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Immunogenicity of C-terminus of *Plasmodium falciparum* Merozoite Surface Protein 1 Expressed as a Non-glycosylated Polypeptide in Yeast

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Abstract The C-terminal region of the merozoite surface protein 1 ($MSP1_{19}$) is one of the most promising vaccine candidates against the erythrocytic forms of malaria. In the present study, a gene encoding *Plasmodium falciparum* $MSP1_{19}$ was expressed in yeast *Pichia pastoris*. A non-glycosylated form of the recombinant protein $MSP1_{19}$ was purified from culture medium. This recombinant protein maintains its antigenicity. Significant immune responses were seen in C57BL/6 mice after the second immunization. Moreover, the specific antibodies recognized the native antigens of *P. falciparum*. The prevailing isotypes of immunoglobulin (Ig) G associated with immunization were IgG1, IgG2a and IgG2b. The antibodies isolated from mouse sera immunized with $MSP1_{19}$ can inhibit parasite growth *in vitro*. Based on these immunological studies, we concluded that $MSP1_{19}$ deserves further evaluation in pre-clinical immunizations against *P. falciparum*.

Key words *Plasmodium falciparum*; C-terminal region of merozoite surface protein 1 ($MSP1_{19}$); *Pichia pastoris*; immunity

Malaria is a major public health problem, resulting in approximately 300–500 million clinical cases and estimated 1–3 million deaths each year [1]. Development of a vaccine against *Plasmodium falciparum*, the parasite responsible for the most severe form of malaria, is an urgent priority, particularly because resistance to most traditional drugs is widespread. Identification of the targets of naturally acquired protective immunity is an essential component of vaccine development.

Among the blood-stage antigens identified, the major surface protein 1 (MSP1) has been implicated as a target for protective immunity by various criteria in studies of *P. falciparum* in nonhuman primates, *in vitro* studies of laboratory parasite host models and epidemiological studies of naturally acquired immunity [2–4].The C terminus of MSP1, from which $MSP1_{19}$ is derived, has been studied in most detail as the target of a protective immune response [5]. The available evidence suggests that antibody against $MSP1_{19}$ can inhibit parasite growth *in vivo* and *in vitro* [6]. Thus, MSP1, especially $MSP1_{19}$ of *P. falciparum*, is also regarded as a leading vaccine candidate for malaria.

An abundant source of recombinant protein, which was produced in a secreted soluble form with a conformation resembling the native protein, would greatly facilitate pre-clinical vaccination studies using $MSP1_{19}$. We chose the *Pichia pastoris* system not only because it is a eukaryote, which has many advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and post-translational modification, but also because it is manipulated as easily as *Escherichia coli* or *Saccharomyces cerevisiae* [7]. It is faster, easier and less expensive to use than other eukaryotic expression systems, such as *Baculovirus* or mammalian tissue cultures, and generally gives higher expression levels. In many cases, it provides a proper folding for recombinant proteins without the hyperglycosylation in *S. cerevisiae* [8].

In this study, high-level expression of recombinant proteins were obtained in *P. pastoris* and most of them were in non-glycosylated form under the optimized expression condition. Here, we describe some of the immunogenic properties of recombinant protein $MSP1_{19}$ produced as a

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secreted non-glycosylated protein in *P. pastoris*.

Materials and Methods

Plasmid and bacteria

Expression plasmid pPIC9k/MSP1$_{19}$ containing the His6 tag and coding sequence was constructed in our laboratory [9]. *E. coli* DH5α was used as a host for cloning and *P. pastoris* GS115 was used for protein expression.

Enzymes, reagents and animals

Restriction enzymes were purchased from New England Biolabs (Beverly, England). Protein markers were purchased from Takara Biotechnology (Takara, Japan). Yeast nitrogen base (YNB) was purchased from Invitrogen (Carlsbad, USA). The horseradish peroxidase-conjugated goat antimouse immunoglobulin (Ig) G, IgG1, IgG2a, IgG2b and IgG3 were purchased from Jingmei Bio-tech (Shanghai, China). RPMI 1640 medium and L-glutamine were obtained from Life Technologies (Carlsbad, USA). Penicillin, streptomycin and normal human serum AB were obtained from Sigma-Aldrich (St. Louis, USA). C57BL/6 mice were purchased from the Laboratory Center of Henan Medical University (Zhengzhou, China). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

Expression of the recombinant protein MSP1$_{19}$

Transformation and screening for multiple inserts in GS115 were performed as previously described [9]. A Mut^S multiple copy transformant was initially grown overnight in 5 ml of MGY medium (1.34% YNB, 1% glycerol, 4 μg/ml biotin) in a 100 ml baffled flask, then was grown at 28–30 ºC in a shaking incubator (300 rpm) for approximately 24 h (A_{600}≈6). This 5 ml of culture was inoculated to 1 L of MGY medium in a 5 L baffled flask and grown at 30 ºC with shaking (300 rpm) until the culture reached log phase growth (A_{600}≈4). Cells were harvested by centrifugation at 1500 *g* for 5 min at room temperature, re-suspended in 250 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 1.34% YNB, 4 μg/ml biotin, 1.0% methanol) in a 1 L baffled flask, and grown at 30 ºC with shaking. Methanol was added to the culture at a final concentration of 1.0% with a 24 h interval. After induction for 96 h, cells were removed by centrifugation at 1500 *g* and supernatants were collected for protein purification.

Protein purification and immunoblotting analysis

MSP1$_{19}$ protein was purified from the fermentation medium using a Ni-nitrilotriacetic acid (NTA) column (Qiagen, Hilden, Germany). The supernatant was extensively dialyzed against phosphate buffer at 4 ºC then applied to the Ni-NTA column. The column was washed three times with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted proteins were pooled and passed through a Concanavalin A (ConA)-Sepharose column (Sigma-Aldrich) pre-equilibrated with 20 mM Tris-HCl (pH 7.0), 100 mM NaCl and 1 mM $MgCl_2$ to remove the possible glycosylated form of MSP1$_{19}$. The fractions containing the target protein were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The protein concentration was measured by the Bradford method [10].

Protein bands were transferred from the gel to a nitrocellulose membrane using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules,USA). The membranes were saturated for 2 h in phosphate-buffered saline (PBS) containing 3% bovine serum albumin at room temperature, then incubated with biotinylated ConA at a final dilution of 1:50. One hour later, membranes were washed three times with PBS containing 0.05 % (*V*/*V*) Tween 20. Peroxidase-conjugated streptavidin was added to the membranes at a final dilution of 1:1000. After 1 h of incubation at room temperature, the reaction was developed using a chemiluminescence detection assay.

Animal immunization

The diluted antigen solution was formulated with CFA or IFA adjuvant at a ratio of 3:7 using an homogenizer at 2500 rpm for 5 min. The quality of the emulsion was controlled by the droplet test. BALB/c female mice (6–8 weeks old and 18–20 g in weight) were used in this study. The mice (four per group) were immunized subcutaneously with 0.2 ml of the emulsion containing 100 μg of MSP1$_{19}$ antigen. Three injections were given at 3-week intervals. Ten to fourteen days after each immunization, approximately 100 μl of blood was taken for analysis of titers. All the blood samples were placed at room temperature for 5–10 h, then kept at 4 ºC overnight. Sera were isolated from the blood samples by centrifugation at 1500 *g* for 20 min, then stored at –20 ºC.

Enzyme-linked immunosorbent assay (ELISA) for detection of mouse antibody titers to MSP1$_{19}$

Ninety-six-well plates were coated with 100 μl of native

antigen solution (1 μg/ml) diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubated at 37 ºC for 1 h. Plates were blocked with PBS containing 3% skim milk at 37 ºC for 1 h. The sera were diluted before use and 100 μl of serially diluted immune sera was added to each well. Horseradish peroxidase-conjugated goat anti-mouse IgG with 1:1000 dilution in PBS containing 3% skim milk was added to the plates for a further 1 h of incubation at 37 ºC. For every step, plates were washed three times with PBS. After color reaction of tetramethylbenzidin, 50 μl of 2 M H_2SO_4 was added to each well to stop the reaction, and the absorbance of optical density was measured at 490 nm (A_{490}) using an ELISA microplate reader.

ELISA for the detection of mouse IgG subclasses was carried out as described above, except that the secondary antibodies were antibodies specific for mouse IgG1, IgG2a, IgG2b and IgG3 at 1:1000 dilution.

Indirect immunofluorescent assay

Thin blood smears containing the schizont of *P. falciparum* were prepared, air dried, and fixed in 100% methanol for 2 min at –20 ºC. The slides were rinsed in PBS and immersed in blocking buffer (PBS containing 1% bovine serum albumin and 0.1% Triton X-100) at 37 ºC for 30 min in a humid chamber. The smears were incubated with pre-immunized serum at 1:200 dilution at 37 ºC for 30 min, followed by two 5-min washes in PBS. They were then incubated with fluorescein-isothiocyanate-labeled goat anti-mouse IgG (Sigma-Aldrich) with 1:1000 dilution in PBS for 30 min. The parasite nuclei are stained with 4′-6-diamidino-2-phenylindole (0.5 μg/ml) (Molecular Probes, Eugene, USA). Fluorescence images were captured using a digital camera and fluorescence microscope.

Growth inhibition assay

Function of specific antibodies was determined by detecting their ability to inhibit growth of the parasite *in vitro*. Parasites of the FCC1/HN isolate were maintained in RPMI 1640 medium containing 15% rabbit serum. To isolate specific antibodies for the inhibition assay, purified recombinant proteins were immobilized on CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The antibodies eluted from the columns were extensively dialyzed before use. The inhibition assay was carried out with the starting culture containing 2% hematocrit and approximately 0.5% parasitemia with the majority being late trophozoites and schizonts. One hundred and seventy microliters of the culture suspension and 30 μl of various concentrations of antibodies were added in triplicate wells to 96-well flat-bottomed plates and incubated at 37 ºC for 24 h. Thin blood smears were prepared to determine parasitemia. The inhibition rate was determined according to the following equation:

$$\text{Inhibition rate (\%)} = \frac{Pc - Pt}{Pc} \times 100\%$$

where *Pc* is the number of parasitemia of IgG isolated from pre-immunized sera and *Pt* is the number of parasitemia of IgG from immunized sera.

Cell-mediated immunity

C57BL/6 mice were immunized as described above with recombinant proteins emulsified in CFA. Twelve to fourteen days after immunization, cells were obtained by draining the lymph nodes of three animals. Cells were washed three times in plain RPMI medium and re-suspended in 1 ml of cell culture containing RPMI 1640 medium (pH 7.4) supplemented with 2 mM L-glutamine, 10 mM HEPES, 0.2% sodium bicarbonate, 1% non-essential amino acid solution, 59 μg/ml of penicillin, 133 μg/ml of streptomycin, 1 mM sodium pyruvate, 5×10^{-5} M beta-mercaptoethanol, and 2% normal human serum AB. The viability of cells was evaluated using 0.2% trypan blue dye exclusion to discriminate between live and dead cells. Cell concentration was estimated with the aid of a Neubauer counting chamber (Pharmacia Biotech) and adjusted to 1.25×10^6 cells/ml in cell culture medium. For cytokine determination, 4×10^6 lymph node cells were cultivated in flat-bottom 96-well plates in a final volume of 200 μl in triplicate. The recombinant proteins were added to the cultures at a final concentration of 10 μg/ml. After 4 d, the supernatants were collected for cytokine determination. The supernatants were diluted up to 10 times for a precise estimate of cytokine concentration. Cytokine concentration in each sample was determined from standard curves performed in parallel with known concentrations of recombinant mouse interferon-γ (IFN-γ).

Statistical analysis

Student's *t* test and one-way ANOVA were used to determine the differences between different groups.

Results

Expression and purification of $MSP1_{19}$

After transforming *Pichia* GS115 cells, approximately 300 His^+ clones transformed with the plasmid pPIC9K/

$MSP1_{19}$ were screened for high copy number integration by G418 selection. Of these clones, six were resistant to 4 mg/ml G418. The culture supernatant of yeast *P. pastoris* GS115 transformed with pPIC9K/$MSP1_{19}$ was harvested and concentrated. The target protein was purified by the Ni-NTA chromatograghy method from the supernatant. To separate the glycosylated and non-glycosylated forms of $MSP1_{19}$, the purified protein was applied to a ConA-Sepharose column. Most of the proteins flowed through and the fraction obtained included non-glycosylated forms. The fractions containing the target protein were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis [**Fig. 1(A)**] and by immunoblotting using biotinylated ConA [**Fig. 1(B)**]. There was a small amount of the glycosylated form of $MSP1_{19}$ in the Ni-NTA purified protein [**Fig. 1(B)**, lane 1]. The non-glycosylated form of $MSP1_{19}$ (ConA-unbound fraction), with a molecular weight of approximately 19 kDa, completely failed to react with ConA-biotin [**Fig. 1(B)**, lane 2].

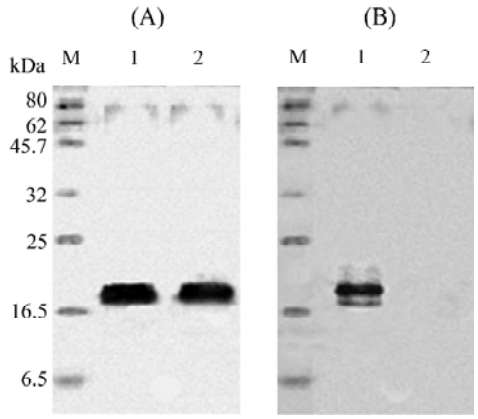

Fig. 1 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (A) and immunoblotting (B) analysis of yeast-secreted recombinant merozoite surface protein 1 ($MSP1_{19}$)

Biotinylated Concanavalin A (ConA) was used in immunoblotting analysis. M, molecular mass marker; 1, $MSP1_{19}$ after Ni-nitrilotriacetic acid (Ni-NTA) agarose purification; 2, recombinant $MSP1_{19}$ after Ni-NTA agarose and ConA-Sepharose column purification (ConA-unbounded fraction).

Evaluation of mouse immune responses elicited by immunization with $MSP1_{19}$

The immunogenicity of $MSP1_{19}$ was evaluated after immunization of C57BL/6 mice. As shown in **Fig. 2**, after the second immunization, the specific antibody levels from C57BL/6 were significantly increased. Control animals immunized with the adjuvants only had a negligible antibody immune response to antigen (T=7.648, *P*=0.0016).

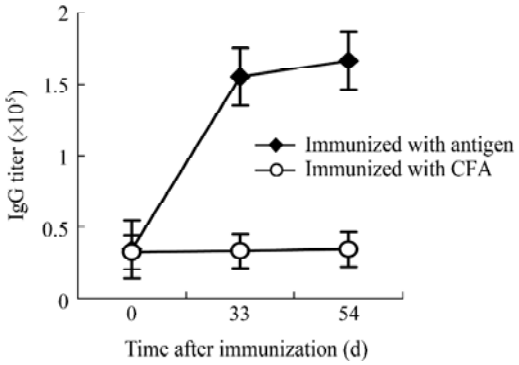

Fig. 2 Antibody immune response of mice immunized with the purified non-glycosylated form of merozoite surface protein 1 ($MSP1_{19}$)

Antibody titers were measured at each time point by enzyme-linked immunosorbent assay. Titer of antibody immunized with CFA/IFA was used as a control. CFA, complete Freund's adjuvant. IFA, incomplete Freund's adjuvant.

IgG isotype analysis in mice

As shown in **Fig. 3**, four subclasses of IgG against $MSP1_{19}$ were induced in C57BL/6 mice, IgG1, IgG2a and IgG2b isotypes were the prevailing subclasses. The high-lower sequence of four IgG subclasses was IgG1, IgG2a, IgG2b and IgG3 (F=19.1244, *P*=0.0001). The IgG1, IgG2a and IgG2b titers induced in mice were notably higher than IgG3 titer (q=10.1162, *P*=0.0001; q=8.1024, *P*=0.0003; and q=6.2588, *P*=0.0009, respectively).

Interaction of specific antibodies with native antigens on surface of parasite

Fluorescen merozoites were clearly visible (green) after incubation with the immune sera, which indicated that parasites could be recognized by the anti-$MSP1_{19}$ antisera

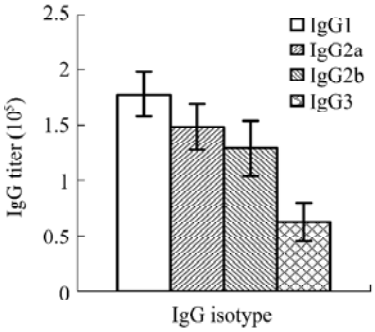

Fig. 3 Immunoglobulin (Ig)G isotype titers in merozoite surface protein 1 ($MSP1_{19}$)-immunized mice

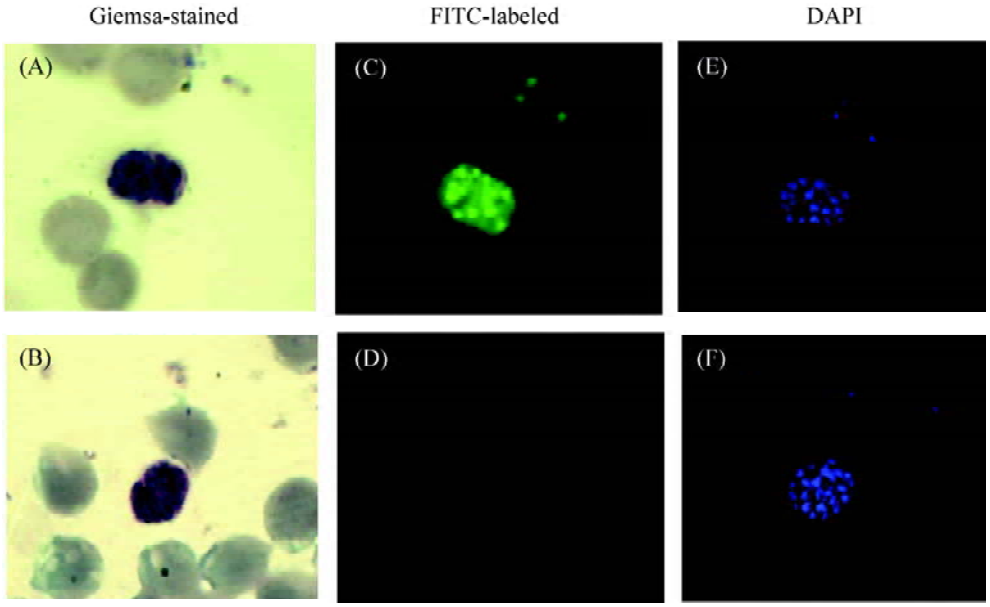

Fig. 4 Giemsa-stained smears (A and B) and immunofluorescence reactivity against native $MSP1_{19}$ (C–F)

(A,B) Giemsa-stained smears containing the schizont of *P. falciparum*. (C,E) Immunofluorescence reactivity of antisera against $MSP1_{19}$ with FITC-labeled goat anti-mouse IgG (green) and parasite nuclei were stained with DAPI (blue), respectively. (D,F) Immunofluorescence reactivity of pre-immune sera with FITC-labeled goat anti-mouse IgG (no green) and parasite nuclei were stained with DAPI (blue), respectively.

[**Fig. 4(C)**]; and the controls using pre-immune sera had no green fluorescence [**Fig. 4(D)**] indicating no positive reaction. Parasite nuclei were stained with DAPI [**Fig. 4 (E,F)**, blue]. Antibodies to $MSP1_{19}$ recognized the native antigens of *P. falciparum* strain FCC1/HN.

In vitro growth inhibition assay

To analyze the function of antibodies against $MSP1_{19}$, isolated IgG was detected by its ability to inhibit the invasion of parasites *in vitro*. As shown in **Fig. 5**, IgG isolated from the sera of mice immunized with $MSP1_{19}$ inhibited parasite growth *in vitro* in a dose-dependent manner, whereas the IgG from pre-immunized sera had no effect on parasite growth. At concentrations of 0.98 mg/ml, the antibodies isolated from mouse sera inhibited *P. falciparum* strain FCC1/HN growth by 75.7%.

Production of intracellular cytokines

The fact that C57BL/6 mice responded well after immunization with $MSP1_{19}$ suggested that $MSP1_{19}$ contained epitopes recognized by T cells which provided help for antibody production. To confirm that the $MSP1_{19}$ presented epitopes recognized by mouse T lymphocytes, we evaluated the proliferative response and IFN-γ secretion of lymph node cells of C57BL/6 mice immunized with $MSP1_{19}$ emulsified in CFA. We observed that upon *in vitro* stimulation with $MSP1_{19}$, these cells proliferated and secreted IFN-γ (**Fig. 6**). The immune response was specific because lymph node cells from CFA-immunized animals did not proliferate or secrete IFN-γ. This result

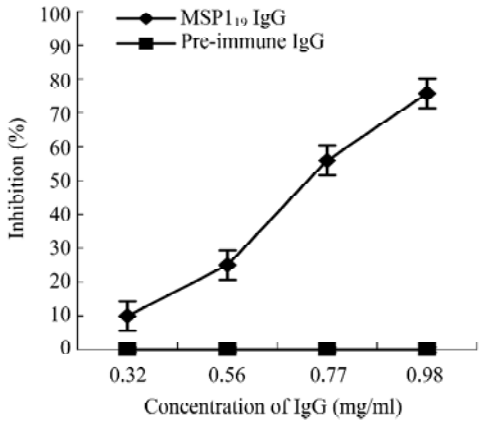

Fig. 5 Inhibition of *Plasmodium falciparum* parasite growth in mice *in vitro* by antibodies against merozoite surface protein 1 ($MSP1_{19}$)

Concentration of IgG was detected based on its ability to inhibit the growth of *P. falciparum* strain FCC1/HN *in vitro* as described in "Materials and Methods". The IgG isolated from pre-immune sera was used as a negative control for the inhibition assay. IgG, immunoglobulin G.

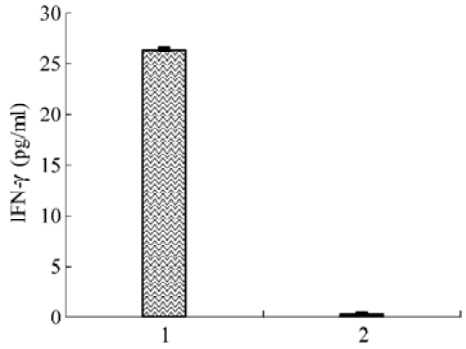

Fig. 6 Cell-mediated immune response of mice with purified merozoite surface protein 1 ($MSP1_{19}$)

1, interferon-γ was estimated in the supernatants of lymph node cells from mice immunized with $MSP1_{19}$/complete Freund's adjuvant (CFA) 96 h after stimulation with 10 μg/ml indicated antigen; 2, control (immunized with CFA only).

demonstrates that $MSP1_{19}$ contains epitopes recognized by lymph node cells of C57BL/6 mice.

Discussion

Plasmodium merozoites are major targets of blood-stage malaria vaccine development because they are the only form that is exposed to the host immune system during this stage. Intervention in merozoite invasion of erythrocytes would, in principle, block the erythrocytic life cycle of the parasite and prevent the clinical manifestations of infection. *P. falciparum* $MSP1_{19}$ is a very important asexual blood-stage antigen, its right protein folding and mild post-translational modification are required for functionality [11, 12]. The *P. pastoris* system has the potential not only for high-level expression of foreign genes but also for production of correctly folded, fully functional products [13–15], which is induced by methanol and repressed by other carbon sources such as glucose, glycerol, and ethanol [16]. Another important feature of this system is its ability to achieve extremely high cell densities, enabling efficient protein production and secretion [17].

Numerous studies have demonstrated that humoral immunity plays a crucial role in the protection against blood-stage malaria parasites [18,19]. Specific antibodies to $MSP1_{19}$ can mediate the protective immunity, but it is necessary to induce high levels of the antibodies [20–23]. As shown in this study, after immunization of C57BL/6 mice with recombinant protein of $MSP1_{19}$ produced as a secreted non-glycosylated polypeptide from *P. pastoris*, we observed that protein $MSP1_{19}$ induced high levels of specific antibodies in mice after the second immunization. The protective efficacy of humoral immunity in blood-stage malaria was not only correlated to the level of IgG, but also associated with isotypes of IgG. Many studies on serology conducted in malaria endemic regions have demonstrated that cytophilic IgG1 and IgG3 were major isotypes that associated with protective immunity in humans [24–27]. In this study, the prevailing isotypes of IgG induced by $MSP1_{19}$ in mice were IgG1, IgG2a and IgG2b, implying that $MSP1_{19}$ antigen can induce protective immunity. Moreover, the antibodies isolated from mouse sera immunized with $MSP1_{19}$ can inhibit parasite growth *in vitro*. These data provide strong support for $MSP1_{19}$ against blood-stage parasites.

We also observed that upon *in vitro* stimulation with recombinant $MSP1_{19}$, mouse T lymphocytes proliferated and secreted high levels of IFN-γ. These results suggest that the epitopes present in $MSP1_{19}$ were recognized by T lymphocytes capable of providing antibody help. Diallo *et al.* [28] examined the levels and distribution of IgG antibodies to $MSP1_{19}$ in plasma from *P. falciparum* immune adult Senegalese and the capacity of the peripheral blood mononuclear cells from these patients to either proliferate or secrete IFN-γ *in vitro*. Forty-four percent of mononuclear cells were proved to be capable of proliferating *in vitro* and IFN-γ was detected in 37% of culture supernatants. HENSMANN *et al.* [29] observed that T-cell responses to reduced recombinant proteins and linear peptides were more prevalent than responses to disulfide-bonded proteins, suggesting that the complex disulfide-bonded structure of native $MSP1_{19}$ might inhibit antigen processing or presentation. Cunha *et al.* [30] observed that, upon *in vitro* stimulation with GST-$MSP1_{19}$, His6-$MSP1_{19}$, the proliferative response and IFN-γ secretion of lymph node cells of C57BL/6 mice immunized with His6-$MSP1_{19}$ were higher than GST-$MSP1_{19}$. Further studies are needed to analyze cell-mediated immunogenicity of the recombinant $MSP1_{19}$.

In summary, we have expressed recombinant $MSP1_{19}$ as a secreted non-glycosylated polypeptide from *P. pastoris*. Antibodies against $MSP1_{19}$ can recognize the native protein. The protein was highly immunogenic in all mice tested.

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