Cross-Reactive Immune Responses as Primary Drivers of Malaria Chronicity

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The within-host dynamics of an infection with the malaria parasite *Plasmodium falciparum* are the result of a complex interplay between the host immune system and parasite. Continual variation of the *P. falciparum* erythrocyte membrane protein (PfEMP1) antigens displayed on the surface of infected red blood cells enables the parasite to evade the immune system and prolong infection. Despite the importance of antigenic variation in generating the dynamics of infection, our understanding of the mechanisms by which antigenic variation generates long-term chronic infections is still limited. We developed a model to examine the role of cross-reactivity in generating infection dynamics that are comparable to those of experimental infections. The hybrid computational model we developed is attuned to the biology of malaria by mixing discrete replication events, which mimics the synchrony of parasite replication and invasion, with continuous interaction with the immune system. Using simulations, we evaluated the dynamics of a single malaria infection over time. We then examined three major mechanisms by which the dynamics of a malaria infection can be structured: cross-reactivity of the immune response to PfEMP1, differences in parasite clearance rates, and heterogeneity in the rate at which antigens switch. The results of our simulations demonstrate that cross-reactive immune responses play a primary role in generating the dynamics observed in experimentally untreated infections and in lengthening the period of infection. Importantly, we also find that it is the primary response to the initially expressed PfEMP1, or small subset thereof, that structures the cascading cross-immune dynamics and allows for elongation of the infection.

Naïve individuals infected with the malaria parasite *Plasmodium falciparum* for the first time invariably develop significant clinical symptoms. Most individuals survive the initial acute infection but then generally remain chronically infected with low and diminishing levels of parasitemia for many months. Maintenance of these long asymptomatic infections is believed to result from the parasite's ability to continually evade the immune system by constantly varying the expression of proteins on the surface of infected red blood cells (RBCs) (1). This process is termed antigenic variation and centers predominantly on the expression of the *var* multigene family, which is spread across all 14 chromosomes and encodes the variant surface antigen *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (2). Each haploid genome contains approximately 50 to 60 distinct *var* genes (2) that have been grouped into four major groups (A, B, C, and E) and two intermediate groups (B/A and B/C) based on their upstream region, chromosomal organization, and similarities in coding and noncoding regions, as well as in the domain structure of the encoded proteins (3, 4).

P. falciparum parasites express *var* genes in a mutually exclusive fashion (5). However, while multiple *var* genes may be expressed by different parasites within a host at any one time, the dynamics of infections are characterized by sequential waves of parasitemia that are dominated by the expression of a specific *var* gene (6–8). The decrease in frequency of expression of a particular *var* gene is associated with the production of antibodies specific to that *var* gene product, PfEMP1. However, evidence suggests that a particular threshold density must be reached before a long-lasting immune response against a particular PfEMP1 protein is induced (9). After clearance of parasites expressing a particular PfEMP1 protein, antibodies generated in response gradually decrease, al-

though immunological memory remains and is rapidly restored upon reexposure (10).

Antigenic variation of the expressed PfEMP1 proteins is believed to be the main mechanism by which the parasite evades the immune system's response and prolongs the duration of infection (1); however, the process by which the parasite switches which *var* gene is expressed is still not well understood. Rates of switching appear to be variable and controlled epigenetically (11, 12). Theoretical models and *in vitro* data suggest that either differences in the rate that the parasite switches the expressed *var* gene (13, 14) or differences in the growth rates of the parasites expressing different *var* genes (15) constitute the main mechanism by which the parasite avoids exhausting its entire repertoire of *var* genes early in the infection. However, unlike other organisms that use antigenic variation to evade the immune system, *P. falciparum* has only a limited number of *var* genes per haploid genome. Because at the peak of a primary acute infection there are typically between 10⁹

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and 10^{13} asexual parasites (16, 17), even low rates of switching would result in the display of all variants to the immune system early in an infection.

An alternative suggestion is that cross-reactive immune responses play a significant role in structuring the dynamics and length of malaria infections (18). Cross-reactivity has been suggested as a mechanism to evade the immune response in a number of human pathogens, including influenza virus (19), *Streptococcus pneumoniae* (20), and schistosomiasis (21). In this study, we describe a model that combines variant switching with cross-reactive immune responses to determine the most effective and likely manner for producing chronic asymptomatic infections despite a limited *var* gene repertoire. Incorporating both the immune system and the parasite's time-specific reproduction rate allows us to explore how well differential switch rates, differential clearance rates, and cross-reactive immune responses produce infection patterns that are qualitatively similar to experimental *P. falciparum* infections. Our results suggest that cross-reactive immune responses, as proposed by others (18), rather than differences in growth or switch rates play the most significant role in structuring the chronic dynamics of a *P. falciparum* infection. Significantly, we also find that the initial immune response is a key aspect in generating the cascade of dominantly expressed *var* genes and the resulting structure of the dynamics.

MATERIALS AND METHODS

The within-host dynamics of malaria or antigenic variation have generally been modeled using ordinary differential equations (22, 23). Since these models have used continuous time to describe the interaction of the parasite with the immune system, they fail to describe the discrete reproduction of the parasite, which occurs in a synchronized fashion approximately every 48 h (24). Thus, the existing models largely neglect the fact that new variants can only switch on after a minimum of 48 h. In this paper, we use an approach that combines both mechanisms of modeling (25–27); the interaction of the parasite and the immune system occurs in continuous time punctuated by the replication of the parasite at discrete intervals.

We assume that parasites that survive the 48-h period of replication without being cleared by the immune system produce 18 new merozoites (24, 28, 29). We further assume that a fraction of these new merozoites infects RBCs. The fraction is assumed to be dependent on the density of the parasite. Thus, as parasite density increases, increased immune activity, including fever and resource constraints, limit the fraction of new merozoites that infect RBCs. This density-dependent mechanism, which has been shown to be important in structuring the dynamics of malaria infections (30, 31), impacts the parasite most significantly during the initial parasitemia, when unrestrained growth leads to significant parasite density. This is consistent with both theoretical (32) and murine models, which have demonstrated that an innate immune response is necessary for controlling the initial level of parasitemia (33), but antibody-specific mechanisms of the adaptive immune response are necessary for eliminating the parasite thereafter (34).

The discrete reproduction events of the parasite are described by the following equation:

$$P' = \frac{K\lambda P}{K + \lambda P} \quad (1)$$

where P' is the number of new parasites, λ is the number of new parasites produced per parasite, and K is the carrying capacity.

Between discrete replication events, the parasite interacts with the immune system in a continuous manner. We assume that parasitized cells are removed by the spleen or other mechanisms of the innate immune system at a constant rate, μ , while the adaptive immune effectors (A) and

memory cells (M) that are specific for that antigen remove parasitized cells in a mass-action manner related to the concentration of both parasite and immune effector (γ).

Undifferentiated adaptive immune effectors are activated by the parasite at a rate that approximates their concentration in the blood (κ). Once activated, they become specific for the removal of parasites expressing that antigen, and the removal of those parasites stimulates the production of new adaptive immune effectors at rate ρ , inducing a positive feedback loop that is characteristic of the immune system (35). Adaptive immune effectors are assumed to have a short life span and wane at rate δ . However, once the immune system is activated, it is assumed to remain active until the infection is contained. Thus, the removal of parasites is assumed to stimulate the production of cytokines (V) that keep the adaptive immune effectors from degrading and from generating memory cells. Cytokines have a short life span and degrade quickly at rate θ . Thus, as interactions between parasites expressing a particular *var* gene and the adaptive immune effectors specific for that *var* gene PfEMP1 product wane, the immune response will downregulate.

As the immune response downregulates, a proportion of the generated effectors (ϵ) become memory cells (M), which are not assumed to wane (36), though they are lost when activated by the parasite. Compared to naive immune effectors, memory cells have higher sensitivity to antigenic stimulation and provide significantly more positive stimulatory feedback to the immune system (37). Thus, when a particular PfEMP1 protein is expressed again (in the same or a new infection), memory cells are assumed to rapidly produce new adaptive immune effectors at a significantly higher rate (π). To ensure that memory cells are only created as the immune response is downregulated, we instituted a binary function, $\epsilon(A_{t=0})$, where t is time of 48-h cycle, which regulates the production of memory cells. At the beginning of a 48-h cycle, if the production of new immune effectors is outpacing their destruction, which only occurs when the immune response is actively increasing (i.e., $\epsilon > 0$), no memory cells are produced. The dynamics of the interaction between the parasite and the immune system between replication events are described by the following set of ordinary differential equations.

$$\begin{aligned} \dot{P} &= -\mu P - \gamma PA - \gamma PM \\ \dot{A} &= \kappa P + \gamma PA\rho + \gamma PM\pi - \delta Ae^{-V} \\ \dot{M} &= \delta Ae^{-V} \epsilon(A_{t=0}) - \gamma PM \\ \dot{V} &= \gamma P(A + M)\omega - V\theta \end{aligned} \quad (2)$$

Antigenic variation. The primary benefit of the hybrid approach is that we can analyze the major choice that the parasite makes at each replication event: (i) become merozoite clones expressing the same *var* gene; (ii) switch to express a different *var* gene; or (iii) become a gametocyte (the sexual stage of the parasite that is transmitted to mosquitoes). We ignore the role of gametocytes in the model, as they account for only a small fraction of the newly produced merozoites, and the adaptive immune system is not generally targeted at gametocytes (38). An additional implication of the model is that we can simulate the extirpation of parasites expressing a particular *var* gene product. We assume that once a parasite expressing a certain PfEMP1 protein has fallen below a threshold value and antibodies specific for that PfEMP1 protein exist, it will be cleared by the immune system with no further switches or replications. *P. falciparum* antibodies are capable of reacting with PfEMP1 proteins expressed by heterologous isolates (39–41), suggesting that antibodies for specific variants are cross-reactive with other variants. Thus, we introduced cross-reactivity: immune effector cells and memory cells specific for a particular PfEMP1 protein are assumed to be capable of eliminating parasites expressing other PfEMP1 proteins but at a reduced rate depending on the relatedness of each PfEMP1 protein.

While there are other multigene families that have been associated with variant surface protein expression and undergo antigenic variation (2, 42), we limit the variant surface antigens undergoing antigenic varia-

FIG 1 PfEMP1 protein similarity. Predicted protein sequences were obtained from PlasmoDB (<http://www.plasmodb.org>) for 62 PfEMP1 *var* gene products in the 3D7 reference strain. The sequences were then aligned, and an identity matrix was calculated using Clustal X (gap opening, 10; gap extension, 0.2). The resulting matrix is organized by *var* gene group, A, B/A, B, B/C, C, and *var2csa*. Group A *var* gene products are the least similar to each other and the most varied, which is consistent with their predicted extracellular domains. *var2csa* is also significantly dissimilar to other proteins, as would be expected by its alternative binding patterns. Groups B and C are more similar to each other than to the other groups, although they are slightly more similar within each group.

tion to the PfEMP1 protein expressed by the *var* genes. The structure of PfEMP1 is similar across groups and plays a significant role in adhesion and binding properties, as well as in immune evasion (43). Divergence in the protein sequence is related to variant proteins that bind alternative receptors linked to cerebral and placental malaria (43). Thus, we use relatedness of the expressed protein sequences (σ) as a proxy for calculating the potential cross-reactivity of specific PfEMP1 proteins.

Predicted protein sequences were obtained from PlasmoDB (<http://www.plasmodb.org>) for 62 *var* genes in the 3D7 reference strain

(44), which has been shown to be representative of typical sequence diversity across both regions and strains (45). The sequences were then aligned and an identity matrix calculated using Clustal X2 (46). The resulting identity matrix is based on protein sequence similarity (Fig. 1) and organized by *var* gene group. A similar analysis was done for each of the first three Duffy binding-like and cysteine-rich interdomain region domains, with the similar result that average relatedness was lowest and variation was greatest in the protein products of the group A *var* genes (see Table S1 in the supplemental material). In addition, analysis of the full

model using these alternative matrices was similar to the results produced from the full protein sequences. Thus, in our analysis, we calculated the cross-reactivity, or ability to bind, of an adaptive immune effector specific for PfEMP1 protein sequence j (termed PfEMP1 j) to a parasite expressing PfEMP1 i based on the relatedness of the full protein sequence using the following functional relationship:

$$\sigma_{ij} = e^{(-\beta_{ij}D)} \quad (3)$$

where β_{ij} is the distance between protein sequences i and j , measured as the proportion of homologous amino acids, and D is the e-folding distance, or the exponential rate at which cross-reactivity wanes. Cross-reactivity is assumed to be due to heterogeneity in the affinity of immune effectors for alternative PfEMP1 proteins. Thus, when a parasite expressing PfEMP1 j is cleared by an immune effector specific for PfEMP1 i , the assumption is that this is due to a subdominant epitope that is more specific for PfEMP1 j . Due to the positive feedback inherent in the immune system (35), we assume that this results in the production of more immune effectors specific for PfEMP1 j . This approach is used to account for the role of subdominant epitopes that were originally stimulated by the expression of an alternative PfEMP1 protein but may be more efficient at binding to a different PfEMP1 protein. Memory cells are assumed to function similarly. Thus, the full model of parasite interaction with the immune system between replication events can be described by the following set of ordinary differential equations:

$$\begin{aligned} \dot{P}_i &= -\mu P_i - \gamma P_i \left[\sum_j A_j \sigma_{ij} + \sum_j M_j \sigma_{ij} \right] \\ \dot{A}_i &= \kappa P_i + \gamma P_i A_i \rho + \gamma P_i \left[\sum_j A_j \sigma_{ij} + \pi \sum_j M_j \sigma_{ij} \right] - \delta A_i e^{-V_i} \\ \dot{M}_i &= \delta A_i e^{-V_i} \varepsilon(A_{i,t=0}) - \gamma M_i \sum_j P_j \sigma_{ij} \\ \dot{V}_i &= \gamma P_i (A_i + M_i) \omega + \gamma A_i \sum_j P_j \sigma_{ij} + \gamma M_i \sum_j P_j \sigma_{ij} - V_i \theta \end{aligned} \quad (4)$$

where i refers to each specific PfEMP1 protein. Variation in the expressed antigen is assumed to occur in a structured manner, where the order of switching is determined randomly at the onset of infection and then the parasite loops through that pattern until the infection is cleared. The discrete reproduction equation for the full model is

$$P'_i = \left(\frac{K \lambda P_i}{K + \lambda \sum_j P_j} \right) \quad (5)$$

for which each PfEMP1 protein is calculated separately, but density dependence is measured based on total parasite density; thus, j refers to all actively expressed PfEMP1 proteins, including i .

Each simulation is assumed to be an infection of a naive individual (except where we examine reinfection) by a sporozoite that produces on the order of 50,000 genetically identical merozoites that all burst from the liver on day 0 and express the same *var* gene, although we relax this assumption later. The initial parasitemia corresponds to a parasite density of 0.01/μl in an adult with a 5.0-liter blood volume, which is approximately 2,000 times less than the microscopic detection level of 20/μl. At each replication event a proportion of the replicating merozoites, 2% in the base simulation, switches to express an alternative *var* gene. Once an immune response has been initiated to a particular expressed PfEMP1 protein, it will be eliminated when the density is less than 0.0002/μl, although it can reappear later once the immune effectors have waned and other *var* genes switch expression to match that of this particular *var* gene. An infection ends when the immune system has completely eliminated all parasites, although we also determine the last day that parasites are visible at or above the microscopic detection level. Parameter specifications for the full model can be found in Table 1.

Lastly, we ran each simulation until the parasite had been eliminated and all adaptive immune effectors had waned, leaving only memory cells. We then initiated a second infection but assumed an alternative switching order. Thus, the parasite expressed a different *var* gene at the outset of the

TABLE 1 Parameter values used in the analysis

Parameter	Value	Description
λ	18	No. of merozoites per merozoite (i.e., growth rate)
γ	10^{-4}	The rate of removal of infected red blood cells per innate immune effector per day
δ	$1/10 \text{ day}^{-1}$	Death rate of immune effector
κ	10^{-3}	Rate of interaction between parasite and undifferentiated immune effector
μ	0.01	Death rate of parasite in the spleen
ε	0.05	Percent immune cells that become memory cells
ρ	4	No. of immune cells produced per effector-parasite interaction
θ	$1/2 \text{ day}^{-1}$	Cytokine rate of decline
π	10,000	No. of immune cells produced per memory cell-parasite interaction
ω	10,000	Upregulation of immune system per immune cell-parasite interaction
D	21	The e-folding distance (the exponential rate at which cross-protection wanes)
Blood vol	5 liters	Blood volume
K	800,000 parasites/μl	Parasite carrying capacity

infection than in the initial infection and had a different switching order. This allowed us to determine if the parasite was able to reinfect individuals and to determine its capability in generating a second clinical infection.

RESULTS

We first calibrated the model with a parasite that was unable to vary its antigens to ensure that the peak parasitemia reaches a density consistent with that of experimental *P. falciparum* infections (47) before being cleared by the immune response. After an initial exponential increase in the density of the parasite, the immune system controls the infection, eliminates the pathogen, and dispatches subsequent challenges relatively quickly (Fig. 2).

We examined the basic mechanism by which cross-reactivity generates infection dynamics using a hypothetical parasite with only three *var* genes. We simulated the 3-*var* model 60,000 times, assuming differing levels of cross-reactivity between each PfEMP1 protein. In the majority of simulations, the overall infection length was nearly indistinguishable from that of a system with no cross-reactivity. However, we found that certain combinations of cross-reactivity generated infections that were more than four times longer than infections with no cross-reactivity (Fig. 3). This result occurs when the binding between the immune effectors is mismatched so that the immune effectors for one PfEMP1 protein strongly bind only one of the other PfEMP1 proteins. For example, immune effectors for PfEMP1₁ and PfEMP1₂ are strongly cross-reactive, and so are immune effectors for PfEMP1₂ and PfEMP1₃, but immune effectors for PfEMP1₁ and PfEMP1₃ are not. Thus, when PfEMP1₁ is expressed by parasites at the outset of an infection, immune effectors can reduce parasites expressing PfEMP1₂. A strong immune response against PfEMP1₂ is not generated, which means that there is only a small impact of cross-reactivity on parasites expressing PfEMP1₃. However, after PfEMP1₁ and PfEMP1₂ are suppressed, there are only a limited number of parasites expressing PfEMP1₃. This means that, while weak, the cross-reactive responses generated by the immune effec-

FIG 2 Calibration of immune system dynamics for a nonvarying pathogen. To calibrate the immune response, a generic pathogen was introduced that had infection dynamics similar to those of malaria but did not possess the ability to vary its displayed antigens. After an initial inoculation, the immune system is able to control the parasite by about day 14 and generates immunological memory. A second infection was introduced at day 400 to ensure that the immune system can control future infections. The parasite was introduced with the same initial density as the original infection, but the rapid proliferation of immune effectors (which proliferate from the memory cells remaining) kept the parasite from exceeding the limit of microscopic detection (which is approximately 20 parasites per μl) and rapidly eliminated the parasite.

tors produced against PfEMP1₁ and PfEMP1₂ keep the parasite level low enough that the immune system does not generate a strong response against PfEMP1₃, allowing parasites expressing PfEMP1₃ to recrudescence and persist at low levels.

The three-*var* model provides the intuition to understand the dynamics of an infection with a parasite with 62 *var* genes. In the model with 62 *var* genes, we find that an infection with cross-reactive responses can structure the qualitative dynamics of the infection to be similar to the dynamics from experimental chronic infections (47) (see Fig. S1 in the supplemental material). Without cross-reactivity, the infection is shorter and the density of the parasites stays nearly constant at a level near the initial maximum parasitemia throughout the course of the infection (Fig. 4A). Conversely, with cross-reactivity, the infection peaks and then drops significantly and stabilizes around the microscopic detection level before dying out, a result qualitatively similar to the dynamics observed in natural infections (47) (Fig. 4B). In addition, similar to the sequential wave-like pattern exhibited in natural infections (6–8), the peaks of the infection with cross-reactive responses are dominated by different PfEMP1 proteins (Fig. 5).

The specific dynamics produced by cross-reactive immune responses in the model depends on the order and mechanism of switching. To account for the stochasticity inherent in this process, we simulated 5,000 infections with randomly generated switch orders. The average infection length, measured as the last day the infection was above 20 parasites/ μl , or the detectable limit of microscopic evaluation, which is how infections were analyzed in experimental infections of individuals (47), was 282 days (standard deviations [SD], 71 days), which was only slightly longer than the approximate duration of 222 days (SD, 154) of infection

measured in experimental infections of individuals (47). Infections in the model were capable of persisting for a much longer period below detectable limits. The longest infection was approximately 5 years. Although atypical, reports of infections lasting for several years after subjects leave a zone in which malaria is endemic have occasionally been reported in the literature (48).

Within the context of the model, we examined a number of different alternatives to compare the dynamics generated. First, we examined how differential switch rates structured infections by assuming that the parasite had variable switch rates and no cross-reactive responses. Switch rates were assumed to vary from 0.2% to as high as 16% (approximating the vast differences noted in switch rates [49]) and were randomly generated. Over thousands of simulations, we found that while the infection dynamics fluctuated more than in the case with a constant switch rate, they still approximated the same dynamics as those with no cross-reactivity (Fig. 6A). In addition, we tried numerous variations, such as high switch rates for *var* type A genes and low rates for *var* type B and C genes as well as vice versa, with the same general results in all cases. Second, parasites expressing different PfEMP1 proteins may have different rates of clearance due to both heterogeneous adherence phenotypes and differential immunogenicity. Thus, we examined how differences in the clearance rate structured the infection dynamics. The resulting dynamics produced long infections, but not until after a prolonged period of high parasitemia that is not consistent with experimental infection dynamics (Fig. 6B).

Lastly, we examined the mechanism of switching. In our primary model, we assumed that each *var* gene switched to express a single different *var* gene in an ordered pattern determined at the onset of the infection. Therefore, we varied this in two ways. First, we assumed that each *var* gene switched to express a single random *var* gene in each generation. In the absence of cross-reactivity, the resulting infection was typically short and was quickly extinguished. Including cross-reactivity resulted in long-lasting infections similar to those of our primary model; however, the rate at which parasitemia levels dropped was more precipitous than that of our initial model, and the model tended to oscillate in a more regular manner (Fig. 6C). Recent evidence suggests that rather than switching randomly, parasite antigens switch in a structured manner from a group of dominant *var* genes through intermediaries, finally resulting in a new dominantly expressed *var* gene (50). Under these assumptions, we found similar dynamics: without cross-reactivity, the exposure of multiple PfEMP1 proteins quickly led to the parasite being eliminated, while cross-reactivity increased the longevity of the infection significantly. Notably, in both cases, the number of actively expressed *var* genes rose at a higher rate than in the primary model, with the majority of *var* genes being expressed by day 10 and all of them expressed by day 14.

These results suggest that increasing the number of *var* genes expressed at the onset of infection determines how rapidly the parasite enters the chronic stage. To test this, we examined how the dynamics of an infection were structured when more than one *var* gene was expressed at a time, assuming that each expressed *var* gene started at the same initial concentration. We found that an increase in the number of expressed *var* genes at the onset of infection significantly reduced the probability that an infection would be cut short due to a switch to an antigen that was too similar antigenically. While expressing a few different *var* genes had no significant impact on the dynamics, as more *var* genes were expressed the qualitative dynamics became less and less similar to

FIG 3 Dynamics of a hypothetical infection with only three variant surface antigens. (A) To understand the dynamics of cross-reactivity within the model, we first examined a hypothetical infection consisting of only three antigenic variants. These infections generally lasted a very short period of time. However, in a small minority of cases, antigenic variation increased the length of the infection significantly. The inset panels B and C show a single infection lasting approximately 120 days. In this example, PfEMP1₁ and PfEMP1₂ are closely related; thus, the generation of a strong immune response to the initial PfEMP1₁ is also capable of clearing PfEMP1₂. However, the presence of a third PfEMP1 that is only slightly cross-reactive with PfEMP1₁ but strongly cross-reactive with PfEMP1₂ inhibits the immune system from mounting a memory response that is capable of eliminating PfEMP1₃ initially. This results in an incomplete immune response. As the immune response begins to downregulate with the collapse in parasite levels, the parasites expressing PfEMP1₃ are able to increase, with the result being a recrudescing infection.

those of experimental infections as the initial crash in the population became more and more significant and reduced the probability the infection would persist. The dynamics reached a threshold at approximately five expressed *var* genes at infection onset, after which the infection never persisted. We also examined the dynamics when all 62 *var* genes were expressed initially at differing levels, and again the infection never persists in our model. This suggests that the initial *var* gene or a limited group of *var* genes initially expressed by the parasite plays a key role in initiating a cascade of cross-immunity to the resulting PfEMP1 proteins, which structures the response of the immune system.

A secondary benefit for the parasite from an incomplete immune response is the ability to reinfect the same individual, causing a new, long-lasting chronic infection (Fig. 6D). For each simulation, we allowed all adaptive immune effectors to wane, leaving only memory cells, and then reinfected individuals with the same parasite but with an alternative switching order. In this case, we found that the parasite could reinfect individuals, but while infections could persist for a significant time period below microscopic detection levels, they were never capable of generating a clinical infection. Thus, in line with field (51, 52) and experimental results

(53), the immunity conferred by a primary infection does not prevent future infection, rather it reduces the probability of clinical symptoms and shortens the length of new infections. This ability to reinvade suggests that incomplete immune responses due to cross-reactivity allow specific *var* genes to persist in the population for long periods of time, a result consistent with those of other studies examining cross-immunity between genotypes (54). Alternative research has suggested that parasites switch in a structured manner to allow it to evade immune responses from prior infections (50). This result is not inconsistent with the current research but suggests alternative ways in which the parasite can express PfEMP1 proteins so as to avoid the immune system; however, cross-reactivity would still be required to produce a long-lasting chronic infection.

DISCUSSION

A major question in the biology of malaria infections is how the limited repertoire of *var* genes (~60) in a single malaria parasite is capable of maintaining a chronic infection. We present a hybrid model that mixes continuous interaction between the parasite and the immune system with discrete replication events. The inclusion

FIG 4 Example parasite invasion. The dynamics of infection differ significantly between a parasite with no cross-reactivity (A) and one with cross-reactivity (B) between PfEMP1 proteins. Without cross-reactivity, the infection lasts ~200 days but does not conform qualitatively with the expected dynamics of a long-lasting chronic malaria infection. On the other hand, cross-reactivity of PfEMP1 generates an infection that is qualitatively similar to long-term chronic infections of malaria in both dynamics and duration.

of both long- and short-term immunity, as well as antigenic switching and heterogeneous clearance rates, provides a robust framework for examining different possible mechanisms for prolonging infection. From our results, we suggest that not only are cross-reactive immune responses central to prolonging infection, and that the resulting dynamics are qualitatively similar to experimental infections (47), but also that it is the initial immune re-

sponse that is important for structuring the long chronic phase of the infection.

The generation of a strong immune response is a key element for eliminating a pathogen and for developing a memory of the pathogen. However, *P. falciparum* appears to take advantage of this strong immune response. High initial parasite levels, which result from replication in the liver, induce the immune system to

FIG 5 Sequential dominance of different variant surface antigens. The densities of each PfEMP1-expressing parasite in the infection shown in the figure are color coded, and each peak is labeled with the dominant PfEMP1 protein. Each number corresponds to a particular *var* gene.

FIG 6 Alternative methods of structuring *P. falciparum* infections. (A) Differential switch rates. While a mixture of slow and fast switch rates increases the fluctuations of the parasite levels, without cross-reactivity the dynamics are still not consistent with experimental infections. (B) Differential clearance rates. Variance in patterns of adherence or differential antigenicity can result in differential clearance rates of parasites expressing different *var* genes; however, the limited impact on the dynamics suggests that this is not a central mechanism in structuring the dynamics. (C) Random switch patterns. Instead of an ordered progression, parasites may switch randomly in each generation to different *var* genes. The dynamics in this case, with cross-reactivity, seem consistent with experimental infections and are similar to those of ordered switching. Thus, the model cannot discriminate between the best switching methods. (D) Reinfection. An infection with the same strain but with an alternative switching order can produce a chronic infection that may only occasionally rise to the level of microscopic detection.

produce specific immune effectors against one or a few PfEMP1 proteins that are initially expressed. Our model suggests that this strategy is central to generating the cascade of sequential antigens. Because the initial response of the immune system is significant, as the parasite switches to express other *var* genes, even weak cross-reactivity between *var* gene PfEMP1 protein products limits the growth of other parasites expressing other PfEMP1 proteins. However, this response controls but does not eliminate parasites expressing other PfEMP1 proteins, resulting in an incomplete immune response to these other PfEMP1-expressing RBCs. The infection then is able to recrudesce as the response to the first PfEMP1 wanes. Thus, while a strong, specific primary response may cause significant harm to the host, it may also provide a mechanism by which the parasite can persist chronically.

Our results suggest that if more than approximately five *var* genes are expressed at once, the parasite is unable to take advantage of cross-immunity and the immune system is able to eliminate the infection after a short period. On the other hand, if only a single *var* gene or up to approximately five genes are initially expressed, the immune system response against the initial *var* gene product(s) weakens the response against other cross-reactive PfEMP1 proteins. As the first subset of *var*-expressing parasites wane, parasites expressing other *var* genes are able to proliferate.

This suggests that the parasite should maximize the long shadow of cross-reactivity by switching to express PfEMP1 proteins that are not too similar antigenically. Evidence from experimental infections of individuals suggests that immune acquisition of antibodies at the outset of an infection is variable between humans and point to limited initial *var* gene repertoires or at least a mechanism that approximates that type of immune stimulation (41). One mechanism that has been suggested for generating an immune response against only a limited *var* gene repertoire at the outset is through a structured switch pathway in which one *var* gene switches to many *var* genes, and then all of those *var* genes have a high probability of switching to the same *var* gene (50). Whatever the switch mechanism, a limited number of initially expressed *var* genes is not inconsistent with experimental infection data showing that most, if not all, *var* genes are expressed by day 10 to 12 in an infection (55), as even low rates of switching per generation would activate all *var* genes by about day 10 to 12 and in even shorter time using different switching mechanisms.

Prior models examining the role of antigenic variation in *P. falciparum* have proposed a range of possibilities for how *var* genes generate chronicity. Molineaux et al. (15) generated fairly realistic patterns of asexual parasitemia; however, they relied upon differences in the intrinsic growth rates of parasites expressing different

variant surface antigens due ostensibly to infection with multiple clones. As part of their model, they assumed that the immune system generated both variant-specific immunity and a variant-transcending nonspecific immunity that is essentially a form of cross-reactivity, though they did not term it that. A more recent paper used a similar method to include cross-reactivity and the appearance of rare variants through differential growth rates (56). While no genetic data are available from the era of malaria therapy, it seems unlikely that infections were made up of 50 to 60 different clonal variants with different growth rates. It is also not clear why specific variants would have intrinsically different growth rates that were not related to immune function. Paget-McNicol et al. (13) criticized the different-growth-rate model and proposed instead that variation in the rate that different *var* genes switch on and off, which has been shown experimentally (14), produced chronic infections. However, their model relied upon switch rates that were much lower than have been found experimentally.

Cross-reactivity was explicitly proposed as a means to generate long chronic infections in malaria by Recker et al. (18). They used a mechanistic model that assumed that there were both variant-specific immune responses, which were long-lasting, and short-lived cross-reactive responses. While their model was able to produce long-lasting chronic infections, it was mechanistic in nature and did not include key biological aspects, such as variant switching and immune memory. While the model assumed that the cross-reactivity was driven by the generation of minor epitopes that generated immune responses that were nonunique to the variant, it did not suggest an underlying reason why these cross-reactive responses are short lived (57). One possibility suggested for the transiency of these cross-reactive immune responses is immune exhaustion (57), where as an infection progresses, pathogen-specific T cells lose functional activity (58). This mechanism has recently been shown to play a role in *P. yoelii* and possibly *P. chabaudi* infections, and there is also some evidence that it affects infections with *P. falciparum* (59). Johnson et al. (57) suggest that the continued stimulation of the immune response through cross-reactivity results in exhaustion of the response as well as its transiency.

While immune exhaustion may play a role in determining the dynamics, in our model we have shown that immune exhaustion is not a necessary component to produce chronic infections. Rather, we find that the transiency of cross-reactivity is a function of waning responses to a dominant PfEMP1 protein, and that it is the interplay between multiply expressed PfEMP1 proteins that generates chronicity. In other words, even though one PfEMP1 protein is generally dominant (6–8), infections are composed of parasites expressing a multitude of PfEMP1 proteins (55). An immune response to the PfEMP1 *var* gene product is hypothesized to cross-react with other PfEMP1-expressing parasites, which results in their suppression but not elimination. Because a variant is being suppressed due to cross-reactivity, a variant-specific response is not generated, and as the immune response to the other variant wanes, the suppressed variant now can expand.

By including both variant switching and immune memory, we were able to examine different hypotheses regarding the generation of chronic infections. While our analysis suggests that cross-immune responses play a dominant role in generating a chronic infection, this is not to suggest that differential immunogenicity or heterogeneous adherence properties play no role in structuring the dynamics of an infection, as certain *var* genes are selectively expressed in pregnant women (*var2csa*), while others have been

associated with severe malaria syndromes that portend poor outcomes (60–62), such as cerebral malaria (63, 64), where the parasites adhere to the endothelial cells in the brain, leading to hemorrhaging (65). However, we found that without some cross-reactivity, the resulting dynamics do not conform to observed *in vivo* infection dynamics. Our model also does not discount the role of differential switch rates or immune exhaustion as factors affecting the dynamics, but we suggest that they play more minor roles in generating the response and that it is likely that cross-reactivity is the driving force in generating long-lasting chronic malaria infections. This conclusion suggests that the timing of treatment will have no effect on the generation of cross-reactivity (as Johnson et al. suggest would happen with immune exhaustion [57]). On the other hand, it does suggest that reductions in parasitemia levels (which reduce the likelihood of clinical disease) would increase with both the number of infections received and how related new infections are to prior infections, but that infection risk would never completely disappear as long as *var* gene diversity was high enough. Two empirical observations conform to these hypotheses. First, in high-transmission areas, *var* gene diversity is significantly higher than that in lower transmission areas (66). Second, a recent study showed that while the probability of clinical infection decreased with age (due presumably to repeated infection), there was no association between age and the probability of becoming infected (52).

In addition to structuring the dynamics of the infection, we also found that cross-reactive responses may influence the rate that immunity to particular PfEMP1 proteins is acquired over multiple infections. Because the proteins encoded by the group A *var* genes tend to be less similar and have greater variability in how similar they are to each other than to other groups, cross-reactivity does not dampen their growth as significantly. Thus, during the long chronic stage of the infection, they tend to generate a larger number of parasites, which means a larger number of specific immune effectors. While in a single infection the initially expressed PfEMP1 protein generates the largest response, in repeated infections the higher parasitemia generated by the expression of the group A *var* genes would generate stronger immune responses after fewer infections, a result consistent with field data (67). Although higher levels of parasitemia would likely contribute to the perceived link between virulence and certain PfEMP1 proteins, other factors, such as differences in adherence or antigenicity, will likely play a larger role.

Lastly, the structured dynamics that result from cross-reactive responses suggest some factors involved in the ecology and epidemiology of the disease. Faster growth rates, which increase the probability of clinical symptoms (68), would generate a more robust immune response and a longer cross-immune shadow that retards the formation of an immune response to other PfEMP1 proteins, and it would allow the parasite to maintain a chronic infection longer. In addition, higher growth rates allow the parasite to switch expressed *var* genes at a higher rate, which is useful in individuals already partially immune. While infections with multiple different parasites would take longer to clear, on average, even if the parasites were clearing independently, our model suggests an additional reason why infections have been shown to be greatly lengthened by the presence of multiple infections (69): an increasing number of infections would result in a greater number of different *var* genes being expressed at any particular time. Because antibodies are cross-reactive, they reduce the density of

other PfEMP1-expressing parasites, which reduces the rate at which undifferentiated immune effectors encounter PfEMP1 proteins and slows the positive feedback loop of antibody creation. Thus, the generation of an immune response capable of eliminating each PfEMP1-expressing parasite takes longer to develop, and the overall length of the infection is increased.

While the results from our model are robust for a wide range of parameters, the model is only an abstraction of the complicated interaction between the parasite and the immune system. In particular, we assumed that the parasite replicated in synchrony every 48 h, and that each invasion produced an equal number of merozoites with each replication. Evidence suggests that synchronous replication is generally maintained in the earlier stages of an infection (51), and we feel that deviations that may occur later in the infection would not significantly impact the described dynamics, as by that point, the infection in our system is primarily regulated by PfEMP1-specific immune effectors, and mechanisms that slow growth (which mimic the role of the innate immune response) would play, at most, a very minor role. Thus, asynchrony would only impact the timing of the late peaks and would not alter the fundamental interaction between the immune system and the parasite. The constant replication rate is consistent with the *in vitro* evidence (24), and the density-dependent mechanism we include, which changes the percentage of parasites that infect RBCs, approximates the fold changes in parasite growth both in the malaria therapy data (47) and in experimental infections (29). Changes in the growth rate parameter did not affect the qualitative results. In addition, our model is only an approximation of the human immune system, and we made a number of simplifications, chief among them that immune effectors are generated immediately after interacting with the parasite rather than after a delayed period. However, there is evidence that this type of model better approximates the exponential increase of immune effectors as opposed to a strict delay model, because the amount of interaction an undifferentiated immune effector has with a pathogen is important in determining the effector growth rate (70).

We also make the simplifying assumption that cross-immunity is specifically related to similarities in protein sequences, despite the fact that protein sequence similarity is no guarantee that the proteins are structurally similar or that the immune response is likely to cross-react with these structures. However, we feel this is a good approximation, as strong structural similarities mediated by conserved regions of DNA have been shown to exist between PfEMP1 variants and are predicted to mediate cross-reactivity of certain epitopes (71), protein structure has played a significant part in how *var* genes have been classified, suggesting a strong link between sequence and structure (3, 4, 44), and some functional differences have been observed between groups (43, 72), suggesting that variant proteins are more similar within groups than across groups and are targeted similarly by the immune system, increasing the likelihood of cross-reactive responses. In addition, our analysis of sequence similarity generated patterns that would be expected based on the extracellular domain structure, and the results were similar when we looked only at a single domain. While the model assumes that cross-reactive responses are due to subdominant epitopes that were originally stimulated by an alternative PfEMP1 but are more efficient at binding to a different PfEMP1 protein, the implementation of the model groups all PfEMP1-specific immune effectors together. Thus, to account for the fact that these subdominant epitopes are binding more effec-

tively, we assume that when they are stimulated, the positive feedback of the immune system makes them more effective at targeting the new PfEMP1 protein; thus, we group them there. The result is that some PfEMP1-specific immune effectors are created through these cross-immune responses (see Fig. S2 in the supplemental material). While this model lacks some of the complexity of subdominant epitopes, we feel that it best represents the way that cross-immunity functions in this simplified system. Lastly, our results were qualitatively robust in terms of large variations in merozoite replication number, switching mechanisms and rates, e-folding distance (see Materials and Methods), and immune system parameters, suggesting that the magnitude of cross-reactivity is not as important in generating chronicity as the fact that the system possesses cross-reactivity. However, we examined only a single infection in a naive individual. Additional protective effects (and possible cross-reactive responses) can also be generated by the surface antigens of the merozoite or by coinfections with other organisms. However, both are likely to generate a sustained, broad cross-reactivity (because they are highly conserved) or sustained suppression of the immune system, resulting in broad changes in the response to infection rather than changing the inherent dynamics generated by PfEMP1 variation or the strength of within-malaria cross-reactivity (73).

Understanding how immunity to *P. falciparum* develops is crucial for progress in the quest for an effective vaccine. Models of the within-host dynamics of malaria infections have generally been unable to capture the complex dynamics of the interaction between host and parasite without relying on complicated assumptions about the biology of the parasite (13, 15). We believe our model, on the other hand, is robust in terms of large variations in the biological assumptions. The model also provides a good mathematical framework for understanding how the malaria parasite generates long-term chronic infections with a limited antigenic repertoire. Significantly, we suggest that it is the expression of only one or a very limited number of antigens at the onset of an infection that drives the cascade of cross-immune responses required to establish a lasting infection, suggesting that a vaccine targeted at immune effector generation could reduce the severity of symptoms even if it was incapable of protecting an individual from infection.

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