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Evaluation of immune responses to a *Plasmodium vivax* CSP-based recombinant protein vaccine candidate in combination with second-generation adjuvants in mice

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ABSTRACT

Plasmodium vivax is the major cause of malaria outside of sub-Saharan Africa and causes morbidity and results in significant economic impact in developing countries. In order to produce a *P. vivax* vaccine for global use, we have previously reported the development of VMP001, based on the circumsporozoite protein (CSP) of *P. vivax*. 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, groups of C57BL/6J mice were immunized subcutaneously three times with VMP001 emulsified with synthetic TLR4 (GLA) or TLR7/8 (R848) agonist in stable emulsion (SE), a combination of the TLR4 and TLR7/8 agonists, or SE alone. Sera and splenocytes were tested for the presence of antigen-specific humoral and cellular responses, respectively. All groups of mice generated high titers of anti-*P. vivax* IgG antibodies as detected by ELISA and immunofluorescence assay. GLA-SE promoted a shift in the antibody response to a Th1 profile, as demonstrated by the change in IgG2c/IgG1 ratio. In addition, GLA-SE induced a strong cellular immune response characterized by multi-functional, antigen-specific $CD4^+$ T cells secreting IL-2, TNF and IFN-γ. In contrast, mice immunized with SE or R848-SE produced low numbers of antigen-specific $CD4^+$ T cells, and these T cells secreted IL-2 and TNF, but not IFN-γ. Finally, R848-SE did not enhance the immune response compared to GLA-SE alone. Based on these results, we conclude that the combination of VMP001 and GLA-SE is highly immunogenic in mice and may serve as a potential second-generation vaccine candidate against vivax malaria.

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1. Introduction

Plasmodium vivax is the major cause of malaria outside of sub-Saharan Africa, leading to morbidity and significant economic impact in developing countries. In order to produce a *P. vivax* vaccine for global use, we developed VMP001, based on the circumsporozoite protein (CSP) of *P. vivax* [1,2]. VMP001 is a novel recombinant protein encompassing the N- and C-terminal regions flanking a chimeric repeat region representing VK210 and VK247, the two major alleles of *P. vivax* CSP.

It is generally accepted that subunit vaccines require adjuvants to induce long-lasting protective immune responses. Until 2009, the aluminum salt (alum) adjuvants were the only adjuvants used in U.S.-licensed vaccine formulations. While alum has a good safety record, it is a relatively weak adjuvant and often induces suboptimal immunity for protection against many pathogens. Recent insights into how immune responses are activated have facilitated the discovery of new and improved adjuvants. Many pathogen-derived molecules have pathogen-associated molecular patterns that stimulate innate immune responses via toll-like receptors (TLRs) [3]; these molecules are being explored for their roles as adjuvants.

TLR4 is expressed on many cell types including macrophages, dendritic cells, neutrophils, mast cells, and B cells [4]. TLR4 recognizes the lipopolysaccharide (LPS) of gram-negative bacteria [5] and signaling through TLR4 results in an up-regulation

Abbreviations: CSP, circumsporozoite protein; DC, dendritic cells; GLA, glucopyranosyl lipid adjuvant; MPL, monophosphoryl lipid A; Pv, *P. vivax*; SE, stable emulsion; TLR, toll-like receptor.

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of co-stimulatory molecules, cell-surface receptors, cytokines and chemokines [6]. The TLR4 ligand MPL®, a detoxified 4′-monophosphoryl lipid A derivative of LPS obtained from *Salmonella minnesota*, has proven effective as a vaccine adjuvant in human clinical trials for infectious disease, cancer and allergy vaccines [7–9] and is the first TLR4 agonist to be approved for use in a human vaccine (FENDrix™ vaccine for hepatitis B in Europe, Cervarix™ vaccine for human papilloma virus in USA). Glucopyranosyl lipid adjuvant (GLA) is a synthetic lipid A analog of MPL® that shows adjuvant activity similar to or better than MPL® in mouse models of tuberculosis [10–12], influenza [13], malaria [14] and leishmaniasis [15] and is currently being evaluated in humans for use in influenza (Clinicaltrial.gov NCT01147068) and visceral leishmaniasis (Clinicaltrials.gov NCT01484548) vaccines.

In humans, TLR7 is expressed predominantly in B cells and plasmacytoid DC [16,17]. By contrast, TLR8 is expressed predominantly in myeloid DC, monocyte-derived DC monocytes/macrophages and neutrophils [17–19]. In mice, TLR7 is expressed in immune cells more broadly than in humans, including monocytes/macrophages and CD8α$^+$ DC [19,20]. Although mice express TLR8, it appears that it is not activated by agonists that stimulate human TLR8 except under limited circumstances [21]. Imidazoquinolines such as imiquimod and resiquimod (R848) are a family of synthetic, small organic molecules that are TLR7 and 8 agonists [22]. These compounds stimulate DC to secrete cytokines, up-regulate co-stimulatory molecule expression, and enhance antigen presentation to T cells [20,23]. Imidazoquinolines also polyclonally activate B cells to proliferate and differentiate into antibody-secreting cells and can, in some cases, induce class switching [24,25]. Imidazoquinolines act as vaccine adjuvants in a number of models in mice [26–30], guinea pigs [31,32] and monkeys [33,34], and resiquimods are currently being evaluated for their immunostimulatory properties in humans (Clinicaltrials.gov NCT01421017).

Several TLRs are expressed on different immune cells; therefore, combining TLR agonists or combining them with other stimuli may provide additive or synergistic effects. For example, studies in mice have shown that combining R848 with either LPS or polyI:C enhances production of cytokines such as IL-6 and IL-12, expression of co-stimulatory molecules, and enhancement of CD4$^+$ and CD8$^+$ responses in vivo [35].

In this study we tested VMP001 in combination with GLA and/or R848 formulated in an oil-in-water emulsion to determine which adjuvant system was most immunogenic. Our data indicates that in mice, GLA-SE enhances both humoral and cellular immune responses, while R848-SE, either alone, or in combination with GLA-SE does not induce a strong immune response to VMP001.

2. Materials and methods

2.1. Animals

Female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at The Walter Reed Army Institute of Medical Research (WRAIR) animal facility and handled according to institutional guidelines. All procedures were reviewed and approved by the WRAIR Animal Care and Use Committee and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Antigens

The novel chimeric recombinant vaccine VMP001 has been previously described [1,2]. Briefly, this synthetic vaccine is based on the CSP of *P. vivax* and 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 [1]. VMP001 was produced in *Escherichia coli* and purified to >95% homogeneity using a three-column purification scheme and had low endotoxin levels as determined by an in vitro test, as well as an in vivo rabbit pyrogenicity assay [2].

2.3. Adjuvant formulations

Proprietary IDRI adjuvants based on squalene-in-water stable emulsions (SE) to which various synthetic adjuvants are added were used. GLA-SE contains a synthetic monophosphoryl hexaacylated TLR4 agonist [10,11]. R848-SE (R848 (Resiquimod) was kindly provided by the 3 M Drug Delivery Systems Division, St. Paul, MN) contains the imidazoquinoline resiquimod, a synthetic agonist of TLR7/8 [22].

2.4. Immunizations

Based on previous results [1] C57BL/6J mice (5 per group) were immunized with 1 μg VMP001 in PBS, or mixed with SE, GLA-SE (1, 5, 10 or 20 μg of agonist), R848-SE (1, 5 μg of agonist) or combinations of GLA-SE (1, 5 μg of agonist) and R848-SE (1 μg of agonist). Montanide ISA 720 was used as a positive control for some experiments. The mice were immunized three times by subcutaneous injection of 0.1 ml of vaccine in the inguinal area at 3-week intervals. As a control, mice were given 5 μg GLA-SE or 1 μg R848-SE alone. Blood samples were collected 2 weeks after each immunization and sera were stored at −40 °C until analysis.

2.5. Antibody ELISA

To test for the presence of antibodies, Immulon 2HB plates (Dynatech, Alexandria, VA) were coated overnight at 22 °C with either 0.4 μg/ml VMP001 protein, or the N- and C-terminal regions of *P. vivax* CSP expressed in *E. coli* or a peptide corresponding to four copies of the Type 1 (VK210) repeat of *P. vivax*. Plates were blocked with PBS–casein (Pierce, Rockford, IL) for 1 h prior to incubation with serum from individual mice diluted in PBS–casein for 2 h, followed by horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The reaction was developed with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] and read after 60 min at A_{414}. ELISA titers are defined as the serum dilution that gives an optical density (OD) of 1. To detect VMP001-specific IgG subclasses, ELISAs using sera from individual mice were performed using secondary antibodies specific for goat anti-mouse IgG1, IgG2b, and IgG2c (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

2.6. Immunofluorescence

Sporozoites were obtained from the salivary glands of *Anopheles dirus* mosquitoes approximately 17–21 days after an infected blood meal and typed for the strain of *P. vivax* (*P. vivax* strains VK210 and VK247). Sporozoites were coated onto multiwell slides, air dried, and fixed with acetone. Slides were blocked with BSA diluted to 1% in PBS (PBS–BSA) for 30 min. Mouse 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. Sera were tested up to a dilution of 1:20,500. The slides were washed with PBS and fluorescein isothiocyanate-labeled goat anti-mouse antibody (Promega) was added for 30 min at room temperature. Slides were washed, mounted in Fluoromount, and viewed on an Olympus microscope at 100× magnification. For live IFA, freshly dissected sporozoites were incubated with a 1:1 dilution of serum and secondary antibody and observed under the microscope.

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