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Development of a Viral Vaccine Vector Expressing a *P. falciparum* Erythrocyte Membrane Protein Domain to Target Severe Malaria

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ABSTRACT

There are an estimated 300-500 million cases of malaria every year, resulting in approximately 2 million deaths. Malaria mortality occurs primarily in children under the age of five, and is resultant from the development of severe malaria. Attempts to control *P. falciparum* malaria face rapidly escalating levels of resistance on two fronts: the parasite to anti-malarial drugs, and the mosquito vector to insecticides. Despite current prevention efforts, the global incidence of malaria continues to rise and there is a clear and urgent need for a malaria vaccine. Malaria vaccine efforts thus far have been largely unsuccessful in eliciting robust and long-lasting immunity. While adults living in malaria endemic regions have not been shown to naturally develop protective immunity to uncomplicated malaria, they do appear to develop protective immunity to severe malaria by around the age of five, raising the possibility that a vaccine specifically targeting severe malaria may be a more attainable goal of malaria vaccine efforts in the immediate future. There is mounting evidence that the pathogenesis of severe malaria is directly related to cytoadhesion and rosetting of parasitized red blood cells. The *P. falciparum* protein PfEMP1, which is expressed on the surface of parasitized red blood cells, appears to have a direct role in mediating cytoadhesion and rosetting. The DBL1α domain of PfEMP1 specifically been implicated in these interactions, and is also one of the only regions of PfEMP1 that is highly conserved, making it a promising target antigen for malaria vaccine development. Here we show that immunization with recombinant vesicular stomatitis virus vaccine vectors expressing DBL1α induce immune responses that are able to disrupt rosetting of parasitized RBC. This response was comparable with that seen in adults from malaria endemic regions.

ACKNOWLEDGEMENTS

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INTRODUCTION

Epidemiology and Global Impact of Malaria

Approximately 40% of the world's population lives in areas at risk for malaria. There are

four species of Plasmodium that cause human malaria, with *P. falciparum* being

responsible for the greatest burden of morbidity and mortality. The WHO estimates that

there are up to 500 million cases of malaria every year, resulting in 1-2 million deaths.

Older children and adults appear to gain some degree of protective immunity that renders

them less susceptible to severe infections that carry high mortality rates, however malaria

is currently the third leading cause of death in children under five in developing

countries. Malaria is both a disease of poverty and a cause of poverty, and is a significant

barrier to economic development in malaria endemic countries. Globally, there is a

striking overlap of malaria burden and regional poverty. It is estimated that malaria is

responsible for a "growth penalty" of up to 1.3% per year in regions of high transmission,

which, over time can lead to substantial retardation of economic growth in the region[1]. In

addition, malaria is estimated to account for up to 40% of public health expenditures,

50% of inpatient admissions, and 50% of outpatient visits in countries with a high

malaria burden, putting an enormous strain on already overtaxed health care systems.

The transmission vector for *P. falciparum* is the female *Anopheles* mosquitos. *P.*

falciparum gametocytes are ingested with a blood-meal taken from an infected human.

Male and female gametocytes fuse and mature into ookinetes in the mosquito gut, and

subsequently into oocysts in the gut wall. The oocysts rupture to release sporozoites that

migrate to the mosquito salivary glands. The sporozoites are then injected into the skin

of a new human host during the mosquito's next blood meal. Once in the host, malaria

parasites undergo two phases of development: the liver or exoerythrocytic phase, and the

blood or erythrocytic phase. Injected sporozoites initially migrate through the

bloodstream to the liver where they infect hepatocytes. Within the hepatocytes, the

sporozoites mature into merozoites over a period of 1-2 weeks. Upon rupture of infected

hepatocytes, large numbers of merozoites are released into the blood and infect red blood

cells [2]. During the blood stage of the infection, merozoites replicate asexually in RBCs

going through the trophozoite or ring form and the schizont form before the RBC

ruptures releasing many mature merozoites that can then go on to infect more RBCs,

continuing the cycle. A small proportion of merozoites mature into male or female

gametocytes after invading RBCs. The gametocytes are released into the blood stream

with the rupture of infected RBCs. They can then be consumed during a blood meal and

mature into sporozoites within the mosquito vector, eventually being transmitted to

another host.

Importance of Malaria Vaccine Development

Attempts to control *P. falciparum* malaria are currently facing rapidly escalating levels of

resistance on two fronts: resistance of the parasite to anti-malarial drugs, and resistance of

the mosquito vector to insecticides. Many areas of the world are seeing increasing

malaria rates due to immigration and foreign travel, in addition to changing climate

conditions that favor multiplication of the mosquito vector. Given the extremely high

public health burden of malaria treatment in endemic areas, it seems clear that prevention is the most cost effective approach. This is especially true because natural protective immunity is very slow to develop, leaving individuals susceptible to repeated infections requiring treatment. In addition, prevention would be thought to mitigate the economic costs of malaria endemicity to a greater extent than treatment if acute attacks of malaria can be prevented before they manifest clinically and lead to significant decreases in current and future productivity. Major strategies for prevention include vector control, insecticide treated bed nets, and indoor spraying of insecticides. These methods have been in use for extended periods of time, and while they are effective, global malaria mortality and morbidity continue to increase despite their use and there is a consensus that a malaria vaccine is urgently needed [3]. No malaria vaccine in late stage development has demonstrated strong (>50%) protection, or a durable protection (>1 year). Much has been learned, however, from first generation malaria vaccine development efforts that can be applied to future efforts.

The majority of malaria vaccines currently in development are based on a very small number of *Plasmodium falciparum* antigens [4]. Few of these antigens have been identified using human or primate studies, most being discovered using mouse models. The relevance of these models is increasingly being questioned due to the non-natural host parasite interaction seen in these models, as well as their demonstrated failure to accurately predict human responses [5]. It will be preferable for future efforts to rely on targets that have been identified or validated in human or primate, studies.

Experiences during clinical trials of several of the first generation malaria vaccines have highlighted the critical importance of understanding the interplay between malaria parasites and the immune system [4], and future vaccine efforts need to additionally focus on exploring how to rationally shape the immune response elicited to malaria target antigens based on our increasing understanding of the host-parasite interactions. There is also an emerging consensus that a multi-stage, multi-antigen vaccine will ultimately be necessary to achieve a high degree of protective efficacy[6]. There are a small number of such efforts currently in early stage development, however these vaccines are likely to be complex to execute and are likely many years away, particularly given the potential for antigenic interference and our current lack of understanding of the requirements for an effective adjuvant. One important, and likely more rapidly attainable, goal of a malaria vaccine development is to improve rates of malaria mortality, particularly in children, by specifically targeting the development of severe malaria. While immunity to uncomplicated malaria develops very slowly and is rarely fully protective, immunity to severe malaria appears to develop with significantly greater rapidity, possibly reflecting a higher degree of antigenic homogeneity in the virulence factors of parasites responsible for severe disease [7]. Antigens implicated in the development of severe malaria are thus potentially attractive vaccine candidates because they may face fewer of the difficulties that have been encountered in eliciting protective immunity to uncomplicated *P. falciparum* malaria.

Vesicular Stomatitis Virus Vectors in Malaria Vaccine Development

A viral vector approach to a *P. falciparum* malaria vaccine is currently receiving a great deal of interest [8]. Vaccines based on live viruses induce life-long immunity through induction of durable memory B and T cell responses. Such vaccines have been responsible for extensive control or even complete elimination of viral diseases. Some have proposed that viral vector-based vaccines may be the most promising approach to generating protective immunity to parasites, and that the specific choice of viral vector appears to be crucial in determining vaccine efficacy [9]. While many, if not most, of the malaria vaccines currently under development that have shown the best success incorporate a viral vector, there has been very little effort specifically targeted at identifying and using the most potent vaccine vectors currently available.

Vesicular stomatitis virus (VSV) is an enveloped negative strand RNA virus in the family Rhabdoviridae. VSV has previously been demonstrated to be a highly effective vaccine vector for multiple pathogens such as HIV-1 [10 11], SARS [12, 13], RSV [14, 15], measles [16], and *Yersinis pestis* [17]. Vaccine vectors based on recombinant, attenuated VSV have been used extensively as experimental vaccines and will be used in clinical trials scheduled to begin in 2011. These experimental VSV vaccines have induced long-term immunity to both viral and bacterial pathogens in animal models, and should be further explored for use in parasite vaccine development.

Pathogenesis of Severe Malaria

Clinically, malaria can be broadly classified as uncomplicated or severe. Symptoms of

uncomplicated malaria can include a cyclical fever recurring every 36-48 hours or a continual low-grade fever, as well as anemia, malaise, arthralgias, vomiting, and rigors. Uncomplicated malaria is important in terms of burden on health care systems and decreasing productivity, however it is rarely fatal. Severe malaria, in contrast, is frquently fatal if untreated. Complications of severe malaria include cerebral malaria, severe anemia, acute renal failure, pulmonary edema, acidosis, and hypoglycemia, and any of these complications can be fatal within days or even hours (World Health Organization, 2000). Risk factors for severe malaria include age less than 5 years or greater than 65 years, non-immune status, pregnancy, delay in treatment initiation, and the presence of pre-existing medical conditions [18] [19] [20]. A major cause of malaria mortality, particularly in young children, is cerebral malaria resulting from occlusion of cerebral microvascular by agglutinated parasitized red blood cells (pRBCs). Untreated cerebral malaria is almost universally fatal, and even with treatment the mortality rate remains around 20% [21]. Surviving patients are at increased risk for developmental delays, neurological deficits, epilepsy, and behavioral problems. Cerebral malaria is a leading cause of childhood neurodisability in African children [21], which has significant long-term implications for future productivity.

The development of severe malaria has been strongly linked to *P. falciparum* virulence factors that mediate RBC cytoadherance and rosetting [22] [23] [24] [25] [26] [27] [28]. Expression of *P. falciparum* proteins on the surface of parasitized erythrocytes mediates binding to the vascular endothelium and to uninfected erythrocytes, resulting in the accumulation of parasitized RBCs in capillary microvasculature. This is compounded by the altered

mechanical properties of pRBCs that cause decreased flexibility of the cell membrane, which makes it difficult for the cell to pass through the microvasculature [29]. The resultant microangiopathic hemolytic anemia, which serves to exacerbate the pre-existent hemolytic anemia from parasite induced rupture of pRBCs, can precipitate acute renal failure, secondary to massive hemoglobinuria [29]. The vasocclusive properties of pRBCs also lead to tissue hypoxia, ultimately resulting in end-organ damage and other clinical manifestations of severe malaria [29]. Finally, the sequestration of pRBCs in the microvascular stimulates cytokine production that can cause direct tissue damage [2]. The major factor mediating pRBC cytoadherence to various ligands on endothelial cells other pRBCs has been established to be the parasite protein *P. falciparum* Erythrocyte Membrane Protein-1 [30].

PfEMP1 and the Development of Severe Malaria

PfEMP1 is a high molecular weight (over 200 kD) protein that is expressed on the cell membrane of parasitized red blood cells (pRBCs). PfEMP1 is composed of several extracellular Duffy binding-like domains (DBL 1 to 5), with one to two cysteine-rich interdomain regions (CIDRs) distributed in between the DBL domains, a transmembrane [31] region, and an intracellular acidic segment [32] [33] [1]. PfEMP1 undergoes extensive clonal antigenic variation and is encoded by a multigene family of variable surface antigen [4] genes with at least 50 copies per genome [34]. The most conserved regions of PfEMP1 are located in both the N- and C-terminal domains. There is a semi-conserved head structure that includes the DBL-1α and CIDR1α domains, and there several regions of DBL-1α are

almost 100% conserved [1]. It is thought that PfEMP1 has a central role in malaria

virulence and the development of clinically severe disease due to its key roles in

rosetting, cytoadhesion, and immune evasion [35]. PfEMP1 is considered by some to be the

"Achilles heel" [36] of *P. falciparum* because it is responsible for so many key aspects of

malaria pathology, and is expressed on the surface of pRBCs making it vulnerable host

immune attack. This is presumably why PfEMP1 has evolved to be highly polymorphic

and demonstrates such extensive clonal antigenic variation.

The region of PfEMP1 mainly responsible for mediating cytoadhesion is thought to be

the extracellular N-terminal adhesive domain DBL1α [37,38], which is relatively conserved

compared to other PfEMP1 adhesive domains [30]. In studies using both rats [39,40] and

primates [40], it has been shown that anti-DBL1α antibodies can disrupt preformed rosettes

and block cytoadhesion in vivo [39]. DBL1α expressed in recombinant Semiliki forest

virus vectored vaccine induced antibodies that prevented cytoadhesion and disrupted

rosettes in a mouse model [39]. More importantly, these associations have been validated in

human studies. The presence of antibodies against PfEMP1 have been associated with

protection against severe disease in various studies of populations living in malaria

endemic areas [41,28,27]. For example, in a study in Gambia, 88% of children with

uncomplicated malaria had anti-rosetting antibodies compared to the 22% of children

with severe malaria that had anti-rosetting antibodies [28].

STATEMENT OF PURPOSE

The vast majority of malaria mortality is attributable to complications of severe malaria. There is compelling evidence that the cytoadhesive properties of pRBCs are a key factor determining the development of severe malaria, and has been linked to the expression of PfEMP1 on the surface of pRBCs. While immunity to uncomplicated malaria develops very slowly or not at all, immunity to severe malaria appears to develop with significantly greater rapidity, possibly reflecting a higher degree of antigenic homogeneity in the virulence factors of parasites responsible for severe disease [7]. Generating protective immunity to severe malaria may be a more immediately achievable goal than immunity to uncomplicated malaria as that PfEMP1 variants of the severe subtypes tend to be more immunogenic [40], and individuals develop protective immunity to severe, but not uncomplicated, malaria after natural infection. PfEMP1 has been validated as a potential target antigen in both animal and human models. The DBL1α domain of PfEMP1 may be a promising vaccine target to reduce morbidity and mortality from severe malaria. DBL1α is one of the few highly conserved regions of PfEMP1, and it appears to be a region of PfEMP1 that is directly responsible for mediating cytoadhesion and rosetting [42][37]. In addition, it is expressed on the surface of pRBCs making it vulnerable host immune attack as opposed to many malaria antigens, which are sequestered in RBCs making them less vulnerable to both antibody and cell mediated immunity.

The first goal of the proposed experiments is to evaluate vesicular stomatitis virus vectors as a platform for a vaccine incorporating the DBL1α domain of PfEMP1. The second goal of the proposed experiments is to evaluate the effectiveness of VSV vectors expressing DBL1α in disrupting rosette formation of pRBCs.

Aim 1. Construct and characterize VSV vectors expressing the *P. falciparum* DBL1α domain of PfEMP1 (VSV-mDBL1α).

> This aim will determine if VSV vectors are able to induce expression of an antigen constructed from the the *P. falciparum* protein PfEMP1 and the PfEMP1 functional domain DBL1α.

Aim 2: Test the effectiveness of VSV-mDBL1α vectors in a rosette disruption assay.

> This aim will determine if the constructed VSV-mDBL1α vectors are able to induce immune responses in mice that are able to decrease or disrupt rosette formation of human pRBCs.

METHODS

Plasmids

We designed a codon-optimized 1.7kb gene construct encoding the PfEMP1 N-terminal sequence (NTS, 67aa), DBL1α domain (348aa), transmembrane region (TM, 36aa), and acid-terminal sequence (ATS, 59aa). The VSV-G signal sequence (16aa) was added to the N-terminus and a cMyc tag (11aa) was added to the C-terminus (Figure 1). Upstream XhoI and downstream NheI sites were introduced to allow for directional cloning into VSV vectors. The gene product was cloned into the fifth position of VSV vectors encoding either the Indiana or New Jersey serotype G-proteins to make VSV-mDBL1α (Figure 2).

*All of the above experiments were preformed by the author, except for the synthesis of the DBL1α gene construct with was preformed by the company MrGene.

Recombinant viruses

BHK-21 cells were grown to 50% confluence and infected with recombinant vaccinia virus expressing T7 RNA polymerase and a multiplicity of infection (MOI) of 10. The cells were incubated for 1 h in serum-free Dulbecco's modified Eagle's medium (DMEM). The cells were then co-transfected with the plasmid expressing the recombinant VSV anti-genome with expression vectors for the VSV N, P, and L proteins under the control of a T7 promoter. Supernatants were collected 40 hours post-transfection, filtered through a 0.2μm-pore filter to remove vaccinia virus, and passaged

onto fresh BHK-21 cells. Medium was collected immediately after cytopathic effects

were observed (~48 hours) and filtered through a 0.1μm-pore filter. Recombinant VSV-

DBL1α was then plaque purified and expanded, and the VSV- DBL1α was stored at

−80°C. Titers were determined on thawed samples to be 2.5×10^8.

*All of the above experiments were preformed by the author.

Detection of DBL1α expression

Immunoflorescence studies were preformed using BHK-21 cells infected with VSV-

DBL1α (MOI of 10), with uninfected and wild type VSV-infected cells used as controls.

Medium was collected 6h after infection, cells were washed with phosphate-buffered

saline (PBS) and lysed with 2× SDS sample buffer. Proteins were separated on a 10%

SDS gel, transferred to a nitrocellulose membrane, probed with anti-cMyc antibody, and

detected with secondary antibody using chemiluminesence. The sample was also

incubated with PNGase in glycoprotein denaturing buffer for 10 minutes at 100°C and

then for one hour at 37°C in order to deglycosylate the protein.

For immunofluorescence microscopy, BHK-21 cells were grown on glass coverslips and

infected with VSV-DBL1α (MOI of 10), with uninfected and VSV-infected cells as

controls. After 5 hours, cells were fixed with 2% paraformaldehyde and then blocked for

1 hour in PBS containing 10% fetal bovine serum. Cells were then incubated with either

anti-cMyc antibody (Santa Cruz Biotechnology; 1:50) or anti-VSV-G antibody (1:200)

for 30 min at 37°C. Cells were washed with PBS following incubation with primary

antibody and then incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (Invitrogen; 1:1,000) for 30 min at 37°C. After washing, cells were mounted onto microscope slides with ProLong Gold antifade reagent containing DAPI (4′,6-diamidino-2-phenylindole; Invitrogen). A Nikon Eclipse 80i fluorescence microscope equipped with a Nikon Plan Apochromat 40x or 60X oil objective and a Photometrics CoolSnap camera was used to capture images.

    *All of the above experiments were preformed by the author.

Immunizations and sera collection

Female Balb/c mice at 10 weeks of age were purchased from Charles River (Wilmington, MA) and housed at the Yale University School of Medicine animal facilities. All experiments were performed in accordance with Yale Institutional Animal Care and Use Committee-approved procedures. Single intramuscular (i.m.) inoculations of VSV-mDBL1α (10^8 PFU) were administered in a 50-µl volume. A VSV vector expressing HIV gag (VSV-41g, 10^8 PFU) was used as a control. All priming injections were preformed with the VSV-Indiana strain. Boost inoculations were preformed 30 days post-prime with the VSV-New Jersey strain of both VSV-DBL1α (10^8 PFU) and VSV-41g (10^8 PFU). Sera were collected at Day 0 (pre-prime), Day 30 (pre-boost), and Day 60.

    *All of the above experiments were preformed by the author except for sera collection, which was preformed by the Yale Animal Research Facility technicians.

Rosette disruption assay

Rosette disruption assays were performed using the DBL1α domain from the highly virulent FCR3S1.2 strain of *P. falciparum*. A 25μl volume of FCR3S1.2 culture (5% hematocrit, with an 80% rosetting rate) was mixed with an equal volume of sera from immunized animal at three different dilutions (1:5, 1:10 and 1:20) and incubated at 37 °C for 1 h. Aliquots were then mixed with 1μl of ethidium bromide (0.5μg/ml) and the percentage of mature pRBCs in rosettes (defined as the binding of two or more labeled RBCs) was examined using fluorescent microscopy.

 *All of the above experiments were performed by collaborators from the lab of Mats Wahlgren at the Swedish Institute for Infectious Disease Control in Stockholm, Sweden.

RESULTS

Construction mDBL1α gene and recovery of VSV vectors expressing mDBL1α

In order to generate effective immune responses to target antigens derived from PfEMP1,

it is thought that there must be expression of the antigen at the cell surface [39]. Due to the

size and significant variability of PfEMP1, expression of the full-length protein at the cell

surface for use as an immunogen is not feasible. For this reason, Chen et al [39] introduced

the concept of creating a 'mini' PfEMP1 gene construct that could be used to express

individual functional domains of the PfEMP1 protein being employed as target antigens

at the cell surface by incorporating PfEMP1 structural domains. The construct we

created using this model, mDBL1α (Figure 1), consists of the NTS (67aa), the DBL1α

domain (348aa), the transmembrane region (24 aa) and part of the ATS-domain (59aa).

The VSV signal sequence (16aa) was inserted upstream to the NTS in order to target the

protein into the exocytic pathway and potentially to the cell surface, and a cMyc tag

(EQKLISEEDL) was inserted downstream of the ATS to facilitate detection of the

protein. Chen et al did not find a significant effect on immunogenicity due to the

presence or absence of the NTS, however others [43] have found that the presence of the

NTS upstream to the DBL1α domain significantly increased the ability to induce an

antibody response that disrupts rosetting of pRBCs. The DNA sequence encoding this

protein was synthesized (GeneMan) with upstream and downstream cloning sites.

mDBL1α was inserted into VSV vectors between the VSV-L and VSV-G proteins

(Figure 2), and successful cloning was verified by restriction digest. Recombinant VSV-

mDBL1α particles were generated in BHK21 cells at titers of 10^8/ml. Cloning of the

mDBL1α gene into VSV and virus recoveries were performed for both vectors expressing the Indiana (I) and New Jeresy [44] [44] serotype G-proteins for use in a prime-boost inoculation regimen. Neutralizing immune responses directed at the VSV vector induced by the priming vaccination can be avoided by switching to a G-protein from a different VSV serotype for the boost vaccination [6].
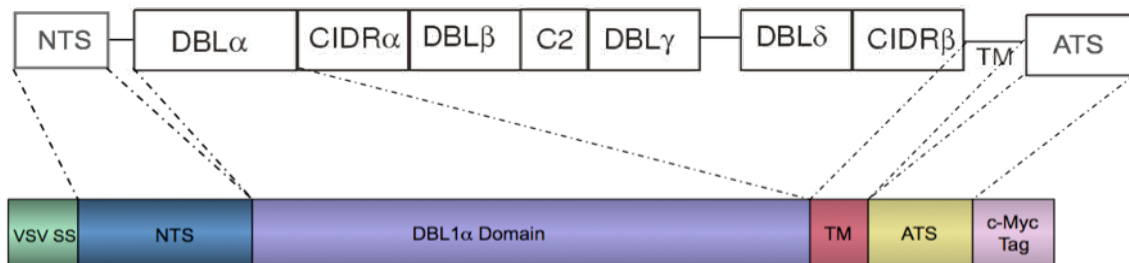
Figure 1. Schematic representation of the structure of the mDBL1α construct derived from PfEMP1 of the highly virulent FCR3S1.2 strain of *P. falciparum*. The N-terminal sequence (NTS) and DBL1α domain from the PfEMP1 gene were joined to a sequence encoding the PfEMP1 transmembrane region [31] and portion of the acid terminal sequence [32]. The vesicular stomatitis virus G-protein signal sequence (VSV SS) was inserted upstream of the NTS to target the protein to the exocytic pathway. A cMyc tag was inserted downstream of the ATS to facilitate detection of the gene product.

Figure 2. Sequence of mDBL1a. DNA sequence is shown on the top line in the 5' to 3' direction. Amino acid sequence is shown on the bottom line. Regions shown in Figure 1 are labeled.

A

B
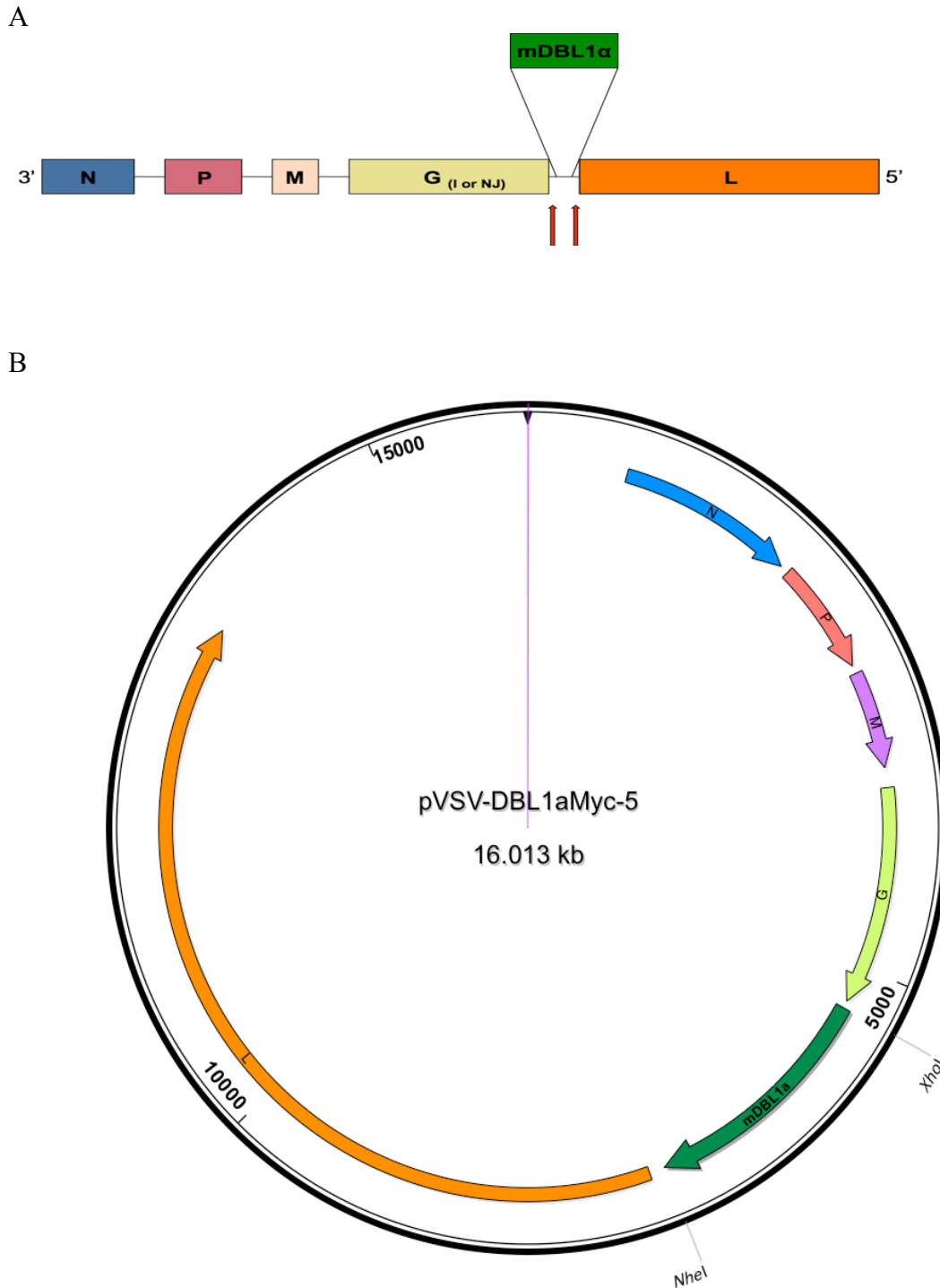
Figure 3. Insertion of mDBL1α into the vesicular stromatitis virus (VSV) genome. (A) Schematic representation of the VSV-mDBL1α genome. The site of mDBL1α gene insertion and gene order are shown in the 3′ to 5′ direction on the negative strand RNA genome. Red arrows indicate the restriction enzyme sites used for cloning the mDBL1α DNA. VSV-G is indicated as either the I or NJ serotypes. (B) Vector map of pVSV-mDBL1α. XhoI and NheI sites used for cloning are shown.

Expression and cell localization of DBL1α

Immunoflorescence microscopy performed to examine expression and localization of c-Myc tagged mDBL1α. The results showed that BHK21 cells infected with VSV-mDBL1[6] expressed mDBL1α (Figure 4c). Anti-cMyc antibodies did not cross react with uninfected cells or with cells infected with wild-type VSV (Figures 4a and 4b). VSV-mDBL1α infected cells also expressed VSV-G protein (Figure 4d). Figure 3e shows VSV-mDBL1α infected cells stained with anti-cMyc antibodies at 60x magnification. Expression of mDBL1α at the cell surface was not detected using immunoflorescence microscopy. The majority of expression appeared to be in a reticular ER-like pattern. Western blot analysis on whole cell extracts of infected cells demonstrated a the presence of a ~62kDa protein with the mobility expected of mDBL1α in cells infected with VSV-mDBL1α, but not in control cells infected with wild type VSV (Figure 5). DBL1a has 3 predicted sites for N-linked glycosylation [45]. If mDBL1α progressed to the secretory pathway, the protein should have been glycosylated. Therefore, to determine whether mDBL1α did enter the secretory pathway, we treated the protein with an N-glycosidase. After glycosidase treatment, a mobility shift was seen, with a sharper band observed for the deglycosylated protein (Figure 5), suggesting that mDBL1a was successfully targeted to the secretory pathway.
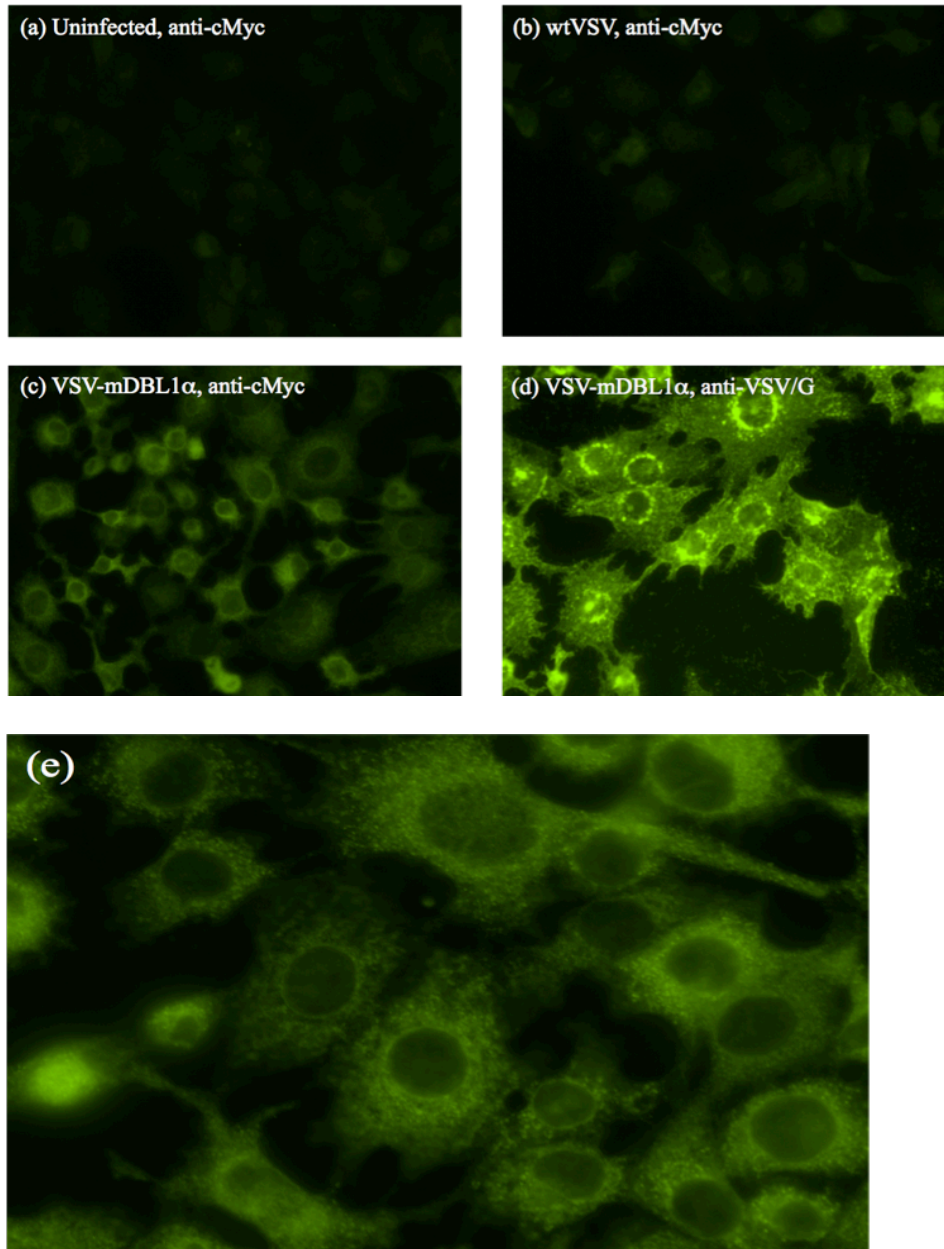
Figure 4. Indirect immunofluorescence microscopy of mDBL1α and VSV-G protein in cells infected with VSV-mDBL1α and wild type VSV (wtVSV). *(a)* Uninfected BHK21 cells stained with anti-cMyc antibodies (40x objective). *(b)* BHK21 cells infected with wtVSV and stained with anti-cMyc antibodies (40x objective). *(c)* BHK21 cells infected with VSV-mDBL1α and stained with anti-cMyc antibodies (40x objective). *(d)* BHK21 cells infected with VSV-mDBL1α and stained with antibodies recognizing VSV-G protein (40x objective). (e) BHK21 cells infected with VSV-mDBL1α stained with anti-cMyc antibody (60x objective).
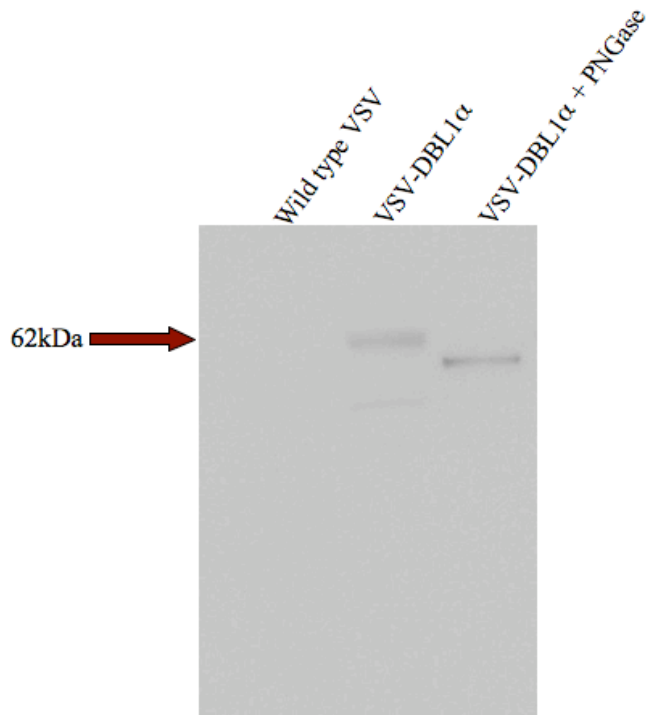
Figure 5. Western blot of mDBL1α. Western blot analysis was performed on whole cell extracts prepared from BHK-21 cells infected with either VSV-mDBL1a or wild type VSV as a control and probed with anti-cMyc antibody. Samples were also cleaved to remove N-linked glycans. (VSV-mDBL1α + PGNase).

Immunization with VSV-DBL1α reduces rosetting of human pRBCs

After prime-boost immunization with VSV-mDBL1α, sera of immunized mice dose-dependently disrupted rosettes formed by human RBCs infected with the highly virulent FCR3S1.2 strain of *P. falciparum* (Figure 6a). Immunization with VSV-mDBL1α generated a response that reduced rosetting by 58% (Figure 6b). The differences in the mean rosette disruption rates between the sera of mice immunized with VSV-mDBL1α and mice immunized with the control vector VSV-41g were statistically significant, $P<0.01$ (Students unpaired *T*-test).

A

B
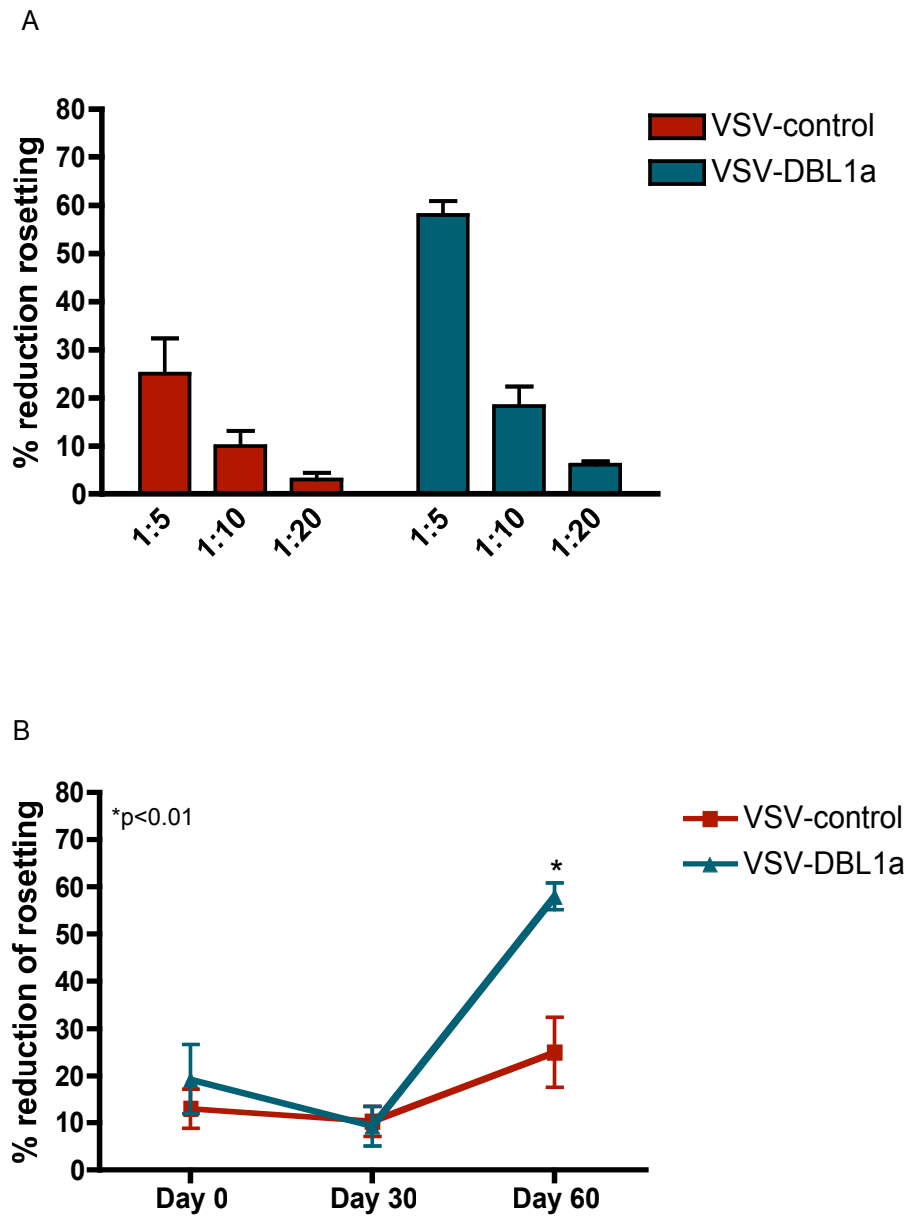
Figure 6. Means of rosette-disrupting activity of the sera generated by the prime-boost immunization with VSV-mDBL1α in mice. Sample of rosetting FCR3S1.2 (rosetting rate of >80%) was mixed with sera diluted from 1:5 to 1:20 and the rosetting rate was scored in incident UV-light. (a) Dose response inhibition of rosetting by serum from mice immunized with VSV-mDBL1α. (b) Sera from mice immunized with VSV-mDBL1α showed significantly ($P<0.01$, Students unpaired T-test) greater rosette-disruption activity than serum from mice immunized with the control vector VSV-41g (VSV-control). Baseline responses are shown at Day 0, post-prime responses were measured at Day 30, post-boost responses were measured at Day 60.

DISCUSSION

Immunization with VSV-mDBL1α induces a robust anti-rosetting response

We have shown that immunization with VSV vectors expressing the DBL1α domain of

the *P. falciparum* protein PfEMP1 generates an immune response that is able to disrupt

rosette formation in pRBCs. Sera of mice immunized with VSV-mDBL1α were able to

reduce rosetting by 58%. For comparison, a serum pool from adults living in a malaria

hyperendemic region has previously been shown to produce a 60% reduction in rosetting

[40]. While older children and adults from malaria endemic areas do not develop complete

protective immunity to uncomplicated malaria, they appear to develop robust immunity

to severe malaria, and malarial mortality is rate after the age of 5 in endemic areas.

Rosette disruption assay results obtained with VSV-mDBL1α vectors compare favorably

with results from other studies using DBL1α as a target antigen. Chen et al., 2004

vaccinated mice with a DBL1α construct (DBL1α-TM-ATS) expressed in Semliki Forest

Virus (SFV) vectors, and showed a 40% reduction in rosetting [39]. Improved rosette

disruption seen with VSV-mDBL1α vectors may be related to the viral vector used to

express DBL1α, but may also be related to expression of the NTS as that a recent article

has show optimal immunogenicity with NTS-DBL1α [43]. In addition, the study by Cheng

et al. primed with SFV-DBL1α but boosted with recombinant protein in combination

with complete Freunds adjuvant rather than boosting with a second round of viral vector.

Moll et al (2007) similarly showed 40% rosette disruption using DBL1α expressed in

SVF vectors in monkeys [40]. In this study, vaccinations were given with one prime and

two boosts with SFV-DBL1α particles, and an additional boost with DBL1α recombinant

protein. It does not appear that boosting with additional rounds of viral vectors as opposed to solely recombinant protein improved rosette-disruption ability, however it is difficult to directly compare as that this study was done in primates rather than rodents.

Mini-PfEMP1 expression in mammalian cells

Codon optimized 'mini' PfEMP1 consisting of the structural regions of the PfEMP1 protein (the NTS, TM, and ATS regions) and the functional DBL1α domain responsible for cytoadhesion were used in this study in an attempt to maximize cell surface expression of DBL1α. The mDBL1α gene construct was codon optimized because there are significant differences in protozoan and mammalian protein synthesis, with the plasmodium genome containing a high A/T content as well as frequent lysine and arginine repeats, and codon-optimization has been shown to significantly increase the level of expression of *P. falciparum* antigens in mammalian cells [8 46 47].

The VSV signal sequence was added to the N-terminal end of mDBL1α in order to target mDBL1α to the exocytic pathway. The glycosylation of mDBL1α suggests that it was present in the secretory pathway, however the immunoflorescence microscopy studies show mDBL1α expression in an ER-like pattern, which could indicate possible problems with protein folding and transport. Historically, there have been reports of poor processing and suboptimal transport of Plasmodial surface proteins in mamallian cells, and it has been suggested that replacement of the Plasmodial GPI signal sequence with a mammalian sequence would improve the immunogenicity of antigens that require cell

surface expression [48]. The effect of using a VSV signal sequence on surface expression is unclear from these experiments as that no direct comparisons can be made. It has been thought that expression of DBL1α at the cell surface is important to the generation of anti-rosetting antibodies [35]. The induction of a robust anti-rosetting response by VSV-mDBL1α vectors, despite their apparent failure to induce surface expression of DBL1α, suggests that cell surface expression is not necessarily requisite to generating a robust anti-rosetting response.

Since VSV-DBL1α was observed to result in production of a glycosylated protein product, a future avenue for exploration to improve VSV-mDBL1α immunogenicity might be to delete N-glycosylation sites. It has been reported that N-glycosylation of some malaria antigens decreases the strength of the immune response directed toward those antigens [49] [50], and it is currently being studied whether removal of N-glycosylation sequences will improve immune responses [8].

Advantages of vesicular stomatitis vectors in malaria vaccine development

VSV not previously been used in malaria vaccine development, but VSV has several attributes that could make it particularly well suited for use in a malaria vaccine strategy. VSV infects humans very rarely because humans are not a natural host for VSV. As such, there is extremely low seroprevalence in the human population world-wide. This is in contrast to viral vectors being used for malaria vaccines such as adenovirus, which is estimated to have a global seropositivity of up to 50%, with some estimates reaching up

to 90% in developing countries. VSV vectors are also able to be administered

intranasally. A mucosal route is much more feasible than a parenteral route for large

scale administration in developing countries due to the lack of a need for needles –

which, when in short supply, can contribute to the spread of blood borne pathogens

through re-use – as well as the need for a trained healthcare professional to administer the

vaccine. The enormous success of the live polio vaccine was due in part to the fact that it

is administered via a non-injectible route. Multiple studies with malaria parasites have

indicated that mucosal vaccination is effective at inducing protective immune responses

[32] [51] [52].

A future possibility for maximizing effectiveness of VSV-vectored malaria vaccines

could be to use them in a heterologus regimen with a second viral vector, particularly

with regard to improving durability of the immune response. Multiple studies of viral

vectored malaria vaccines have shown that heterologus prime-boost regimens (A→B or

B→A) are more effective than homologous prime boost (A→A or B→B), and recent

studies have demonstrated the superiority of an alternating heterlogous regimen

(A→B→A) in maximizing immune response durability [53] [54]. One study using a

combination of MVA and Fowlpox vectored malaria vaccines demonstrated that while

the responses to heterologous prime-boost vaccination fell to pre-vaccination levels after

270 days, the response to the heterologous alternating vector regimen remained at 84% of

the peak response [54]. One way of moving forward might be to combine VSV-vectored

DBL1α vaccine with a Semliki Forest Virus vectored vaccine that also expresses DBL1α.

Clinical significance of rosette disruption in severe malaria

Rosette disruption assays establish the functional ability of immune sera to prevent

cytoadhesive interactions that have been shown to be associated with the

pathophysiology of severe malaria. Therapeutic interventions that target rosetting may

therefore have potential to decrease the global burden of severe malaria [55] [56]. Human

genetic studies have shown that RBC polymorphisms that reduce rosetting, such as

complement receptor 1 deficiency [37] and blood group O [23], confer protection against

severe malaria [57] [58] possibly by reducing the vaso-occlusive effects of rosetting [59]. The

association of rosetting with severe malaria, taken with the protective effects of human

polymorphisms that reduce rosette formation, support a direct role of rosetting in the

pathogenesis of severe malaria. Further support is provided by the observation that the

presence of antibodies against PfEMP1 is associated with protection against severe

malaria in various studies of populations living in malaria endemic areas [41] [28] [27].

Antibodies to DBL1α from the FCR3S1.2 strain of *P. falciparum* have been shown to be

cross-reactive with multiple rosetting strains of highly virulent *P. falciparum* [40]. A

vaccine that that incorporates targets antigens from the more virulent strains of *P.*

falciparum that are specifically related to the development of severe malaria hold promise

of decreasing malaria mortality, the vast majority of which is secondary to the

development of severe malaria. This will mainly affect children under 5, in whom the

vast majority of severe malarial mortality is observed. In addition, such vaccines could

be an important component of a traveler's vaccine as that travelers are also at increased

risk of developing severe malaria due to their lack of previous exposure and non-immune

status. Such a vaccine will likely not be directly effective in preventing morbidity related to uncomplicated malaria. However it may be able to make a unique contribution towards the eventual development of a vaccine that confers protection to uncomplicated malaria due to the important role of PfEMP1 in parasite immune evasion and immunomodulation.

Failure to induce durable immunologic memory has been one of the most significant obstacles to the success of virtually every malaria vaccine effort to date. The interactions between *P. falciparum* and the mammalian immune system are extremely complex. Not only has the parasite developed sophisticated immune evasion mechanisms, there is compelling evidence that it actively induces host immune suppression. Some of the difficulties in malaria vaccine efforts encountered thus far in inducing a durable immune response may be partially addressed by designing vaccines with these interactions in mind. In general, malaria vaccine efforts have thus far focused almost exclusively on the antigen selection process – which is understandable given that the Plasmodium genome contains over 5,000 genes. Interestingly, however, the genome of *P falciparum* contains more genes related to host immune evasion and immune suppression than it does enzymes. Experience during clinical trials of several of the first generation malaria vaccines has highlighted the critical importance of adjuvant selection to the ability of a vaccine to induce a durable immune response, or even any immune response at all [4]. This suggests that the specific immune microenvironment at the time of vaccination may have an importance approaching that of the actual choice of target antigen(s). Rather than simply continuing the strategy of attempting to identify more antigens, future vaccine

efforts need to additionally focus on exploring how to rationally shape the immune response elicited to malaria target antigens based on our increasing understanding of the interactions of the malaria parasite with the host immune system, and targeting PfEMP1 could play an important role in this process.

Future directions: PfEMP1 and parasite induced immune modulation and evasion

Malaria parasites have evolved various mechanisms to evade host immune defenses: 1) The most basic mechanism of immune escape is the intracellular parasitism of RBCs, allowing the parasites avoid humoral immune responses while simultaneously evading recognition by CD8+ T-cells due to the lack of expression of MHC I molecules on the RBC surface. 2) There is a high degree of antigenic polymorphism in malarial parasites, and the low immunogenicity and high heterogeneity of malaria antigens is thought to drive the immune system to respond to too many targets [60]. Many of these targets contain tandem repeats that provide immunodominant B-cell epitopes, potentially masking the critical epitopes for protective immunity by affecting the affinity maturation of antibodies and inducing T-cell independent B-cell activation [36]. 3) The expression of PfEMP1 on pRBCs allows parasites to adhere to the vascular endothelium, protecting them from splenic clearance. 4) Given the critical importance of PfEMP1 to essential parasite functions such as adhesion, as well as its' vulnerable location exposed on the surface of pRBCs, PfEMP1 has evolved to be extremely polymorphic as well as to exhibit a high degree of clonal antigenic variation. The var gene encoding PfEMP1 is present at over 50 loci in the plasmodium genome, and blood stage parasites are able to switch

expression between these various loci resulting in antigenically distinct waves of parsitemia that effectively allow evasion of the primary antibody response [33].

In addition to immune evasion mechanisms, malaria parasites appear to have the ability to actively induce host immune suppression [36][61]. Patients with active malaria infection often show reduced immune responses not only to malaria parasites, but to other, unrelated, infectious organisms as well as vaccines [62]. This active immune suppression during infection could make it difficult for the host to develop and sustain robust protective immunity. This is seen both in the long latency to acquiring protective immunity in individuals in malaria endemic areas, as well as the difficulties encountered thus far in inducing durable immune responses with vaccines. Recently it has been demonstrated that PfEMP1 can bind to B-cells and dendritic cells, an interaction that has the potential to actively interfere with the host immune system during infection [35]. PfEMP1 has been shown to inhibit the maturation of antigen presenting cells in vitro [63], which could lead to impaired T-cell responses. This interaction with dendritic cells induces upregulation TGF-β as well as induction of T-regulatory cell activation and proliferation. PfEMP1 has additionally been shown to suppress PBMC production of IFN-γ in vitro by up to 20-fold [6]. A clear consensus has not yet been achieved as to the domains of PfEMP1 that are critical to its immune evasion properties. The most recent evidence indicates that – in contrast what has been previously believed – the interaction of PfEMP1 with APCs occurs independently of a direct interaction of the CIDR1 domain with host CD36, and instead may involves the DBL domain [64].

Conclusions

The critical role of PfEMP1 in the development of severe malaria and in *P. falciparum* immune evasion make it an important vaccine candidate. The majority of PfEMP1 is highly polymorphic with the major exception being the DBL-1α domain, regions of which are almost 100% conserved [1]. There appears to be a higher degree of antigenic homogeneity in the virulence factors of parasites responsible for severe disease [7], and cross-reactivity to DBL1α appears to occur between a number of highly virulent strains of *P. falciparum*. There is compelling evidence that the DBL1α domain is responsible for the cytoadhesive and rosetting properties of pRBCs that are central to the development of severe malaria, and may be involved in important aspects of parasite immune evasion. There is evidence from numerous human studies, as well as rodent and primate studies, that strongly support the potential utility of DBL1α as a target antigen, and it is hoped that immunization with vaccine incorporating DBL1α could alleviate some of the burden of severe malarial mortality in the future.

VSV vectors have been shown to be highly effective in inducing potent immune responses in a number of other diseases, and may also be well-suited to malaria vaccine development. We have shown that immunization with DBL1α expressed in VSV vectors induces anti-rosetting activity comparable to that of immune sera from adults living in malaria hyperendemic regions. Future studies should look toward establishing whether the immune response durability of VSV vectored DBL1α vaccines might be optimized by incorporating them into an alternating heterologous prime-boost regimen with other viral vectored DBL1α vaccines such as the SFV-DBL1α vaccine currently in development. In

addition, it future studies should examine whether incorporation of DBL1α into a multi-antigen vaccine targeting uncomplicated malaria might improve vaccine efficacy by interfering with parasite immunomodulation and immune evasion strategies that may be partially responsible for difficulties encountered with current vaccine efforts.

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