Evaluation of the safety and immunogenicity in rhesus monkeys of a recombinant malaria vaccine for \textit{Plasmodium vivax} with a synthetic TLR4 agonist formulated in an emulsion

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Running Title: \textit{P. vivax CSP} vaccine in rhesus monkeys

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ABSTRACT

*Plasmodium vivax* is the major cause of malaria outside of sub-Saharan Africa and inflicts debilitating morbidity and consequent economic impact in developing countries. In order to produce a *P. vivax* vaccine for global use, we have previously reported the development of a novel chimeric recombinant protein VMP001 based on the circumsporozoite protein (CSP) of *P. vivax*. Very few adjuvant formulations are currently available for human use. Our interest is to evaluate second-generation vaccine formulations to identify novel combinations of adjuvants capable of inducing strong, long-lasting immune responses. In this study rhesus monkeys were immunized intramuscularly three times with VMP001 in combination with stable emulsion (SE), or a synthetic TLR4 agonist (GLA) in SE. Sera and PBMCs were tested for the presence of antigen-specific humoral and cellular responses, respectively. All groups of monkeys generated high titers of anti-*P. vivax* IgG antibodies as detected by ELISA and immunofluorescence assays. In addition, all groups generated a cellular immune response characterized by antigen-specific CD4+ T cells secreting predominantly IL-2, and lesser amounts of TNF. We conclude that the combination of VMP001 and GLA-SE is safe and immunogenic in monkeys and may serve as a potential second-generation vaccine candidate against *P. vivax* malaria.
INTRODUCTION

Malaria caused by *Plasmodium vivax* is globally more widely distributed than *P. falciparum* (15) and inflicts debilitating morbidity and consequent economic impact in endemic countries. In addition, parasite drug resistance and mosquito resistance to insecticides and the relapsing behavior of this parasite mean that an effective vaccine against *P. vivax* is urgently needed. We have developed a novel recombinant vaccine antigen, designated *vivax* malaria protein 001 (VMP001), which is based on the circumsporozoite protein (CSP) of *P. vivax*. The recombinant VMP001 molecule encodes a full-length molecule encompassing the N-terminal and C-terminal regions flanking a chimeric repeat region representing VK210 and VK247, the two major alleles of *P. vivax* CSP.

VMP001 formulated in Montanide ISA adjuvant induces a potent immune response in genetically disparate strains of mice (4, 33). However, Montanides may not be suitable for widespread human use because they are difficult to formulate, requiring an extensive and costly emulsification procedure for each antigen. In addition, in several studies, Montanides have produced unacceptable local reactions (25, 32). Thus, efforts are underway to identify alternate adjuvants that are acceptable for human use. Many of the newer adjuvants in development are analogs of pathogen-derived molecules that stimulate innate and adaptive immune responses via Toll-like receptors (TLR). The TLR-4 ligand monophosphoryl lipid A (MPL®) is a chemically heterogeneous detoxified bio-product purified from *Salmonella minnesota* lipid A and has been used as an adjuvant in several safety and immunogenicity clinical trials in humans without detection of systemic toxicity. MPL® is a component of GlaxoSmithKline Biologicals’ Adjuvant Systems (10), and one such formulation, AS01B, is currently being used in Phase 3 studies with RTS,S, a CSP-based vaccine for falciparum malaria (8). Glucopyranosyl lipid A (GLA) is a
synthetic, and therefore homogeneous, form of the TLR-4 agonist Lipid A that, when formulated as a stable oil-in-water emulsion, is called GLA-SE (1). Studies of tuberculosis and leishmaniasis vaccine candidates in mice, guinea pigs and non-human primates have demonstrated that GLA-SE shows adjuvant activity similar to, or better than, MPL-SE (2, 5, 6).

It is generally accepted that studies in nonhuman primate models are useful to further develop vaccine preparations because they are much more closely related phylogenetically to humans than mice. Indeed, rhesus monkeys have helped predict subsequent human immunogenicity of various formulations of the *P. falciparum* malaria vaccine candidate RTS,S (11, 14, 28, 29). In the current study, we evaluated the safety and immunogenicity of the VMP001 vaccine in combination with GLA-SE in rhesus monkeys. The vaccine formulation was found to be safe with no significant local or systemic adverse reactions and induced potent cellular and humoral immune responses.
MATERIALS AND METHODS

Vaccine and immunization: The production and characterization of the synthetic recombinant protein VMP001 has been reported previously (4, 33). Fermentation, purification and vialing were performed under Good Manufacturing Practices at the Pilot Bioproduction Facility, Walter Reed Army Institute of Research. Briefly, the vaccine construct encoding the chimeric protein was expressed in E. coli and purified using three chromatographic steps. Several tests were performed to assess vaccine purity. The vaccine tested negative for the presence of endotoxin by an in vitro chromatographic assay, as well as an in vivo rabbit pyrogenicity test. The adjuvants used in this study were prepared by the Infectious Disease Research Institute and based on a 2% squalene-in-water emulsion (SE). To produce GLA-SE, lipophilic GLA and a surfactant were included during emulsification, as described more completely in refs. (1, 3). Adjuvants and VMP001 protein were sent to AFRMIS in Thailand where they were stored under controlled conditions. The vaccine was prepared by mixing the adjuvant with lyophilized VMP001 immediately prior to administration. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institute’s Animal Care and Use Committee, and performed in a facility accredited by the AAALAC.

A total of 20 laboratory bred Indian rhesus monkeys (Macaca mulatta) were selected and randomized into three groups receiving 15 μg VMP001 plus either SE (Group 1; n = 4), 5 μg GLA-SE (Group 2; n = 8), or 20 μg GLA-SE (Group 3; n = 8). From our previous experience in
rhesus monkeys, neither adjuvant alone nor PBS alone control induced antigen-specific immune response. Therefore, due to the limitation in the number of animals available, adjuvant-only and PBS only control groups were not included in this study. Pre-immunization samples from each monkey served as controls. Each vaccine formulation, containing 15 μg VMP001 in a total volume of 0.5 ml, was given by intramuscular injection into alternate rectus femoris muscles. The monkeys were immunized a total of three times at weeks 0, 4 and 8. Serum and blood cells were collected at baseline on the day of immunization and 14 days after each immunization.

**Safety assessment:** The injection sites were examined for reactions, including skin warmth, erythema, induration, swelling, ulceration, abscess or other abnormalities, at baseline and 1, 2, 3 and 14 days after each vaccine injection. Quantitative measurements were not performed, but each site was subjectively graded throughout the study by the same experienced veterinarian unaware of study group assignment using the following numeric grading scale: 0, absent; 1, mild; 2, moderate; and 3, severe (22). Hematologic (hematocrit, hemoglobin level and white blood cell count) and biochemical (blood urea nitrogen, serum creatinine, total protein, albumin, aspartate aminotransferase, gamma-glutamyl transpeptidase levels and C-reactive protein (CRP)) analyses were performed 15-30 days prior to study start and 0, 1, 2, 3, and 7 days after each vaccination and at the end of the study. CRP levels were measured by ELISA using a kit from Alpha Diagnostic International, San Antonio, TX.

**Antigens:** Whole VMP001 protein and pools of 15-amino acid (aa) peptides covering the VMP001 protein were used in *in vitro* assays, as indicated. The peptides were generated to cover
the entire VMP001 coding sequence and overlapped by 11 aa. The following four peptide pools were used: a pool of 62 peptides corresponding to the entire VMP001 protein; a pool of 22 peptides representing the C-terminal region and part of the type 2 repeat of VMP001; a pool of 19 peptides representing the N-terminal region through the first repeat of VMP001; and a pool of 21 peptides representing the entire repeat region and 5 aa into the start of the C-terminus.

**ELISA:** Wells of Immulux HB plates (Dynex Technologies, Chantilly, VA) were coated overnight at 22°C with 100 μl of 0.4 μg VMP001/ml PBS (pH 7.4) or 1 μg/ml of peptides representing the Type 1, AGDR or Type 2 repeat motifs. After a blocking step with PBS-Casein (Pierce, Rockford, IL), plates were incubated for 2 hours at room temperature with serum that was serially diluted in PBS with 0.05% Tween-20, followed by horseradish peroxidase (HRP)-labeled goat anti-human immunoglobulin G (IgG; Kirkegaard & Perry Laboratories (KPL), Gaithersburg, MD) for 1 hour at room temperature. The reaction was developed with ABTS [2,2’azinobis(3-ethylbenzthiazolinesulfonic acid)], stopped with SDS after one hour, and read at A₄₀₅. Enzyme-linked immunosorbent assay (ELISA) titers are defined as the serum dilution that gives an optical density (OD) of 1. Peptide reactivity was assessed by kinetic ELISA using a single serum dilution (post 3rd immunization) of 1:250, followed by anti-human IgG labeled with Alkaline Phosphatase-Reserve. Reaction was developed with Bluphos substrate (KPL) and plates were read every 30 seconds for one hour at A₆₅₀. Data is reported as V₉₅₀, expressed as milli-units per minute, which is the rate of optical density change per minute. Positive responses are defined as values that are two standard deviations above the mean of negative controls.
**Immunofluorescence assay (IFA):** Sporozoites were obtained from the salivary glands of *Anopheles dirus* mosquitoes approximately 17 to 21 days after an infected blood meal and typed for the strain of *P. vivax* (*P. vivax* strains 210 and 247). Separate multiwell slides were coated with sporozoites of each strain, air dried, and fixed with acetone. Slides were blocked with 1% BSA in PBS (PBS-BSA) for 30 min. Anti-VMP001 serum, diluted in PBS-BSA, was added to the wells, and the slides were incubated in a humidified chamber for 1 hour at room temperature. The slides were washed with PBS and fluorescein isothiocyanate-labeled goat anti-monkey IgG antibody (Sigma) was added for 30 min at room temperature. Slides were washed, mounted in Fluoromount, and viewed on an Olympus microscope at x40 magnification.

**Intracellular cytokine staining assay:** Fresh or cryopreserved PBMC were washed twice and re-suspended to 5 x 10^6 cells/ml in culture medium consisting of RPMI 1640 (Invitrogen) supplemented with 1x Penicillin-Streptomycin-Glutamine (Invitrogen), 10% FetalClone® III (Fetal bovine serum from HyClone), and 50 μM 2-mercaptoethanol (Sigma). Cells were cultured for 18 hours at 37°C in 5% CO₂ in 96 U-bottom wells in a volume of 200 μl (1 x 10^6 cells) with VMP001 (10 μg/ml) or VMP001 peptide pools (5 μg/ml of each peptide) in the presence of 1 μg/ml of anti-CD28 and anti-CD49d (both BD Pharmingen). GolgiPlug (diluted 1:1000 from the stock; BD Pharmingen) was added for the last 16 hours of culture to inhibit cytokine secretion. A negative control (complete media containing anti-CD28 and anti-CD49d) and a positive control (4 μg/ml Staphylococcal Enterotoxin B (SEB); Sigma) were included in each assay. After incubation, cells were washed in FACS buffer (PBS containing 1% FCS and 0.1% sodium azide), and stained for 30 min at 4°C with the following: anti-CD3-Alexa Fluor® 700 (SP34-2;
BD Pharmingen), anti-CD4-PE or anti-CD4-PerCP (L200; BD Pharmingen), anti-CD8-PerCP
(SK1; BD Pharmingen) or anti-CD8-Pacific Orange™ (3B5; Invitrogen), anti-CD95-eFluor™
450 (DX2; eBioscience), anti-CD28-PE-Cy™7 (CD28.2; BD Pharmingen) and LIVE/DEAD®
Fixable Blue Dead Cell Stain Kit (Invitrogen). Cells were then washed in FACS buffer,
permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s
instructions, and stained intracellularly for 30 min at 4°C with anti-TNF-PE (MAb11), anti-IL-2-
APC (MQ1-17H12) and anti-IFN-γ-FITC (B27) (all BD Pharmingen). Cells were subsequently
washed in Perm/Wash buffer (BD Pharmingen), resuspended in FACS buffer, and acquired on a
FACSCalibur or LSRII FACS machine (BD Biosciences). Data were analyzed using FlowJo
software Version 8.8.6 for Macintosh (Tree Star Inc, Ashland, OR). The frequency of cytokine
positive cells was defined as the percentage of cells positive for cytokine minus background
staining from the non-antigen stimulated sample for each subject. A response was considered
positive if the frequency of cytokine positive cells was greater than 0.05% and at least 3-fold
higher than baseline.

Statistical Analysis: Statistical analyses (two-tailed non-parametric t test) were performed
using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). P values
of 0.05 or less were considered significant.
RESULTS

Safety assessment of vaccine: Rhesus monkeys were immunized three times at four-weekly intervals with 15 μg VMP001 plus either SE (Group 1; n=4), 5 μg GLA-SE (Group 2; n=8) or 20 μg GLA-SE (Group 3; n=8). No significant or sustained local or systemic abnormalities were observed in any group after any immunization. One or two monkeys from each group exhibited grade 1 or grade 2 local reactions that resolved without intervention. Similarly, none of the hematological and biochemical parameters showed any sustained abnormal values; most of the values remained within the normal range. The levels of CRP, an acute-phase protein, are known to rise in response to inflammation. Thus, recently, the FDA has suggested testing CRP levels in serum as an indicator of systemic toxicity caused by the vaccine formulation. We measured CRP levels after the 3rd immunization as the toxicity, if any, would be higher following multiple immunizations. As expected, all groups showed slight elevations in CRP levels at day 2 post-immunization, with the increase being statistically significant in the two groups that received GLA. However, these values returned to pre-immunization levels by day 7 post-immunization (Fig. 1).

Assessment of VMP001 and peptide-specific antibody responses: CSP-specific antibodies are thought to play a major role in a protective immune response to a sporozoite challenge (20, 31). To evaluate the antibody levels and importance of multiple immunizations, we examined the kinetics of the anti-VMP001 IgG responses by ELISA (Fig. 2, A-C). All serum samples taken from animals before immunization showed negligible VMP001 reactivity. Two weeks after the first immunization, animals demonstrated seroconversion, which we have defined to be as a greater than three-fold increase of antibody titers from the baseline. Geometric
mean titers (GMT) were significantly higher after the second immunization, and 19/20 monkeys had higher titers after the third immunization demonstrating that a 2nd boost was beneficial for increasing antibody levels. The GMTs ranged from 15,218 post-2nd immunization in the SE group to 84,005 post-3rd immunization in the 5μg GLA-SE group. Compared to the SE adjuvanted vaccine, the GMTs were slightly higher in the GLS-SE groups: 2.6-3.3-fold higher after the 2nd immunization, and 1.6-1.8-fold higher after the 3rd immunization, at corresponding time points. However, these differences did not reach statistical significance.

Antibodies to the repeat region (Fig. 2 D-F), in particular to the AGDR motif within the Type 1 repeat sequence, are thought to play an important role in protection (7, 34). All monkeys generated responses to the Type 1 repeat peptide. Responses to the AGDR peptide were detected in approximately half the monkeys. The magnitude of response is higher with increasing dose of GLA. While only half the monkeys in the SE group generated a response to the Type 2 peptide, 7/8 monkeys in both the GLA groups generated antibodies to the Type 2 peptide, and similar to the AGDR peptide, the magnitude of responses to the Type 2 peptide was higher with higher dose of GLA.

To determine whether the anti-VMP001 antibodies could also detect native protein on the surface of sporozoites, serum from monkeys in each group collected after the 3rd immunization was pooled and dilutions evaluated by IFA against fixed *P. vivax* sporozoites. The pooled sera were screened on sporozoites of the two predominant *P. vivax* strains: VK210 and VK247. Native CSP was recognized by sera from each of the vaccinated groups after a 1:20,500 dilution, the highest serum dilution tested (Fig. 3). The pre-immune serum was negative at the lowest tested dilution of 1:250 (Fig. 3, top panel). Similar results were observed with VK210 strain sporozoites (not shown).
The frequency of IL-2- and IFN-γ-producing cells after immunization: Using flow cytometry, we followed the development of VMP001-specific cellular immune responses in the immunized macaques. Freshly isolated PBMC collected at baseline and two weeks after each immunization were stimulated in vitro with VMP001 protein and then analyzed for expression of CD4, CD8, IL-2 and IFN-γ by intracellular cytokine staining. Lymphocytes were identified using forward (FSC) and side scatter (SSC) properties, and CD4+ and CD8+ cells were gated on CD4+CD8− cells and CD4−CD8+ cells, respectively. CD4+ and CD8+ cells from all monkeys responded to SEB stimulation with robust IL-2 and IFN-γ production. Antigen-specific cytokine responses were considered positive if the frequency of cytokine positive cells was greater than 0.05% and at least 3-fold higher than baseline. CD4+ cells producing cytokines were detected in all groups. For example, IL-2+CD4+ cells were detected in all groups after the first immunization (Fig. 4). The responses of individual monkeys varied, but overall the responses peaked after the second immunization, and declined following the third immunization in 17/20 monkeys. For any given time point, there were no significant differences in the frequency of IL-2+CD4+ cells between any of the groups. Only two monkeys (one each in Groups 1 and 3) had positive IFN-γ responses, and these cells were predominantly IL-2+IFN-γ+ double-positive cells. In contrast to the CD4+ cell results, no IL-2+ or IFN-γ+CD8+ cells were detected after in vitro stimulation with either VMP001 protein or peptide pools in any of the monkeys at the time points tested (data not shown).
Characterization of T cell responses by multiparameter flow cytometry: To extend the analysis of cellular immune responses generated after immunization, nine-color flow cytometry was used to enumerate the frequency and characterize the phenotype of CD4+ T cells producing IL-2, IFN-γ, and TNF after in vitro stimulation of cryopreserved PBMCs with VMP001 protein. Lymphocytes were first gated via FSC versus SSC, then live lymphocytes identified based on negative staining for UV viability dye. T cells were identified by CD3 expression, and then further subdivided into CD4+CD8− (CD4+ T cells) and CD4−CD8+ (CD8+ T cells). Antigen-specific cytokine responses were enumerated according to the criteria outlined above for fresh PBMCs. The data obtained (Fig. 5A) confirmed the experiments performed with fresh PBMCs, in that IL-2+CD4+ T cells were detected in all groups, peaking after the second immunization, and most animals (19/20) lacked IFN-γ-producing CD4+ T cells. There were no significant differences between the groups. The responses were lower in magnitude than those detected with fresh cells, which is likely to reflect reduced antigen-presenting cell function as a result of cryopreservation (12, 13). In addition to IL-2 production, TNF+CD4+ T cells were detected in some monkeys (6/20 were positive with responses ranging from 0.055 to 0.11%), albeit at a lower frequency than IL-2-producing cells. The majority of the TNF+ cells were also IL-2+ (data not shown). Boolean analysis was performed to determine the proportion of cells producing one, two or three cytokines. In all groups single cytokine-producing cells predominated, and there were very few multi-functional cells detected in any group (Fig. 5B).

We further characterized the differentiation stage of the responding CD4+ T cells. Subsets of memory CD4+ T cells were identified based on CD28 and CD95 staining: central memory CD4+ T cells are CD28+CD95+ and effector memory CD4+ T cells are CD28−CD95+ (23). Individual monkeys had different proportions of naïve, central memory and effector memory
CD4+ T cells, likely reflecting their prior antigenic exposure. Representative results from three individual monkeys are shown in Fig. 6. The phenotype of cytokine-producing cells was also determined, and in response to stimulation with SEB, cells were of the memory CD95+ phenotype, although the proportion of central and memory cytokine-positive cells varied with the cytokine analyzed, and between individual monkeys. In contrast, essentially all cells that produced IL-2, TNF or IFN-γ in response to VMP001 stimulation were central memory cells, even in monkeys which had a large number of effector memory cells producing cytokines in response to SEB.

Breadth of the T cell responses: In order to assess the fine-specificity of the immune responses, cryopreserved PBMCs from one monkey from each group were stimulated with either the total peptide pool (peptides 1 to 62, covering the entire VMP001 protein), an N-term peptide pool (peptides 1 to 19, covering the N-terminal region through the first repeat of VMP001), a repeat peptide pool (peptides 20 to 40, covering the entire repeat region and 5 aa of the start of the C-terminus of VMP001), or a C-terminal peptide pool (peptides 41 to 62, covering the C-terminal region and part of the type 2 repeat of VMP001). The production of IL-2 by CD4+ T cells is shown in Fig. 7. Positive responses (>0.05%) were detected to the total pool, N-term pool and C-term pool in the three monkeys examined. In all cases the highest response was to the C-term region, followed by the N-term region. Responses were observed after both the second and third immunization although in each case, the responses were quantitatively less after the 3rd immunization. Consistent with our results using whole VMP001 protein (Fig. 6) IL-2+CD4+ T cells from the monkey representing each group had a predominantly central memory phenotype (data not shown).
DISCUSSION

Malaria is a complex disease in terms of parasitology, pathology and immunology. It is likely that an effective vaccine will need to induce and maintain both humoral and cell mediated immune responses. For the *P. falciparum* vaccine RTS,S both anti-CSP antibody levels and T cell responses have been partially correlated with protection (17, 30). In the present study, we characterized the humoral and cellular immune responses obtained after immunizing rhesus monkeys with the *P. vivax* candidate vaccine VMP001 in either SE (a stable oil-in-water emulsion) or GLA-SE (a TLR4 agonist formulated in the same stable emulsion). This is the first time a candidate malaria vaccine has been tested in non-human primates using the synthetic second generation adjuvant formulation based on GLA, an adjuvant that is at the cusp of being tested in humans under an FDA-approved protocol. The formulations tested were safe and well-tolerated. Both VMP001-specific antibody and CD4+ T cell immune responses were generated: there was a trend towards higher immune responses in groups that were immunized with the TLR4 agonist as compared to those that received SE alone; however, the differences did not show statistical significance. Antibody responses were higher after the third immunization, whereas the CD4+ T cell response peaked with the second immunization in the majority of monkeys. Surprisingly, the VMP001-specific CD4+ T cell response decreased significantly after the third immunization. It is unclear why the final dose of vaccination led to a reduction in the number of VMP001-specific CD4+ T cells in circulating blood, but the observed reduction is consistent with the CD4+ T cell response to *P. falciparum* liver-stage antigen 1 with adjuvant AS01B vaccine in rhesus monkeys (21).
VMP001-specific CD4$^+$ T cells predominantly secreted IL-2, with fewer TNF$^+$ cells, and positive IFN-γ responses were detected in only two monkeys. IFN-γ responses were detected in cynomologus macaques after immunization with GLA-SE in combination with the Fluzone influenza vaccine (9) and the tuberculosis protein ID93 (6); however, in these studies IFN-γ was detected by whole blood cytometric bead arrays, which may be a more sensitive assay than ICS. There are several possibilities why GLA-SE did not induce a strong Th1 activity in this study. It is possible that there is some kind of incompatibility between VMP001 and GLA-SE but, as stated above, the combination of VMP001 and GLA-SE works well in mice (Lumsden et al. manuscript in preparation). In addition, GLA-SE has been shown to be a potent adjuvant for stimulating Th1 responses in a number of different infectious disease mouse models (2, 3, 5, 24). It is also possible that we used either a suboptimal or an excessive dose of either VMP001 or GLA-SE although previous experiments do not make a case for a suboptimal dose (Yadava A, unpublished results).

Several reports have indicated that TLR4 signaling may not be as effective in rhesus monkeys as it is in humans or mice. Ketloy et al (18) demonstrated that, while TLR4 expression in rhesus macaque APCs mirrored that of human APCs, the responsiveness of rhesus macaque APC to the TLR4 ligand LPS differed from that of human APC. Specifically, unlike human monocyte-derived DC, rhesus macaque monocyte-derived DC did not produce bioactive IL-12p70 in response to LPS. In contrast, rhesus macaque monocytes produced TNF in response to LPS stimulation, and B cells secreted IL-6 upon LPS addition. Mehlhop et al (19) observed only low-levels of IL-12 production by LPS-activated rhesus macaque DCs, and LPS induced appreciably weaker phenotypic differentiation and LPS-activated rhesus macaque DCs were less
effective at stimulating T cell activation. It remains to be seen whether rhesus macaque APCs respond to GLA in the same way as LPS.

Although not statistically significant, there was a trend towards higher anti-VMP001 antibody responses in the animals immunized with GLA-SE. Additionally, while all three groups generated high antibody responses to the Type 1 peptide, the magnitude of responses to the Type 2 and the protective AGDR peptides was higher in groups with GLA. Highest responses were observed in the group with the highest dose of GLA. While we did not detect a strong IFN-γ response after immunization with GLA-SE, it is possible that GLA was able to enhance T helper activity to B cells. Alternatively, GLA may act directly on B cells to enhance antibody responses.

Ruprecht and Lanzavecchia have proposed a model whereby TLR stimulation provides a third signal that synergizes with BCR signaling (signal one) and T cell help (signal two) to amplify and sustain specific B-cell responses (26). It has been demonstrated that rhesus macaque B cells express TLR4 and can respond to LPS by producing IL-6 (18).

Seder et al. (27) have proposed a linear differentiation model in which CD4+ T cells progressively gain functionality with increasing differentiation until they reach optimal effector function. Continued antigenic stimulation leads to progressive loss of memory potential resulting in terminal differentiation. According to this model, Th1 CD4+ cells differentiate from IL-2+ and/or TNF+ central memory cells to multifunctional IFN-γIL-2+TNF+ or IFN-γIL-2+TNF+ effector memory cells and ultimately to IFN-γ single-positive terminal effector cells. In this study, VMP001-specific cells produced IL-2 and/or TNF, but not IFN-γ, in response to immunization with VMP001 in GLA-SE. As predicted by the CD4+ linear differentiation model, these cells were shown to be of the central memory phenotype. This suggests that the antigenic
stimulation received was not sufficient or sustained enough to induce multifunctional cells that also produce IFN-γ. This is in contrast to studies with VMP001 in mice (Lumsden et al, manuscript in preparation).

CSP T cell epitopes have not been previously identified in rhesus monkeys. A chimpanzee *Pan troglodytes* (21) was immunized by multiple exposures to the bites of *P. vivax*-infected mosquitoes. The T cell lines and clones derived from the PBL of this sporozoite-immunized chimpanzee were used to identify two epitopes on the *P. vivax* CSP. One CD4+ T cell epitope was contained in a conserved region in the carboxyl terminus of the CS protein that overlaps a sequence homologous to region II of the *P. falciparum* CSP, and another was located in the polymorphic repeat region. In another study, *Aotus lemurinus* were immunized with Multiple Antigen Peptides and two Th epitopes were detected by proliferation (16). In our study, three monkeys responded to peptide pools from both the N-term and C-term regions of the VMP001 protein. These preliminary data suggest that the VMP001 protein contains multiple rhesus T helper cell epitopes and more detailed studies are warranted.

In conclusion, there is need to test new adjuvant formulations capable of inducing strong humoral and cellular immune responses that are suitable for human use. Our study demonstrates for the first time that the malaria vaccine VMP001/GLA-SE is safe with no significant local or systemic adverse safety as assessed by general biochemical analysis, as well as testing of CRP, and is capable of eliciting antibody and CD4+ T cell responses in rhesus monkey model. The safety and immunogenicity profile of this vaccine formulation indicate that it can be tested in humans.
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defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant Mycobacterium tuberculosis. Sci Transl Med 2:53ra74.


FIGURE LEGENDS

Fig. 1. Change in serum CRP levels in the week following immunization. Blood was collected at the indicated time points (day 0, 2, and 7) after the 3rd immunization, and CRP levels were determined by ELISA. The lines represent geometric means with 95% confidence intervals. There is a transient, statistically significant, increase in the CRP levels following immunization in the GLS-SE, but not the SE alone groups, which return to pre-immunization values by day 7 post-immunization.

Fig 2. Kinetics of VMP001 and repeat peptide-specific antibody responses. ELISA was used to assess antibody titers to VMP001(A-C) (defined as serum dilution that gives an OD_{414} of 1.0) in sera obtained either before immunization or 2 weeks after each boost. Antibody titers continue to rise with each immunization. Kinetic ELISA was used to determine anti-Type 1, AGDR, and Type 2 peptide antibodies at a serum dilution of 1:250. V_{Max} values represent the rate of optical density change per minute (milli-OD/min).

Fig. 3. VMP001-specific antibodies generated in vaccinated monkeys recognize native CSP expressed by *P. vivax* sporozoites. Reactivity to native CSP expressed on *P. vivax* VK247 sporozoites is shown for pooled monkey sera after the 3rd immunization at the highest serum dilution tested (1:20,500) from the three immunization groups (Gp 1 = 15μg VMP001+SE; Gp 1 = 15μg VMP001+ 5μg GLA-SE; 15μg VMP001+ 20μg GLA-SE). Preimmune serum was tested at a 1:250 dilution.

Fig 4. PBMC from monkeys immunized with VMP001 produce cytokines after *ex vivo* antigen stimulation. PBMC collected before the primary immunization and 2 weeks after each immunization were evaluated for antigen-specific cytokine production using intracellular
cytokine staining and flow cytometry. Each circle represents the percentage of CD4+ T cells producing either IL-2 alone (black), IFN-γ alone (gray), or both IL-2 and IFN-γ (white) from each monkey PBMC sample. Bars represent the group median. Differences between means within an immunization group were not statistically significant (p>0.05).

Fig 5. Analysis of Th1 cytokine expression in CD4+ T cells. Cryopreserved cells were stimulated in vitro with VMP001 protein and CD4+ T cells were identified by ICS based on CD3 and CD4 expression and counted based on expression of IL-2, TNF and IFN-γ. (A) Percent of cells expressing IL-2 (black), TNF (gray) and IFN-γ (white). Bars represent the group mean ± SEM. (B) Proportion of cells producing one, two or three cytokines.

Fig 6. Antigen-responsive CD4+ T cells were subdivided into central and effector memory phenotypes using CD95 and CD28 expression. Representative plots show overlays of the total CD4+ population (gray) with the indicated cytokine-producing cells (black) after stimulation with VMP001 protein or SEB. Numbers show the percentage of cytokine-positive cells within each CD4+ subgroup.

Fig 7. Responsiveness of IL-2+CD4+ T cells to domains within the VMP001 antigen. Cryopreserved PMBC were stimulated with intact VMP001 protein or peptide pools at the indicated time points after immunization. Each graph represents an individual monkey (nd = not determined).