Evaluation of the Safety and Immunogenicity in Rhesus Monkeys of a Recombinant Malaria Vaccine for Plasmodium vivax with a Synthetic Toll-Like Receptor 4 Agonist Formulated in an Emulsion†‡

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Received 19 April 2011/Returned for modification 17 May 2011/Accepted 8 June 2011

Plasmodium vivax is the major cause of malaria outside sub-Saharan Africa and inflicts debilitating morbidity and consequent economic impacts 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 a stable emulsion (SE) or a synthetic Toll-like receptor 4 (TLR4) agonist (glucopyranosyl lipid A [GLA]) in SE (GLA-SE). Sera and peripheral blood mononuclear cells (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 enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays. In addition, all groups generated a cellular immune response characterized by antigen-specific CD4+ T cells secreting predominantly interleukin-2 (IL-2) and lesser amounts of tumor necrosis factor (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.

Malaria caused by Plasmodium vivax is globally more widely distributed than that caused by Plasmodium falciparum (15) and inflicts debilitating morbidity and consequent economic impacts in countries where the disease is endemic. In addition, parasite drug resistance, 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 under way 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 (TLRs). The TLR4 ligand monophosphoryl lipid A (MPL) is a chemically heterogeneous detoxified bioproduct purified from Salmonella enterica serovar Minnesota lipid A and has been used as an adjuvant in several safety and immunogenicity clinical trials in humans without the detection of systemic toxicity. MPL is a component of GlaxoSmithKline Biologics Adjuvant Systems (10), and one such formulation, AS01g, 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 TLR4 agonist lipid A that, when formulated as a stable oil-in-water emulsion (SE), is called GLA-SE (1). Studies of tuberculosis and leishmaniasis vaccine candidates in mice, guinea pigs, and nonhuman primates have demonstrated that GLA-SE shows...
adjuvant activity similar to, or better than, that of MPL-SE (2, 5, 6).

It is generally accepted that studies with 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 the subsequent human immunogenicities 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 were reported previously (4, 33). Formulation, 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 *Escherichia coli* and purified by using three chromatographic steps. Several tests were performed to assess antigen purity. The antigen tested negative for the presence of endotoxin by an *in vitro* chromato graphic assay as well as an *in vivo* rabbit progency 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 more completely described previously (1, 3). Adjuvants and the VMP001 protein were sent to the Armed Forces Research Institute of Medical Sciences (AFRIMS) 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 adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (21a). All procedures were reviewed and approved by the institute’s Animal Care and Use Committee and performed in a facility accredited by the AALAC.

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 with rhesus monkeys, neither the adjuvant-alone nor the phosphate-buffered saline (PBS)-alone control induced antigen-specific immune responses. Therefore, due to the limitation in the number of animals available, adjuvant-only and PBS-only control groups were not included in the immunization study. All sites were subjectively graded 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 assignments, using the following numeric grading scale: 0, absent; 1, mild; 2, moderate; 3, severe (22). Hematocrit (hematocrit, hemoglobin level, and white blood cell count) and biochemical (blood urea nitrogen, serum creatinine, total protein, albumin, aspartate aminotransferase, gamma-glutamyl transpeptidase, and C-reactive protein [CRP]) analyses were performed 15 to 30 days prior to the start of the study and on 1, 2, 3, and 7 days after each vaccination and at the end of the study. CRP levels were measured by enzyme-linked immunosorbent assay (ELISA) using a kit from Alpha Diagnostic International, San Antonio, TX.

**Antigens.** The whole VMP001 protein and pools of 15-aminio-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 Immulon 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 h 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 h at room temperature. The reaction was developed with ABTS [2,2’-azinobis(3-ethylbenzthiazolsulfonic acid)] and stopped with SDS after 1 h, and the A414 was read. 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 (after the third immunization) of 1:250, followed by anti-human IgG labeled with ReservecAP alkaline phosphatase. The reaction was developed with Bliphon substrate (KPL), and plates were read every 30 s for 1 h at an Amax. Data are reported as Vmax values, expressed as milliliters per minute, which is the ratio of the optical density change per minute. Positive responses are defined as values that are 2 standard deviations above the means of negative controls.

**Immunofluorescence assay (IFA).** Sporozoites were obtained from the salivary glands of *Anopheles darsus* 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 multwell slides were coated with sporozoites of each strain, air dried, and fixed with acetone. Slides were blocked with 1% bovine serum albumin (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 h 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 with an Olympus microscope at a ×40 magnification.

**Intracellular cytokine staining (ICS) assay.** Fresh or cryopreserved peripheral blood mononuclear cells (PBMCs) were washed twice and resuspended to 5 × 10^6 cells/ml in culture medium consisting of RPMI 1640 medium (Invitrogen) supplemented with 1% penicillin-streptomycin-glutamine (Invitrogen), 10% FetalClone III (fetal bovine serum from HyClone), and 50 µM 2-mercaptoethanol (Sigma). Cells were cultured for 18 h at 37°C in 5% CO2 in 96-U-bottom wells in a volume of 200 µl (1 × 10^5 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 from BD Pharmingen). GolgiPlung (diluted 1:1,000 from the stock; BD Pharmingen) was added for the last 16 h of culture to inhibit cytokine secretion. A negative control (complete medium 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 fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% fetal calf serum [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-phycocerythrin (PE) or anti-CD4-peridinin chlorophyll protein (PerCP) (L200; BD Pharmingen), anti-CD8-PerCP (SK1; BD Pharmingen) or anti-CD8-Pacific Orange (3B8; Invitrogen), anti-CD95-fluor-450 (DX2; sIgIos; anti-CD28-PE-Cy7 (CD26.2; BD Pharmingen), and a 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-tumor necrosis factor (TNF)-PE (MAAb11), anti-interleukin-2 (IL-2)–allophycocyanin (MQ1-17H12), and anti-gamma interferon (IFN-γ)-fluorescein isothiocyanate (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 by 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 the baseline.

**Statistical analysis.** Statistical analyses (two-tailed nonparametric *t* tests) were performed by 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 4-week 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 for 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 the testing of CRP levels in serum as an indicator of systemic toxicity caused by the vaccine formulation. We measured CRP levels after the third immunization, as the level of toxicity, if any, would be higher following multiple immunizations. As expected, all groups showed slight elevations in CRP levels at day 2 postimmunization, with the increase being statistically significant for the two groups that received GLA. However, these values returned to preimmunization levels by day 7 postimmunization (Fig. 1).

Assessment of VMP001 and peptide-specific antibody responses. CSP-specific antibodies are thought to play a major role in the 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. 2A to 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 a greater-than-3-fold increase of antibody titers from the baseline. Geometric mean titers (GMTs) were significantly higher after the second immunization, and 19/20 monkeys had higher titers after the third immunization, demonstrating that a second boost was beneficial for increasing antibody levels. The GMTs ranged from 15,218 after the second immunization in the SE group to 84,005 after the third immunization in the 5-μg GLA-SE group. Compared to the SE-adjuvanted vaccine, the GMTs were slightly higher in the GLA-SE groups, 2.6- to 3.3-fold higher after the second immunization and 1.6- to 1.8-fold higher after the third immunization, at corresponding time points. However, these differences did not reach statistical significance.

Antibodies to the repeat region (Fig. 2D to 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 of the monkeys. The magnitude of the response was higher with an increasing dose of GLA. While only half of the monkeys in the SE group generated a response to the type 2 peptide, 7/8 monkeys in both GLA groups generated antibodies to the type 2 peptide, and similar to the AGDR peptide, the magnitudes of responses to the type 2 peptide were higher with a 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 third immunization was pooled, and dilutions were evaluated against fixed P. vivax sporozoites by IFA. 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 preimmune serum was negative at the lowest tested dilution of 1:250 (Fig. 3, top). Similar results were observed with VK210 strain sporozoites (not shown).

**Frequency of IL-2- and IFN-γ-producing cells after immunization.** Using flow cytometry, we monitored the development of VMP001-specific cellular immune responses in the immunized macaques. Freshly isolated PBMCs collected at baseline and 2 weeks after each immunization were stimulated in vitro with the VMP001 protein and then analyzed for the expression of CD4, CD8, IL-2, and IFN-γ by intracellular cytokine staining. Lymphocytes were identified by using forward-scatter (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 the 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 immu-
nization in 17/20 monkeys. For any given time point, there were no significant differences in the frequencies 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 cells. In contrast to the CD4 cell results, no IL-2/CD8 or IFN-γ/CD8 cells were detected after in vitro stimulation with either the 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 the in vitro stimulation of cryopreserved PBMCs with the VMP001 protein. Lymphocytes were first gated via FSC versus SSC, and live lymphocytes were then identified based on negative staining with 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 data from 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 (APC) 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 that of 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 multifunctional 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 four 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 proportions 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 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-terminal 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%) to the total pool, N-terminal pool, and C-terminal pool were detected in the three monkeys examined. In all cases the highest response was to the C-terminal region, followed by the N-terminal region. Responses were observed after both the second and third immunizations, although in each case, the responses were quantitatively less after the third immunization. Consistent with our results using the 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 that a candidate malaria vaccine has been tested in nonhuman 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 toward higher immune responses in groups that were immunized with the TLR4 agonist than in 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 secreted predominantly IL-2, with fewer TNF-\(\alpha\) cells, and positive IFN-\(\gamma\) responses were detected in only two monkeys. IFN-\(\gamma\) responses were detected in cynomolgus macaques after immunization with GLA-SE in combination with the Fluzone influenza vaccine (9) and the tuberculosis protein ID93 (6); however, in those studies IFN-\(\gamma\) was detected by whole-blood cytometric bead arrays, which may be a more sensitive assay than ICS. There are several possibilities as to 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 the combination of VMP001 and GLA-SE works well in mice (J. M. Lumsden et al., unpublished data). In addition, GLA-SE has been shown to be a potent adjuvant for stimulating Th1 responses in a number of different mouse models of infectious disease (2, 3, 5, 24). It is also possible that we used a suboptimal or an excessive dose of either VMP001 or GLA-SE, although previous experiments did not make a case for a suboptimal dose (A. Yadava, unpublished results).

Several reports indicated that TLR4 signaling may not be as effective in rhesus monkeys as it is in humans or mice. Ketloy et al. (18) demonstrated previously that while TLR4 expression in rhesus macaque APCs mirrored that in human APCs, the responsiveness of rhesus macaque APCs to the TLR4 ligand lipopolysaccharide (LPS) differed from that of human APCs. Specifically, unlike human monocyte-derived dendritic cells (DCs), rhesus macaque monocyte-derived DCs 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 the addition of LPS. Mehlhop et al. (19) observed only low levels of IL-12 production by LPS-activated rhesus macaque DCs; 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 toward 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 the groups with GLA. The highest responses were observed for the group with the highest dose of GLA. While we did not detect a strong IFN-\(\gamma\) response after immunization with GLA-SE, it is possible that GLA was able to enhance T helper activity for B cells. Alternatively, GLA may act directly on B cells to enhance antibody responses. Ruprecht and Lanzavecchia proposed a model whereby TLR stimulation provides a third signal that synergizes with B cell receptor (BCR) signaling (signal 1) and T cell help (signal 2) to amplify and sustain specific B cell responses (26). It was demonstrated previously that rhesus macaque B cells express TLR4 and can respond to LPS by producing IL-6 (18).

Seder et al. (27) proposed a linear differentiation model in which CD4\(^+\) T cells progressively gain functionality with increasing differentiation until they reach optimal effector functions. Continued antigenic stimulation leads to a progressive loss of memory potential, resulting in terminal differentiation. According to this model, Th1 CD4\(^+\) cells differentiate from IL-2- and/or TNF-\(\alpha\) central memory cells to multifunctional IFN-\(\gamma\) IL-2- TNF\(^+\) or IFN-\(\gamma\)-IL-2- TNF\(^+\) effector memory cells and ultimately to IFN-\(\gamma\)-single-positive terminal effector cells. In this study, VMP001-specific cells produced IL-2 and/or TNF, but not IFN-\(\gamma\), 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-\(\gamma\). This is in contrast to data from studies with VMP001 in mice (Lumsden et al., unpublished).

CSP T cell epitopes have not been previously identified in rhesus monkeys. A *Pan troglodytes* chimpanzee (21) was previously immunized by multiple exposures to the bites of *P. vivax*-infected mosquitoes. The T cell lines and clones derived from the peripheral blood lymphocytes (PBLs) of this sporozoite-immunized chimpanzee were used to identify two epitopes on *P. vivax* CSP. One CD4\(^+\) T cell epitope was contained in a conserved region in the carboxyl terminus of the CSP 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* monkeys 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-terminal and C-terminal regions of the VMP001 protein. These preliminary data suggest that the VMP001 protein contains multiple rhesus monkey T helper cell epitopes, and more detailed studies are warranted.

In conclusion, there is a 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 reactions, as assessed by general biochemical analyses as well as the testing of CRP, and is capable of eliciting antibody and CD4\(^+\) T cell responses in the rhesus monkey model. The safety
and immunogenicity profile of this vaccine formulation indicate that it can be tested in humans.

ACKNOWLEDGMENTS

We thank Cysha Hall and members of the Malaria Serology Laboratory, Walter Reed Army Institute of Research; the Department of Entomology, AFIRMIS; and the IDRI Formulations team for excellent technical assistance and Magdalini Moutatsi for reviewing the manuscript. This work was supported by the U.S. Army Medical Material Development Activity and the Military Infectious Diseases Research Program (Fort Detrick, MD) and by grant number 42387 from the Bill and Melinda Gates Foundation (S.G.R.).

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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