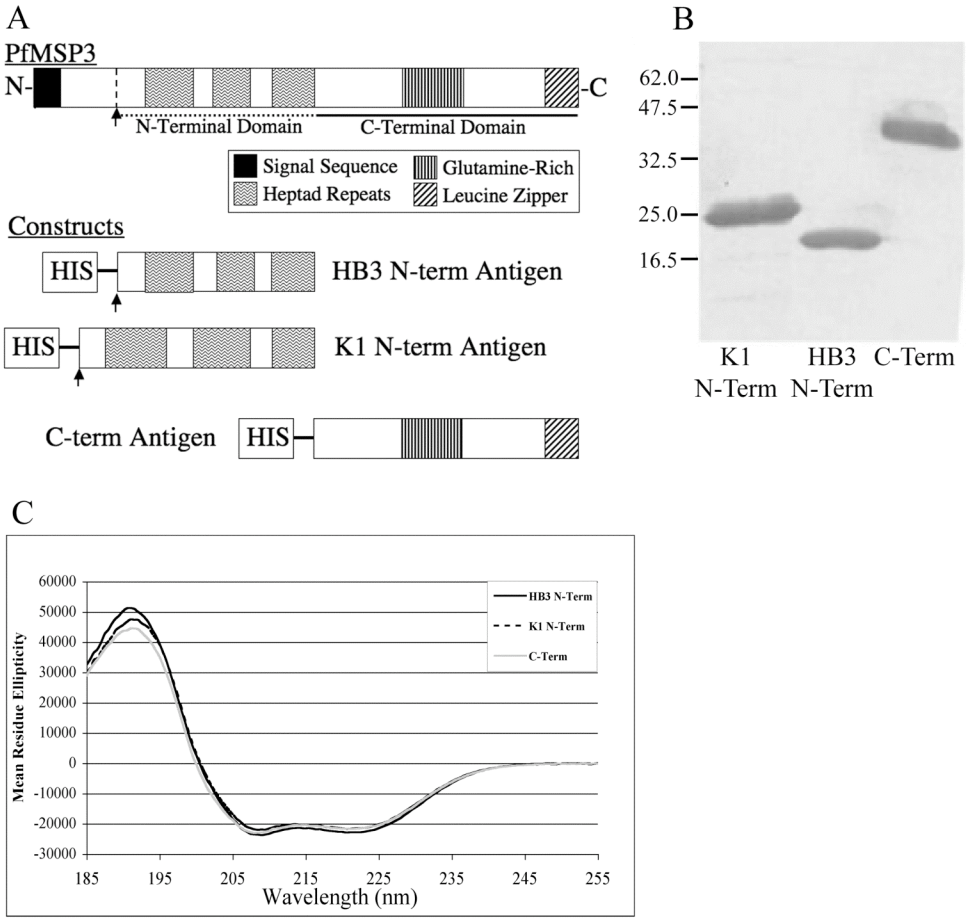

Figure 1. Purity and structural integrity of PfMSP3 domain antigens. (A) Three PfMSP3 constructs were generated to test antibody responses by ELISA: HB3 and K1 allele N-terminal domains and the conserved C-terminal domain. The N-terminal domains began at the proteolytic cleavage site and included all 3 heptad repeats. The C-terminal domain began just C-terminal to the last heptad repeat. (B) Coomassie-blue stained SDS-PAGE demonstrates PfMSP3 construct antigens are > 95% pure as measured by scanning densitometry. (C) Circular dichroism spectroscopy reveals signature peaks at 215nm and 190nm indicative of constructs being comprised largely of alpha-helical structures, consistent with published data for full-length PfMSP3.

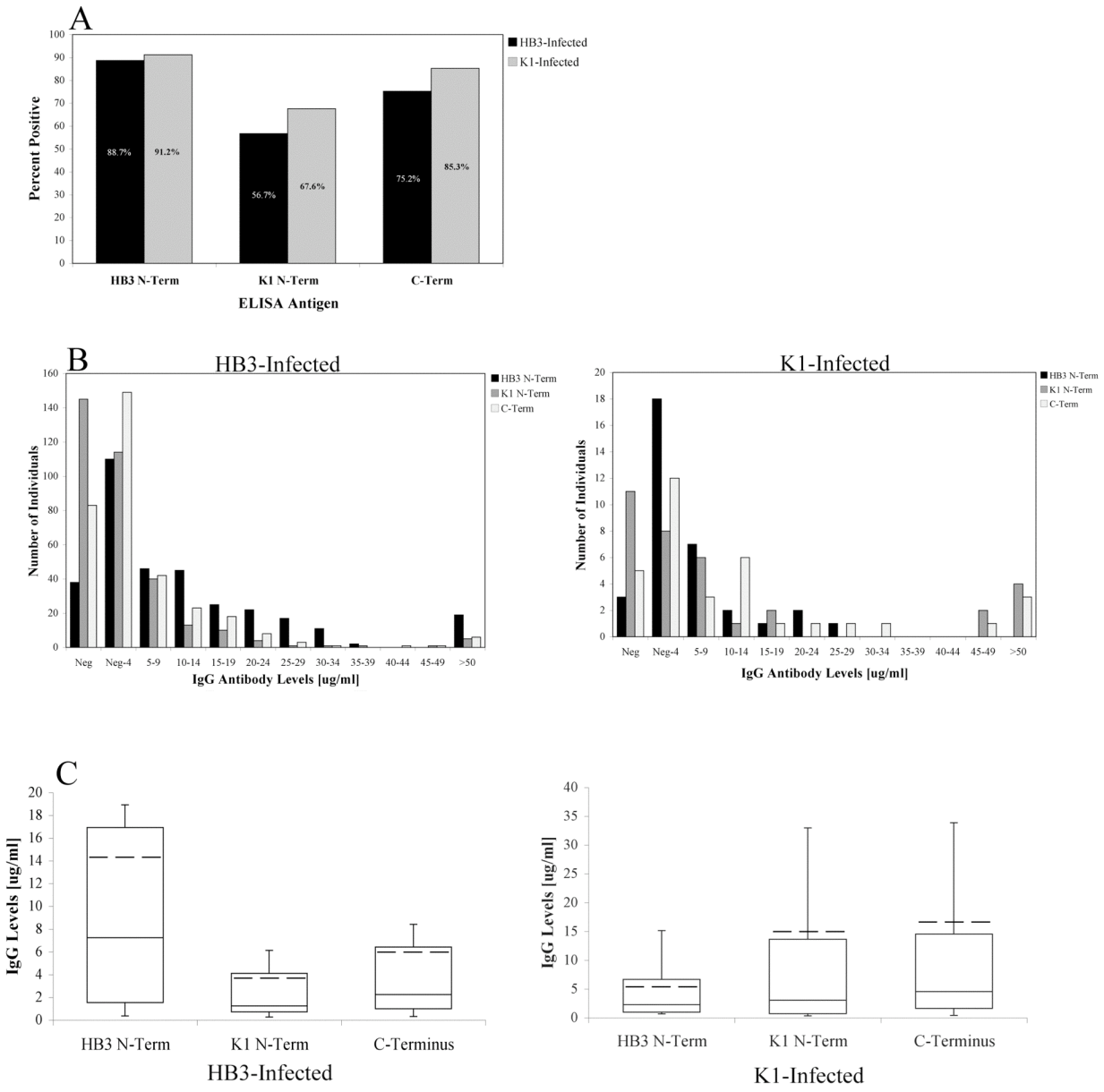

Figure 2. Antibody responses against PfMSP3 domains are not specific to the infecting allele class (A) Percent positive responses, by infected allele, for each PfMSP3 domain antigen. Responses were scored positive if they were three standard deviations above the mean of a negative control pool of serum from individuals who had never been infected with *P. falciparum*. (B) Number of individuals, by allele, who exhibit given IgG antibody levels [μg/ml] against each PfMSP3 domain antigen. (C) IgG antibody level

[μg/ml] distribution in quartiles for each PfMSP3 domain antigen. Whiskers represent upper and lower quartiles, and mean values are indicated by the dashed line. n = 335 for HB3-infected, n = 34 for K1-infected.

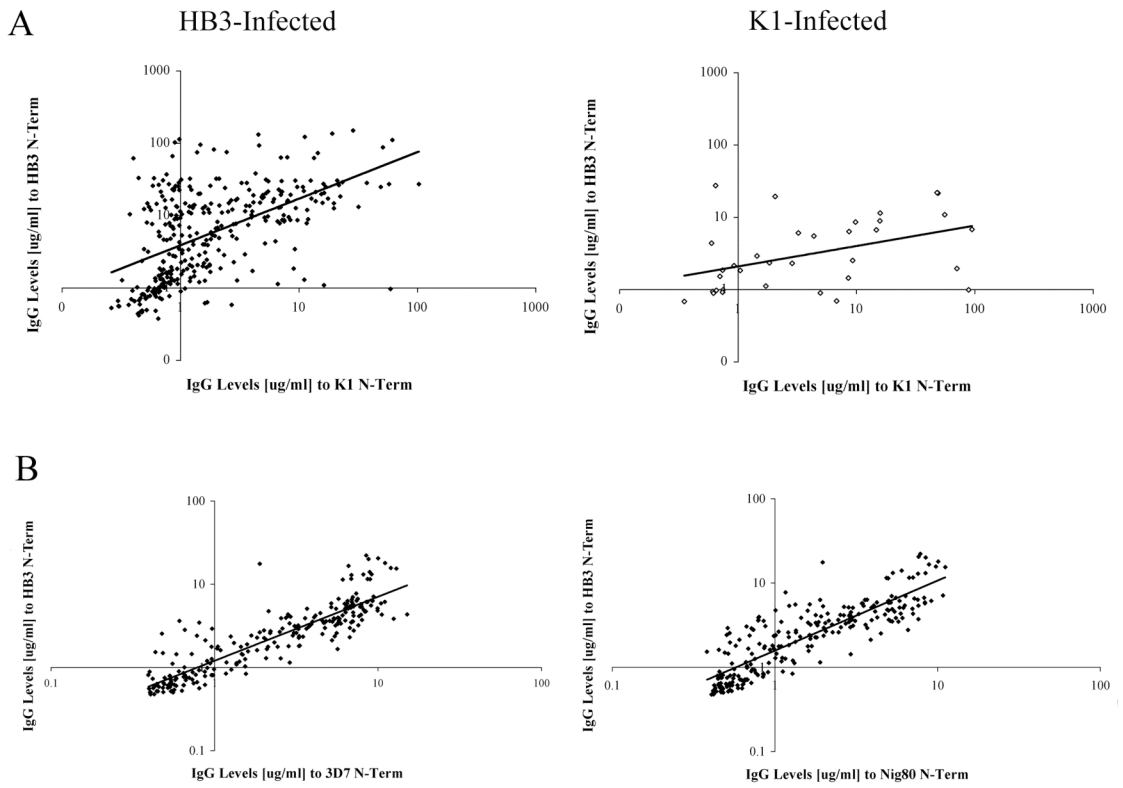

Figure 3. Correlations between anti-N-terminal domain IgG responses both between allele classes, and between polymorphic variants in a single allele class. (A) Pair-wise correlation between anti-HB3 and anti-K1 N-terminal domain responses in individuals separated on the basis of their infecting PfMSP3 allele (Spearman's $\rho = 0.5645$, $p < 0.0001$ for HB3-infected, Spearman's $\rho = 0.4780$, $p = 0.0043$ for K1-infected). The black line indicates a best-fit exponential line. (B) Antiserum from 241ELISA-positive HB3-infected individuals was tested for the ability to recognize other sequence variants within the same allele class; the 3D7 sequence which differs from HB3 at a single SNP, and the more variant Nig80 allele taken from a Nigerian sequence. For 3D7, Spearman's $\rho = 0.8985$, $p < 0.0001$. For Nig80, Spearman's $\rho = 0.8685$, $p < 0.0001$. N = 241 for all samples. The black line indicates a best-fit line.

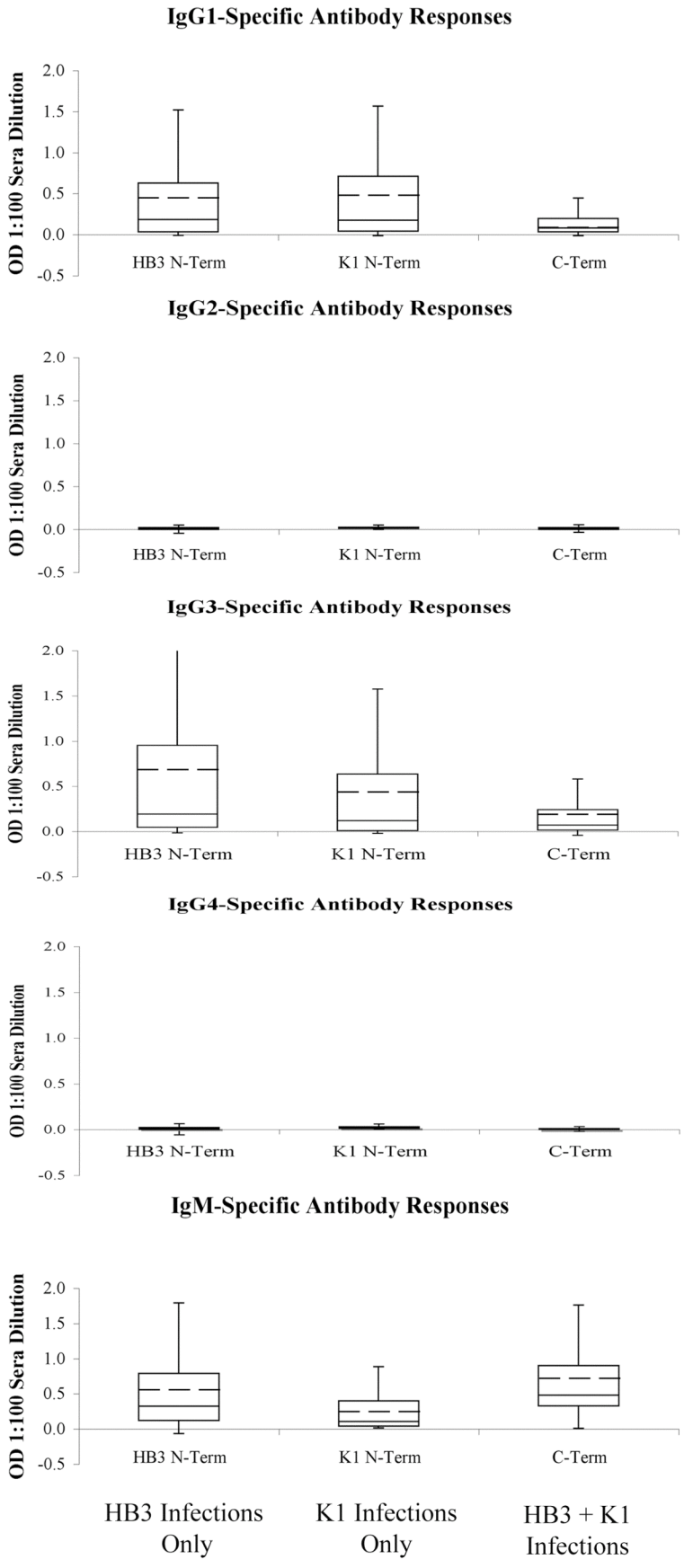
IgG1-Specific Antibody Responses
OD 1:100 Sera Dilution
2.0
1.5
1.0
0.5
0.0
-0.5
HB3 N-Term
K1 N-Term
C-Term
IgG2-Specific Antibody Responses
OD 1:100 Sera Dilution
2.0
1.5
1.0
0.5
0.0
-0.5
HB3 N-Term
K1 N-Term
C-Term
IgG3-Specific Antibody Responses
OD 1:100 Sera Dilution
2.0
1.5
1.0
0.5
0.0
-0.5
HB3 N-Term
K1 N-Term
C-Term
IgG4-Specific Antibody Responses
OD 1:100 Sera Dilution
2.0
1.5
1.0
0.5
0.0
-0.5
HB3 N-Term
K1 N-Term
C-Term
IgM-Specific Antibody Responses
OD 1:100 Sera Dilution
2.0
1.5
1.0
0.5
0.0
-0.5
HB3 N-Term
K1 N-Term
C-Term
HB3 Infections Only
K1 Infections Only
HB3 + K1 Infections

Figure 4. PfMSP3 isotype antibody levels against N-terminal and C-terminal domains. Only homologous N-terminal domain responses were assessed. The whiskers denote the upper and lower quartiles. N = 304 for HB3 N-terminal domain, 22 for K1 N-terminal domain, and 304 for C-terminal domain. The mean is denoted by the dashed line.

SUMMARY DISCUSSION

Plasmodium falciparum is responsible for up to 3 million deaths annually, and an estimated 300-500 million clinical cases of disease. Unfortunately, an effective vaccine is currently not commercially available, although its development remains a top priority. *P. falciparum* Merozoite Surface Protein-3 (PfMSP3) remains one of the leading vaccine candidates although the identification of the optimal domain for inclusion in a PfMSP3-based malaria vaccine has yet to be elucidated. Due to concerns regarding sequence polymorphism, the N-terminal domain has been largely excluded from ongoing vaccine studies despite an absence of hard data supporting this position. Consequently, the current PfMSP3 vaccine consists exclusively of the C-terminal domain in a monovalent subunit vaccine. The work presented in this thesis identifies the optimal PfMSP3 vaccine constituent and lays the groundwork for the development of a more effective PfMSP3-based malaria vaccine.

Genetic diversity within the N-terminal domain is of concern to vaccine development if this domain is unable to generate cross-protective antibody responses, as such a vaccine would only select for escape mutants. In order to characterize the level of genetic variation within PfMSP3, we collaborated with an ongoing cohort study in the Peruvian Amazon, where *P. falciparum* transmission is maintained in a hypoendemic fashion. In this environment, individuals exhibit inoculation rates of 0.4 infections/person/month during the 7-month transmission season (67). Thus, the majority of infections are due to single genotype *P. falciparum* infections and can readily be allele-typed and screened for sequence variation. In Chapter one, we have shown that sequence variation within the N-

terminal domain is very limited in the hypoendemic setting with only 10 mutations being detected in 630 infections spanning 4 transmission seasons. Consequently, this suggests that the observed sequence diversity in hyperendemic environments is likely the result of frequent inoculation of parasites that contain variant sequences rather than the recent development of SNPs in *P. falciparum* genotypes. This has significant implications for PfMSP3-based vaccine research as it reduces concerns about N-terminal domain sequence diversity if, and only if, cross-protection can be demonstrated to occur between sequence variants. This caveat is contingent on cross-reactivity because if cross-reactivity is limited, an N-terminal domain vaccine will exert strong selection pressure for sequence variants that can evade the immune response, commonly referred to as escape mutants.

In addition to studying sequence variation within the N-terminal domain of PfMSP3, we demonstrated that allelic variation was present in the hypoendemic setting, with both PfMSP3 alleles, HB3 and K1, being detected in the population. Interestingly, when comparing the allelic frequency of PfMSP3 between subsequent transmission seasons, we detected a statistically significant shift, with the HB3 allele becoming dominant, which suggests ongoing selection pressure. If this selection pressure is immunologically-mediated, then it must be driven by antibody responses against the N-terminal domain as the C-terminal domain is conserved between alleles. Although we were unable to correlate the presence of either allele or mutant with either an increase in asymptomatic infections or an increase in the time-to-next-infection, which would be indicative of protection, the lack of correlation by no means negates the possibility that such protection does exist. In fact, hypoendemic settings provide sub-optimal environments to assess protection as infrequent infections make assessing correlates of protection difficult.

As mentioned, the benefit of using hypoendemic environments is that they exhibit combined low within-host diversity with high population-level diversity. Consequently, not only does this allow for the analysis of genetic diversity, but it also permits the study of immune-responses to potential vaccine candidates. This benefit results from the low within-host diversity, which allows the immune system to respond against a single antigenic genotype. Thus, as the immune system is naïve to all other strains except the infected genotype, any ability of antibodies to bind heterologous antigens is due exclusively to cross-reactivity. This opportunity is unique to hypoendemic environments and allowed us to conduct a thorough study of both the ability of PfMSP3 domains to induce strong antibody responses and the potential of anti-PfMSP3 antibodies to mediate cross-protection between alleles and between sequence variants.

Through the MIGIA cohort collaboration, we tested 369 individuals against 3 domains of PfMSP3: both N-terminal domain alleles and the C-terminal domain. As *P. falciparum* is endemic in this setting, infected individuals are exposed to wild type full-length PfMSP3, both N-terminal and C-terminal domains. Thus, by detecting the level of antibody responses against either domain, we can characterize each domain's immunogenicity potential. Using ELISA to determine antibody levels against each domain, we demonstrated that the N-terminal domain is significantly more immunogenic than the C-terminal domain, exhibiting a 2.3-fold increase in total IgG levels compared to the C-terminal domain and a 3.6-fold increase in individuals who had total IgG levels greater than 20μg/ml. This parallels work by Osier *et al* in which antibody responses against the N-terminal domain were significantly more immunogenic than against the C-terminal domain, especially in children less than 15 years old (68). Such potent immune responses

against the N-terminal domain might offer an explanation for the presence of genetic diversity in hyperendemic environments.

Certainly the most critical issue regarding N-terminal domain vaccine development is the lack of data characterizing cross-reactivity potential. Osier *et al* assessed cross-reactivity in the hyperendemic environment, but used full-length PfMSP3 in their studies. It is not possible to tease out the contribution of either C-terminal domain antibodies or cross-reacting N-terminal domain antibodies using this methodology, so the question remained largely unanswered. We were greatly interested in this issue and given the hypoendemic study setting, were uniquely positioned to address the question. By comparing antibody responses against the homologous (infecting) and heterologous (non-infecting) N-terminal domains, we found that inter-allele cross-reactive antibody responses were not as potent as the response to the homologous domain, suggesting allele-specificity does exist for anti-N-terminal domain antibodies. Surprisingly, however, we detected that the partial inter-allele cross-reactivity that did exist in these individuals was almost as substantial as the responses to the C-terminal domain. In HB3-infected individuals, for example, 68% of individuals had ELISA positive responses against the K1 N-terminal domain, despite the fact they were not infected with this domain, nor, given the low frequency of K1 alleles in the study site and the low transmission dynamics, were they likely to have been for at least 12 months. This finding is highly significant as it demonstrates that, with respect to cross-reactive antibodies, N-terminal domain allele-specificity is to a large extent offset by the increased immunogenicity of N-terminal domains compared to the C-terminal domain. Additionally, we determined that N-terminal domain antibodies are capable of mediating substantial intra-allele cross-reactivity. By

testing two 3D7-class allele sequence variants, 3D7 and Nig80, we detected significant pair-wise correlation with the responses against the homologous HB3 N-terminal domain, despite the fact that individuals were not currently infected with either the 3D7 or Nig80 sequences. Additionally, it is unlikely that any of these individuals were previously exposed to either variants as, in the case of 3D7, only ten 3D7 PfMSP3 alleles were identified between May-June, 2003 out of 630 individual infections studied spanning four transmission seasons and none of the infections included in this study have previous infections occurring within that time period. In the case of Nig80 sequence, previous exposure is also highly unlikely as this is a PfMSP3 variant found in Africa that has not so far been detected in South America. This indicates that the epitopes for N-terminal domain antibodies are largely restricted to non-polymorphic regions and suggests that antibody targets might be conserved epitopes within the heptad repeat region. Importantly, as the residues that are responsible for stabilizing coil/coil tertiary structure are almost entirely conserved, it is likely that conserved epitopes could be identified and become vaccine constituents. In fact, preliminary studies into identifying N-terminal domain epitopes that mediate cross-reactivity between alleles and the C-terminal domain are described in Appendix A. In these studies, monoclonal antibodies were raised against the HB3 N-terminal domain, and the cross-reactive potential of 20 different murine hybridoma lines was assessed by ELISA. Interestingly, several hybridomas exhibit monoclonal antibodies that cross-react with both the heterologous N-terminal domain, K1, and the C-terminal domain, suggesting that the identification of highly cross-reactive epitopes might be possible.

Correlates of protection against *P. falciparum* malaria are difficult to identify, as the mechanisms that mediate that protection are poorly understood. It is known that antibodies are intimately involved in protection, as IgG alone is capable of recapitulating protection in naïve individuals against a *P. falciparum* challenge. Currently, the strongest immunological characteristic that correlates with effective protection is the presence of high cytophilic IgG isotype responses (36,37,41,69). Cytophilic isotypes, either IgG1 or IgG3, are capable of stimulating monocytes and macrophages through Fcγ receptor signaling, which elicit developmental arrest of *P. falciparum* within the erythrocytes (55,70). Importantly, the potential of vaccine candidates to elicit high IgG1/IgG3 responses must be assessed in order to characterize protection potential before phase II clinical trials are performed. In a hypoendemic setting, we determined, for PfMSP3, that both domains elicit potent IgG1/IgG3 isotype responses. As expected, the isotype levels were significantly higher for antibodies against the N-terminal domain as it is significantly more immunogenic than the C-terminal domain. Importantly, this suggests that antibodies against the N-terminal domain would better correlate with protection than C-terminal domain antibodies, although further studies are warranted to definitively confirm this. As described in Appendix B, preliminary ADCI studies demonstrate significant *P. falciparum* growth inhibition for HB3 N-terminal domain antibodies. Such experiments are ongoing, but will allow us to functionally characterize the potential of PfMSP3 antibodies to confer *in vitro* protection.

Taken together, we demonstrate that the PfMSP3 N-terminal domain elicits higher antibody responses, compared to the C-terminal domain, in *P. falciparum* infected individuals. Although sequence variation exists in N-terminal domains, compounded by

the presence of two allelic isoforms, we have demonstrated that N-terminal domain antibodies are capable of significant intra-allelic cross-reactivity between sequence variants and partial inter-allelic cross-reactivity, and that the level of inter-allelic cross-reactivity is comparable to the level of the response to the C-terminal domain. Thus, we recommend the inclusion of both N-terminal domain alleles as vaccine constituents, in addition to the C-terminal domain. This recommendation is supported by several additional observations. Firstly, although N-terminal domain antibodies demonstrated only partial cross-reactivity between alleles, inclusion of both alleles in a vaccine would likely elicit potent homologous-antigen levels against both domains. A concern with development of a monovalent N-terminal domain vaccine is that vaccination will select for the heterologous allele, due to N-terminal domains exhibiting only partial inter-allele cross-reactivity. Thus, including both N-terminal domains ensures the highest level of protection against both alleles. Secondly, we confirmed that the potent N-terminal domain antibody response is largely IgG1/IgG3 dominant; an isotype profile that correlates with *in vivo* protection.

Importantly, however, we recommend the development of a trivalent PfMSP3-based subunit vaccine, comprised of both N-terminal domain antigens in combination with the C-terminal domain. Although our data suggests that the N-terminal domain cross-reactivity concerns can be largely alleviated by inclusion of both N-terminal domain alleles, inclusion of the C-terminal domain would provide additional protection against escape mutants. Additionally, evidence from hyperendemic environments does demonstrate that antibodies against the C-terminal domain correlate with protection against *P. falciparum*, albeit not as strongly as N-terminal domain antibodies. This “triva-

lent" vaccine could consist of either three individual domains (two N-terminal alleles and the conserved C-terminal domain), or two full-length PfMSP3 antigens, one from each allele class.

In summary, we describe in this thesis the optimal constituent for a PfMSP3-based vaccine. By including both N-terminal domains in addition to the C-terminal domain, this novel trivalent malaria vaccine should elicit potent antibody responses that are dominantly IgG1/IgG3 cytophilic antibodies. Given that full-length PfMSP3 from one allele class has already been produced at NIH and trialed in *Aotus* monkeys, further trials should be possible in the near future.

GENERAL LIST OF REFERENCES

1. Breman JG, Holloway CN, 2007. Malaria surveillance counts. *Am J Trop Med Hyg 77:* 36-47.

2. McElroy PD, ter Kuile FO, Hightower AW, Hawley WA, Phillips-Howard PA, Oloo AJ, Lal AA, Nahlen BL, 2001. All-cause mortality among young children in western Kenya. VI: the Asembo Bay Cohort Project. *Am J Trop Med Hyg 64:* 18-27.

3. Breman JG, Alilio MS, Mills A, 2004. Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am J Trop Med Hyg 71:* 1-15.

4. Ettling MB, Shepard DS, 1991. Economic cost of malaria in Rwanda. *Trop Med Parasitol 42:* 214-218.

5. Jowett M, Miller NJ, 2005. The financial burden of malaria in Tanzania: implications for future government policy. *Int J Health Plann Manage 20:* 67-84.

6. Larson BA, Amin AA, Noor AM, Zurovac D, Snow RW, 2006. The cost of uncomplicated childhood fevers to Kenyan households: implications for reaching international access targets. *BMC Public Health 6:* 314.

7. Guerra CA, Gikandi PW, Tatem AJ, Noor AM, Smith DL, Hay SI, Snow RW, 2008. The limits and intensity of Plasmodium falciparum transmission: implications for malaria control and elimination worldwide. *PLoS Med 5:* e38.

8. Thores OA, 1996. Malaria: Trends Affecting Scotland. *J Travel Med 3:* 122-123.

9. Brown G, Biberfeld P, Christensson B, Mason DY, 1979. The distribution of HLA on human lymphoid, bone marrow and peripheral blood cells. *Eur J Immunol 9:* 272-275.

10. Groux H, Gysin J, 1990. Opsonization as an effector mechanism in human protection against asexual blood stages of Plasmodium falciparum: functional role of IgG subclasses. *Res Immunol 141:* 529-542.

11. COHEN S, McGREGOR IA, CARRINGTON S, 1961. Gamma-globulin and acquired immunity to human malaria. *Nature 192:* 733-737.

12. MCGREGOR IA, WILLIAMS K, GOODWIN LG, 1963. PYRIMETHAMINE AND SULPHADIAZINE IN TREATMENT OF MALARIA. *Br Med J 5359:* 728-729.

13. Clyde DF, 1975. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. *Am J Trop Med Hyg 24:* 397-401.

14. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M, Paul C, Gordon DM, Stoute JA, Church LW, Sedegah M, Heppner DG, Ballou WR, Richie TL, 2002. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis 185:* 1155-1164.

15. Kumar KA, Baxter P, Tarun AS, Kappe SH, Nussenzweig V, 2009. Conserved protective mechanisms in radiation and genetically attenuated uis3(-) and uis4(-) Plasmodium sporozoites. *PLoS One 4:* e4480.

16. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, Sigauque B, Milman J, Mandomando I, Bassat Q, Guinovart C, Espasa M, Corachan S, Lievens M, Navia MM, Dubois MC, Menendez C, Dubovsky F, Cohen J, Thompson R, Ballou WR, 2005. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet 366:* 2012-2018.

17. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B, 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature 419:* 498-511.

18. Joergensen L, Turner L, Magistrado P, Dahlback MA, Vestergaard LS, Lusingu JP, Lemnge M, Salanti A, Theander TG, Jensen AT, 2006. Limited cross-reactivity among domains of the Plasmodium falciparum clone 3D7 erythrocyte membrane protein 1 family. *Infect Immun 74:* 6778-6784.

19. Viebig NK, Gamain B, Scheidig C, Lepolard C, Przyborski J, Lanzer M, Gysin J, Scherf A, 2005. A single member of the Plasmodium falciparum var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep 6:* 775-781.

20. Ndam NT, Deloron P, 2007. Molecular Aspects of Plasmodium falciparum Infection during Pregnancy. *J Biomed Biotechnol 2007:* 43785.

21. Bannister LH, Mitchell GH, Butcher GA, Dennis ED, Cohen S, 1986. Structure and development of the surface coat of erythrocytic merozoites of Plasmodium knowlesi. *Cell Tissue Res 245:* 281-290.

22. Holder AA, Freeman RR, 1984. Protective antigens of rodent and human blood-stage malaria. *Philos Trans R Soc Lond B Biol Sci 307:* 171-177.

23. Heidrich HG, Miettinen-Baumann A, Eckerskorn C, Lottspeich F, 1989. The N-terminal amino acid sequences of the Plasmodium falciparum (FCB1) merozoite surface antigens of 42 and 36 kilodalton, both derived from the 185-195-kilodalton precursor. *Mol Biochem Parasitol 34:* 147-154.

24. Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA, 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med 172:* 379-382.

25. O'Donnell RA, de Koning-Ward TF, Burt RA, Bockarie M, Reeder JC, Cowman AF, Crabb BS, 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. *J Exp Med 193:* 1403-1412.

26. Branch OH, Udhayakumar V, Hightower AW, Oloo AJ, Hawley WA, Nahlen BL, Bloland PB, Kaslow DC, Lal AA, 1998. A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kiloDalton domain of Plasmodium falciparum in pregnant women and infants: associations with febrile illness, parasitemia, and anemia. *Am J Trop Med Hyg 58:* 211-219.

27. O'Donnell RA, Saul A, Cowman AF, Crabb BS, 2000. Functional conservation of the malaria vaccine antigen MSP-119across distantly related Plasmodium species. *Nat Med 6:* 91-95.

28. Okie S, 2005. Betting on a malaria vaccine. *N Engl J Med 353:* 1877-1881.

29. Graves P, Gelband H, 2006. Vaccines for preventing malaria (SPf66). *Cochrane Database Syst Rev* CD005966.

30. Snounou G, Renia L, 2007. The vaccine is dead--long live the vaccine. *Trends Parasitol 23:* 129-132.

31. Gilbert SC, Hill AV, 1998. The SPf66 Malaria Vaccine: Where Do We Go From Here? *Parasitol Today 14:* 251.

32. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, Pinedo-Cancino V, Patz JA, 2006. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of Falciparum malaria in the Peruvian Amazon. *Am J Trop Med Hyg 74:* 3-11.

33. Aramburu Guarda J, Ramal Asayag C, Witzig R, 1999. Malaria reemergence in the Peruvian Amazon region. *Emerg Infect Dis 5:* 209-215.

34. Oeuvray C, Bouharoun-Tayoun H, Gras-Masse H, Bottius E, Kaidoh T, Aikawa M, Filgueira MC, Tartar A, Druilhe P, 1994. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. *Blood 84:* 1594-1602.

35. McColl DJ, Silva A, Foley M, Kun JF, Favaloro JM, Thompson JK, Marshall VM, Coppel RL, Kemp DJ, Anders RF, 1994. Molecular variation in a novel polymorphic antigen associated with Plasmodium falciparum merozoites. *Mol Biochem Parasitol 68:* 53-67.

36. Soe S, Theisen M, Roussilhon C, Aye KS, Druilhe P, 2004. Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. *Infect Immun 72:* 247-252.

37. Bouharoun-Tayoun H, Druilhe P, 1992. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun 60:* 1473-1481.

38. Perraut R, Marrama L, Diouf B, Fontenille D, Tall A, Sokhna C, Trape JF, Garraud O, Mercereau-Puijalon O, 2003. Distinct surrogate markers for protection against Plasmodium falciparum infection and clinical malaria identified in a Senegalese community after radical drug cure. *J Infect Dis 188:* 1940-1950.

39. Eisenhut M, 2007. Immunity to blood stages of Plasmodium falciparum is dependent on a specific pattern of immunoglobulin subclass responses to multiple blood stage antigens. *Med Hypotheses* .

40. Aribot G, Rogier C, Sarthou JL, Trape JF, Balde AT, Druilhe P, Roussilhon C, 1996. Pattern of immunoglobulin isotype response to Plasmodium falciparum blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). *Am J Trop Med Hyg 54:* 449-457.

41. Taylor RR, Allen SJ, Greenwood BM, Riley EM, 1998. IgG3 antibodies to Plasmodium falciparum merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. *Am J Trop Med Hyg 58:* 406-413.

42. Burgess BR, Schuck P, Garboczi DN, 2005. Dissection of merozoite surface protein 3, a representative of a family of plasmodium falciparum surface proteins, reveals an oligomeric and highly elongated molecule. *J Biol Chem 280:* 37236-37245.

43. Rodriguez LE, Curtidor H, Ocampo M, Garcia J, Puentes A, Valbuena J, Vera R, Lopez R, Patarroyo ME, 2005. Identifying Plasmodium falciparum merozoite sur-

face antigen 3 (MSP3) protein peptides that bind specifically to erythrocytes and inhibit merozoite invasion. *Protein Sci 14:* 1778-1786.

44. Mulhern TD, Howlett GJ, Reid GE, Simpson RJ, McColl DJ, Anders RF, Norton RS, 1995. Solution structure of a polypeptide containing four heptad repeat units from a merozoite surface antigen of Plasmodium falciparum. *Biochemistry 34:* 3479-3491.

45. Nomizu M, Utani A, Beck K, Otaka A, Roller PP, Yamada Y, 1996. Mechanism of laminin chain assembly into a triple-stranded coiled-coil structure. *Biochemistry 35:* 2885-2893.

46. Steven AC, Trus BL, Maizel JV, Unser M, Parry DA, Wall JS, Hainfeld JF, Studier FW, 1988. Molecular substructure of a viral receptor-recognition protein. The gp17 tail-fiber of bacteriophage T7. *J Mol Biol 200:* 351-365.

47. Phillips GNJ, Flicker PF, Cohen C, Manjula BN, Fischetti VA, 1981. Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface. *Proc Natl Acad Sci U S A 78:* 4689-4693.

48. Huber W, Felger I, Matile H, Lipps HJ, Steiger S, Beck HP, 1997. Limited sequence polymorphism in the Plasmodium falciparum merozoite surface protein 3. *Mol Biochem Parasitol 87:* 231-234.

49. McColl DJ, Anders RF, 1997. Conservation of structural motifs and antigenic diversity in the Plasmodium falciparum merozoite surface protein-3 (MSP-3). *Mol Biochem Parasitol 90:* 21-31.

50. Pearce JA, Hodder AN, Anders RF, 2004. The alanine-rich heptad repeats are intact in the processed form of Plasmodium falciparum MSP3. *Exp Parasitol 108:* 186-189.

51. Mills KE, Pearce JA, Crabb BS, Cowman AF, 2002. Truncation of merozoite surface protein 3 disrupts its trafficking and that of acidic-basic repeat protein to the surface of Plasmodium falciparum merozoites. *Mol Microbiol 43:* 1401-1411.

52. Oeuvray C, Bouharoun-Tayoun H, Grass-Masse H, Lepers JP, Ralamboranto L, Tartar A, Druilhe P, 1994. A novel merozoite surface antigen of Plasmodium falciparum (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. *Mem Inst Oswaldo Cruz 89 Suppl 2:* 77-80.

53. Badell E, Oeuvray C, Moreno A, Soe S, van Rooijen N, Bouzidi A, Druilhe P, 2000. Human malaria in immunocompromised mice: an in vivo model to study defense mechanisms against Plasmodium falciparum. *J Exp Med 192:* 1653-1660.

54. Hisaeda H, Saul A, Reece JJ, Kennedy MC, Long CA, Miller LH, Stowers AW, 2002. Merozoite surface protein 3 and protection against malaria in Aotus nancymai monkeys. *J Infect Dis 185:* 657-664.

55. Jafarshad A, Dziegiel MH, Lundquist R, Nielsen LK, Singh S, Druilhe PL, 2007. A Novel Antibody-Dependent Cellular Cytotoxicity Mechanism Involved in Defense against Malaria Requires Costimulation of Monocytes Fc(gamma)RII and Fc(gamma)RIII. *J Immunol 178:* 3099-3106.

56. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P, 1990. Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *J Exp Med 172:* 1633-1641.

57. Khusmith S, Druilhe P, 1983. Cooperation between antibodies and monocytes that inhibit in vitro proliferation of Plasmodium falciparum. *Infect Immun 41:* 219-223.

58. Lunel F, Druilhe P, 1989. Effector cells involved in nonspecific and antibody-dependent mechanisms directed against Plasmodium falciparum blood stages in vitro. *Infect Immun 57:* 2043-2049.

59. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P, 1995. Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages. *J Exp Med 182:* 409-418.

60. Naughton JA, Bell A, 2007. Studies on cell-cycle synchronization in the asexual erythrocytic stages of Plasmodium falciparum. *Parasitology 134:* 331-337.

61. Singh S, Soe S, Mejia JP, Roussilhon C, Theisen M, Corradin G, Druilhe P, 2004. Identification of a conserved region of Plasmodium falciparum MSP3 targeted by biologically active antibodies to improve vaccine design. *J Infect Dis 190:* 1010-1018.

62. Audran R, Cachat M, Lurati F, Soe S, Leroy O, Corradin G, Druilhe P, Spertini F, 2005. Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. *Infect Immun 73:* 8017-8026.

63. Druilhe P, Spertini F, Soesoe D, Corradin G, Mejia P, Singh S, Audran R, Bouzidi A, Oeuvray C, Roussilhon C, 2005. A Malaria Vaccine That Elicits in Humans Antibodies Able to Kill Plasmodiumfalciparum. *PLoS Med 2:* e344.

64. Sirima SB, Nebie I, Ouedraogo A, Tiono AB, Konate AT, Gansane A, Derme AI, Diarra A, Ouedraogo A, Soulama I, Cuzzin-Ouattara N, Cousens S, Leroy O, 2006. Safety and immunogenicity of the Plasmodium falciparum merozoite surface protein-3 long synthethic peptide (MSP3-LSP) malaria vaccine in healthy, semi-immune adult males in Burkina Faso, West Africa. *Vaccine* .

65. Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, Bojang KA, Conway DJ, 2007. Plasmodium falciparum Merozoite Surface Protein 3 Is a Target of Allele-Specific Immunity and Alleles Are Maintained by Natural Selection. *J Infect Dis 195:* 279-287.

66. Escalante AA, Lal AA, Ayala FJ, 1998. Genetic polymorphism and natural selection in the malaria parasite Plasmodium falciparum. *Genetics 149:* 189-202.

67. Branch O, Casapia WM, Gamboa DV, Hernandez JN, Alava FF, Roncal N, Alvarez E, Perez EJ, Gotuzzo E, 2005. Clustered local transmission and asymptomatic Plasmodium falciparum and Plasmodium vivax malaria infections in a recently emerged, hypoendemic Peruvian Amazon community. *Malar J 4:* 27.

68. Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, Marsh K, 2007. Naturally acquired antibodies to polymorphic and conserved epitopes of Plasmodium falciparum merozoite surface protein 3. *Parasite Immunol 29:* 387-394.

69. Oeuvray C, Theisen M, Rogier C, Trape JF, Jepsen S, Druilhe P, 2000. Cytophilic immunoglobulin responses to Plasmodium falciparum glutamate-rich protein are correlated with protection against clinical malaria in Dielmo, Senegal. *Infect Immun 68:* 2617-2620.

70. Tebo AE, Kremsner PG, Luty AJ, 2001. Plasmodium falciparum: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. *Exp Parasitol 98:* 20-28.

APPENDIX A

CRYPTIC N-TERMINAL DOMAIN EPITOPES ELICIT CROSS-REACTIVITY AGAINST BOTH HETEROLOGOUS N-TERMINAL AND C-TERMINAL DOMAINS BY MONOCLONAL ANTIBODIES

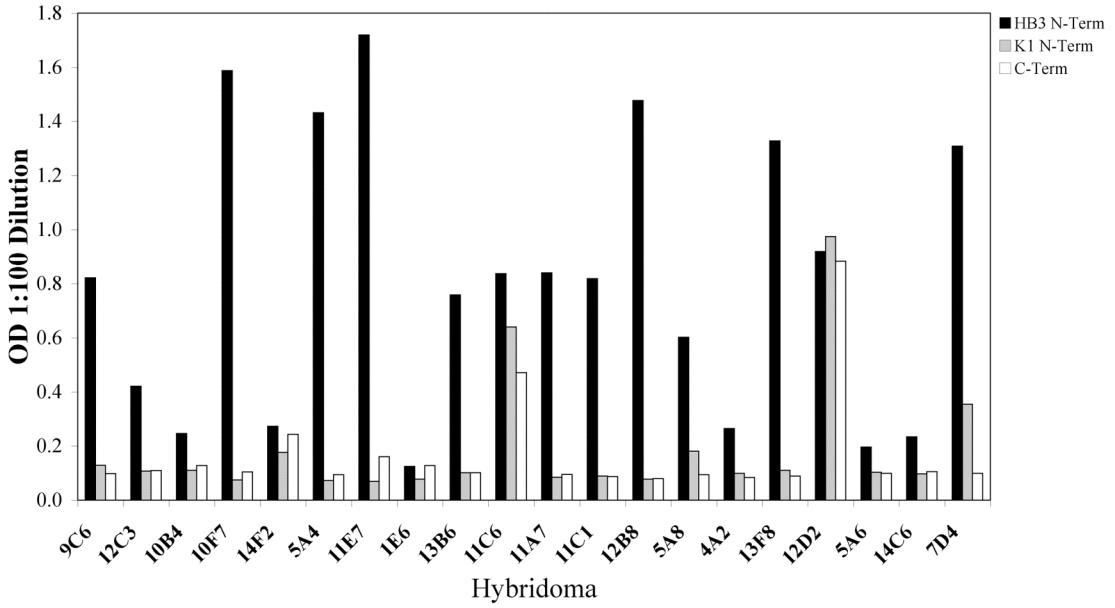

Figure A1. Murine hybridoma monoclonal antibodies raised against the HB3 N-terminal domain exhibit cross-reactivity with the K1 N-terminal domain and the C-terminal domain, by ELISA. Monoclonal antibodies were detected using a HRP-conjugated anti-mouse antibody.

APPENDIX B

CHARACTERIZATION OF *IN VITRO* PROTECTIVE-POTENTIAL FOR PFMSP3 DOMAIN ANTIBODIES THROUGH THE UTILIZATION OF ANTIBODY DEPENDENT CELLULAR INHIBITION ASSAYS

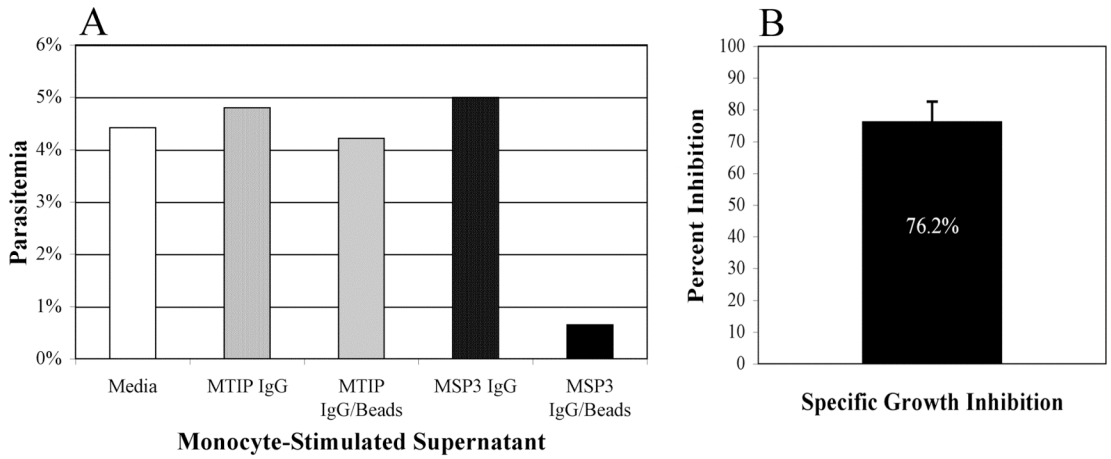

Figure B1. 2-Step Antibody-Dependent Cellular Inhibition Assay using PfMSP3 HB3 N-terminal domain antigen and anti-HB3 N-terminal domain antibodies. Anti-PfMSP3 antibodies were raised in rabbits and affinity purified using HB3 antigen. In the first step, antigen-coated beads (1um in size) were added to THP-1 monocytes in the presence of affinity-purified antibodies (1mg/ml) to facilitate THP-1 cross-linking and incubated for 12 hours. In the second step, the supernatant was removed and added 50% (v/v) to asynchronous *P.falciparum* cultures at 0.5% parasitemia, and cultured for 72 hours. After 72 hours, parasitemia was determined by geimsa-stained thick smears. MTIP, a *P. falciparum* myosin-motor protein is retained within the parasite and is used as a negative control. (A) HB3 N-terminal domain antibodies are capable of significant *P. falciparum* growth via ADCI. (B) HB3 N-terminal domain antibodies confer 76% parasite inhibition by ADCI. Specific growth inhibition normalizes ADCI against non-specific effects of negative control antibodies.

APPENDIX C

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 26, 2008

TO: Julian C. Rayner, B.Sc., Ph.D.
BBRB-567 2170
FAX: 934-5600

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: P. Falciparum Vaccine: Evaulating Candidacy of PfMSP3/PfMSP6 in an Endemic Setting
Sponsor: NIH
Animal Project Number: 080608189

On June 25, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rabbits	C	4

Animal use is scheduled for review one year from June 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080608189 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
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FAX 205.934.1188

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APPENDIX D

IRB APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on January 23, 2012. The Assurance number is FWA00005960.

Principal Investigator:	JORDAN, STEPHEN J
Co-Investigator(s):	
Protocol Number:	**X080403006**
Protocol Title:	*Identification of the Optimal Constituent for a PfMSP3-based Malaria Vaccine (P. falciparum vaccne: Evaluating candidacy of PfMSP3/PfMSP6 in an endemic setting)*

The IRB reviewed and approved the above named project on 5-6-09. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 5-6-09

Date IRB Approval Issued: 5-6-09

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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