MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATIONS OF POTENTIALLY PROTECTIVE BLOOD-STAGE *PLASMODIUM YOELII* ANTIGENS

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Abstract. As malaria remains one of the world's fatal diseases, an effective malaria vaccine is urgently required. Our previous studies have shown that formalin-killed blood-stage *Plasmodium yoelii* (FKPy)-immunized mice can be protected against subsequent challenge by live *P. yoelii* parasites, and two putative immunogenic proteins (~39.6 kDa) were identified. To prove that these proteins are protective antigens, their sequences were determined by LC-MS/MS and a search of the malaria protein databases revealed them to be *P. yoelii* YIR1 and YIR4 surface proteins. Specific primers were designed to amplify regions of *yir1* and *yir4* genes coding for the proteins' ectodomains, which were ligated with a pET28a expression vector and used to transfect *Escherichia coli* BL21-(DE3) and heterologously express recombinant (r)YIR1 (22 kDa) and rYIR4 (31 kDa). Western blotting revealed that recombinant YIR1 and rYIR4 were only mildly antigenic to mouse anti-*P. yoelii* immune serum. Mice immunized with rYIR1 and rYIR4, either separately or together, were not protected against challenges from live, virulent *P. yoelii* YM parasites. Immunization produced a minimal cell-mediated (IFN-γ and IL-4) and weak humoral (IgG and IgG2a) responses. Given that YIR1 and YIR4 are found in FKPy extract, the proteins could constitute components in the development of protein-based subunit malaria vaccines.

Keywords: *Plasmodium yoelii*, immunogenicity, YIR1, YIR4

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INTRODUCTION

Malaria continues to be a major public health concern with a recent report of an estimated 229 million malaria cases and 409,000 deaths, mostly among African children in 2019 (WHO, 2019). So far there has not been a completely effective malaria vaccine except the RTS,S vaccine which is the most advanced malaria vaccine and has been shown to have moderate efficacy (Zavala, 2022; Stanisic and Good, 2023). Several efforts of malaria prevention have shifted toward more appropriate local protection approaches, such as elimination of mosquito breeding areas and the use of insecticide-treated mosquito nets. However, those malaria control measures remain difficult to achieve, in part due to the emergence of insecticide-resistant mosquito vectors and drug-resistant parasites (Aikins *et al*, 1998; Utzinger *et al*, 2001). Therefore, an effective malaria vaccine remains an urgent need.

New vaccine discoveries and novel immunization paradigms are required for successful vaccine development, including whole parasite preparations against homologous and heterologous parasite infection in mice, primates and humans (Koff *et al*, 2013; Stanisic and Good, 2023). Immunization with a low dose of live parasite infection and drug cure has been shown to protect against a high dose re-challenge infection with homologous and heterologous parasites in humans and mice (Pombo *et al*, 2002; Elliott *et al*, 2005). Immunization with low-dose whole-killed parasites formulated with CpG oligodeoxynucleotides (CpG ODN) in alum has also been an effective method to induce protective response to homologous and heterologous parasite infection and effector Th1 cell response (Pinzon-Charry *et al*, 2010).

Immunization with formalin-killed blood-stage *Plasmodium yoelii* (FKPy) successfully protected the mice against homologous challenge (Choolue, 2011). FKPy is formulated with CpG ODN and Montanide ISA720 and injected four times into mice (Hirunpetcharat *et al*, 2003). IgG2a and IgG2b antibodies are

induced at higher titers compared to those in mice immunized with FKPy formulated with only Montanide ISA720 or with CFA/IFA, suggesting that a protective response emerged via Th1 type induction. Western blot analysis shows only the production of IgG2a antibodies in protected, but not in unprotected, mice, which are immunoreactive to a protein of 39.6 kDa obtained from a blood-stage *P. yoelii* soluble extract (Choolue, 2011). Two-dimension gel-electrophoresis and mass spectrometry revealed that this antigen is composed of two proteins of similar sizes (Srimark, 2016).

The aim of the study was to investigate the immunological roles of these two proteins by testing the immunogenicity of heterologously expressed recombinant proteins. Outcomes of this study would benefit the development of protein-based subunit malaria vaccines.

MATERIALS AND METHODS

Parasites and mice

Plasmodium yoelii YM, a lethal murine malaria parasite, was stored at -80°C (Moll *et al*, 2013) at the Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok. Female BALB/c mice, 6-8 weeks of age, were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand. Mice were acclimatized at the Animal Laboratory Research Service Center, Faculty of Tropical Medicine, Mahidol University for at least seven days prior to experimentation.

Animal protocols were approved by the Faculty of Tropical Medicine Animal Care and Use Committee, Mahidol University (approval no. FTM-ACUC 002/2015).

Adjuvants

Adjuvant CpG-ODN 1826 (5' TCCATGACGTTCCTGACGTT 3') was from Ward Medic Ltd Part, Bangkok, Thailand and adjuvant Montanide ISA51 from Seppic Chem Co, Courbevoie, France.

Amplification of *yir1* and *yir4* genes

The amino acid sequences of YIR1 and YIR4 proteins isolated as previously described (Srimark, 2016) were determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein solution was injected into a nano ACQUITY UPLC System equipped with an ACQUITY UPLC BEH column (15 cm x 75 μm, 1.7 μm bead size) (Waters Corp, Milford, MA), coupled to a SYNAPT G2-Si Mass Spectrometer (Waters Corp, Milford, MA) (Richardson *et al*, 2013). The mass spectrometer detected

mass-to-charge ratios (m/z) from 100-2,500 units and the mass spectra were then analyzed by a Mascot program version 2.2.04 (Matrix Science, London, UK) to identify the protein from the NCBI protein database as well as the Universal protein resource (Unipro) and PlasmoDB. Then the amino acid sequences of the identified proteins (YIR1 and YIR4) were converted to nucleotide sequences, and appropriate primers designed (BioDesign Co Ltd, Bangkok, Thailand) to amplify *yir1* and *yir4* genes from *P. yoelii* YM genome:

YIR1F (5' [ATACCATGGGCAT] GGATCCTGAACTGTGT-GAAAATTTTGATATGT-TAATTG 3')

YIR1R (5' [GTGCTCGAGT]CGTGT-TTTCATTAGATTCATATGC 3'), amplifying nt 5076-5616 of *yir1*

Yir4F (5' [ATACCATGGGCAT] GAATAAAGAAGTGTG-TAAAAGTTTTATGAGTG-TATG 3')

Yir4R (5' [GTGCTCGAGT] GTAAATAATTTGTTTGT-CACCGATG 3'), amplifying nt 5076-5844 of *yir4*

Note: sequences in square brackets are the attached restriction enzyme sites to allow incorporation with pET28a expression vector) (Merck, Darmstadt, Germany).

Amplicons were purified using AccuPrep® PCR Purification Kit (Bioneer Corp, Daejeon, Republic of Korea).

Heterologous expression of recombinant (r)YIR1 and rYIR4

The amplicons described above were digested with *Nco*I and *Xho*I and inserted into the pET28a expression vector (Merck KGaA, Darmstadt, Germany), generating recombinant plasmids *yir1*-pET28a and *yir4*-pET28a, the sizes of which were verified by 0.8% agarose gel-electrophoresis. *yir1*-pET28a and *yir4*-pET28a were individually transfected into competent *E. coli* DH5α cells (Merck KGaA, Darmstadt, Germany), which were then grown in Luria-Bertani (LB) medium (Thermo Fisher Scientific, Waltham, MA) with shaking at 37°C for 1 hour. Then samples were cultured on LB agar plate in the presence of 30 µg/ml kanamycin (Sigma-Aldrich, St Louis, MO) overnight at 37°C. Recombinant vectors were extracted from random colonies and transfected into *E. coli* BL21-(DE3) cells (Merck KGaA, Darmstadt, Germany). Heterologous gene expressions were induced by adding 0.4 M isopropyl-β-1-D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, Waltham, MA) and cells further incubated

overnight at 20°C. His6-tagged rYIR1 and rYIR4 were extracted and purified using an anti-His-tag antibody gel column (Ni Sepharose™ 6-Fast Flow; GE Healthcare, Chicago, IL).

Antigenicity of rYIR1 and rYIR4 determined by Western blotting analysis

Recombinant YIR1, rYIR4 and crude soluble *P. yoelii* extract were separated by 12% SDS-PAGE and transferred onto a PVDF membrane as previously described (Srimark, 2016). Protein-bound membranes were incubated with 5% (w/v) skim milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST). Membranes then were incubated with FKPy-immune serum (1:300 dilution in TBST) (Srimark, 2016) or *P. yoelii*-specific hyperimmune serum (HIS) (1:500 dilution in TBST) (Srimark, 2016) for 2 hours at room temperature, followed by incubating with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3000 dilution in TBST) or HRP-conjugated goat anti-mouse IgG2a (1:1000 dilution in TBST) (Invitrogen-Molecular Probes, Eugene, OR) at room temperature for 2 hours. After washing with TBST three times, the immunoreactive proteins were visualized by staining with 1 mg/ml diaminobenzidine (DAB) (Merck, Darmstadt, Germany) solution in 0.1 M Tris-HCl pH 7.4 and 0.06% hydrogen peroxide (Hirunpetcharat *et al*, 2003).

Immunization of mice

Female BALB/c mice were divided into six groups of eight mice, which were immunized as follows: Group I unimmunized; Group II with $3x10^7$ formalin-treated *P. yoelii* YM-infected red blood cells (FKPy-pRBC); Group III by infecting with $1×10^4$ *P. yoelii* YM-infected RBCs and cured with 200 μg of pyrimethamine in saline/mouse after 5 days via intraperitoneal injection for three consecutive days, and this protocol repeated three times; Group IV with 10 μg of rYIR1; Group V with 10 μg of rYIR4; and Group VI with a mixture of 10 μg of rYIR1 and 10 μg of rYIR4 (De Souza, *et al*, 1996). Adjuvant used for immunization of mice in Groups II, IV, V, and VI was a mixture containing 50 ug of CpG ODN1826 in 50 ul of Montanide ISA51. Mice were immunized via subcutaneous injection on day 0 and day 21, followed by intraperitoneal injection on day 42 and day 56 (Hirunpetcharat *et al*, 1997). One day prior to immunization, blood was collected via mouse-tail snip for antibody assay by enzyme-linked immunosorbent assay (ELISA) as previously described (Hirunpetcharat *et al*, 1997; Hirunpetcharat *et al*,

2003). Fourteen days after the last immunization, five randomly chosen mice from each Group were injected intravenously with 1×10^4 *P. yoelii* YM-infected RBCs, and parasitemia and mouse survival were monitored every two days from day 5 to day 23 post-challenge.

Lymphoproliferation assay

Two weeks after the last immunization, the three remaining mice from each Group that were not subjected to live parasite challenge were euthanized with CO_2 gas and individual spleens collected in Minimum Essential Media (MEM) (Gibco, Waltham, MA), cut into pieces, dispersed into cells using an end of syringe puncture pressing repeatedly and gently, washed twice with MEM and 4×10^6 cells/ml suspended in MEM/2-mercaptoethanol solution containing 2% (v/v) normal mouse serum. One hundred-µl aliquots of splenocytes were then incubated in a 96-flat bottom well plate (Corning Inc, Corning, NY) in triplicates at 37°C for three days in a 5% CO_2 incubator wells in the presence of 2×10^6/ml FKPy-pRBC, 2×10^6/ml mouse uninfected RBCs, 30 µg/ml each of rYIR1 and rYIR4, or 10 µg/ml of concanavalin A (ConA) as positive control. Then 10-µl aliquot of 10 mg/ml cell proliferation reagent water-soluble tetrazolium salt-1 (WST-1) (Roche Diagnostics GmbH, Mannheim, Germany) was added to each well and $A_{450\ nm}$ measured with reference $A_{630\ nm}$ at 0, 18 and 24 hours using an ELISA microplate reader (BioTek Instruments Inc, Winooski, VT). Stimulation index (SI) was calculated for each mouse group as follows: SI = ($A_{450\ nm}$ - $A_{630\ nm}$ of antigen-treated cells)/($A_{450\ nm}$ - $A_{630\ nm}$ of untreated cells) (Praditpol, 2014).

Interferon-γ (IFN-γ) and interleukin-4 (IL-4) assays

Splenocytes were cultured as described in the lymphoproliferation assay and supernatants were collected and IFN-γ and IL-4 concentrations were determined using murine IFN-γ and IL-4 ELISA Kits (Petro Tech Inc, Rocky Hill, NJ). Briefly, a 96-well ELISA plate (Nalge Nunc, Rochester, NY, USA) was coated with 100 µl/well of capture antibodies [rabbit anti-mouse IFN-γ or anti-mouse IL-4 antibodies, 1 µg/ml, in phosphate-buffered saline (PBS)], sealed and incubated overnight at 4°C. The plate was washed three times with washing buffer (0.05% Tween 20 in PBS; PBST) and incubated with 3% (w/v) skim milk in PBS at room temperature for 1 hour. After washing with PBST, each well was added with a 100-µl aliquot of cell culture supernatant or that of unimmunized mice (negative control) and the plate was incubated

at 37°C for 1 hour. After washing three times with the washing buffer, each well was added with a 100-µl aliquot of detection antibody (biotinylated antigen-affinity purified rabbit anti-mouse IFN-γ + D-mannitol or biotinylated antigen-affinity purified rabbit anti-mouse IL-4 + D-mannitol) and the plate incubated at 37°C for 1 hour. After washing with PBST, each well then was added with a 100-µl aliquot of avidin-conjugated horseradish peroxidase (HRP) (1:2,000 dilution with 3% (w/v) skim milk in PBS) and the plate was further incubated at 37°C for 30 minutes, washed three times with washing buffer and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution added (KPL, Gaithersburg, MD). After incubating at room temperature in the dark, $A_{405\ nm}$ was measured using an ELISA microplate reader (BioTek Instruments Inc, Winooski, VT). The concentrations of IFN-γ and IL-4 were determined using standard curves of known concentrations of IFN-γ and IL-4 standards (provided in the ELISA Kits).

Statistical analysis

Difference in absorbance or titer of antibodies between experiment and control groups was analyzed using Student's *t*-test or Mann-Whitney rank sum test, with a *p*-value ≤0.05 considered statistically significant.

RESULTS

Identification of amino acid and nucleotide sequences of potentially protective blood-stage *P. yoelii* antigens YIR1 and YIR4

Amino acid sequences of the two *P. yoelii* immunoreactive proteins isolated as previously described (Srimark, 2016) were determined by LC-MS/MS and a Mascot software, resulting in matches to PYYM_1200600 YIR Pseudogene (YIR1) and PYYM_0043200 YIR Pseudogene (YIR4), with deduced nucleotide sequences of 538 and 756 bp respectively (Table 1). Primer pairs were then designed to amplify *yir1* and *yir4* genes, with YIR1F/YIR1R primer pair amplifying *yir1* between nt 5076 and 5616 and YIR4F/YIR4R primer pair amplifying yir4 between nt 5076 to 5844. Primers also contained *Nco*I and *Xho*I restriction sites for ligation into pET28a expression plasmid. The correct insertions were confirmed by size determination of *Nco*I- and *Xho*I-digested recombinant plasmids using 0.8% agarose gel-electrophoresis, generating inserts of 538 and 756 bp of *yir1* and *yir4* respectively, and by restriction mapping of the recombinant

Table 1

Amino acid sequences of and nucleotide sequences encoding YIR1 and YIR4

Protein	Sequence
YIR1, amino acid sequence	MCENFDMLIEQYPDELNNSEECDIHNIDGIEEYCPNGNSGNKCITELD-KINAACLWLLNQNIANRIDDLSNEHVKAFIIYIMIWLNYMLNLKNAG-KINNLNEFYTKHIENNTHYTNCESYGSDCNSTLNDKAGYNNFKE-VIVKNMDFSNISFEDISKFYEAFKLLC
YIR1, nucleotide coding sequence	ATGGATCCTGAACTGTGTGAAAATTTTGATATGTTAATTGAG-CAATATCCCGATGAATTAAACAATTCCGAAGAATGTGATAT-TCATAATATAGATGGTATTGAGGAATACTGCCCTAATGGGAATTCAG-GAAACAAATGCATTACTGAGCTCGATAAAATAAATGCTGCATGT-TTATGGTTGCTCAATCAAAATATTGCTAATAGGATTGATGATTTAAG-TAATGAACATGTTAAAGCGTTTATTATATACATTATGATATGGTTAAAT-TATATGTTAAACCTAAAGAATGCTGGGAAAATCAACAACCTAAAT-GAGTTTTACACTAAACATATAGAAAATAATACGCATTATACTAACT-GTGAAAGTTATGGTAGCGATTGTAATAGTACATTAAATGATAAAG-CGGGATATAATAATTTTAAGGAAGTCATAGTTAAAAATATGGAT-TTTTCGAATATTAGTTTTGAAGATATATCTAAATTTTATGAAGCAT-TTAAATTATTATGTAATGCATATGAATCTAATGAAAACACGATAG
YIR4, amino acid sequence	MNKEVCKSFMSVWDFFPDELEENKYKFNNNNFLDSYCVSNSCDT-DLAKINGGSLYLFNKFFGSSEFFESVANSNINIVEYILIWLSYMLN-LKQQVGDETNLQYFYKMYVLNHEKYKNSIMGVDGYTSYKELID-KKTYFLDMDKKIISNFYEAFKLLCEMYNEFDDNSQFCAKCSENAN-QFINKYREMNKNSDITNNSSYNELLSTLSKDYDNFINKYNNNQH-FKSSPLPAIEEIQTSGQISGDISSSSSVTNKLFT
YIR4, nucleotide coding sequence	ATGAATAAAGAAGTGTGTAAAAGTTTTATGAGTGTATG-GGATTTTTTCCCCGATGAATTAGAAGAAAATAAATATAAAT-TTAATAATAATAATTTTTTAGATAGTTATTGTGTTAGTAATAGTTGT-GATACTGATCTCGCAAAAATTAATGGTGGATCTTTATATCT-TTTTAATAAATTCTTTGGGAGTTCTGAATTTTTTGAGTCTGTTGCAAAT-AGTAACATCAATATTGTTGAGTACATTTTGATATGGTTAAGTTATATGT-TAAACCTAAAGCAACAAGTAGGAGATGAGACCAATCTACAATACT-TTTATAAAATGTATGTACTGAATCATGAAAAGTATAAAAATTCTATAAT-GGGTGTTGATGGGTATACTAGTTATAAGGAGCTTATAGATAAAAAAA-CATATTTTTTGGATATGGATAAAAAAATTATATCTAATTTTTATGAAG-CATTTAAATTATTATGTGAAATGTATAATGAATTTGATGATAACT-CACAATTTTGCGCAAAATGTTCGGAAAATGCTAATCAATTTAT-TAATAAATATAGAGAAATGAATAAAAATTCTGATATTACTAATAATA-GTTCCTATAATGAACTATTGTCTACTTTATCAAAAGATTATGATAAT-TTTATAAATAAATATAATAATAATCAACATTTCAAATCTTCACCTCT-TCCAGCGATAGAAGAAATACAAACATCTGGACAAATTTCTGGAGA-TATATCATCAAGTTCATCGGTGACAAACAAATTATTTACA

plasmids. Recombinant *yir1*-pET28a and *yir4*-pET28a plasmids were then used to individually transform *E. coli* DH5α cells, which were grown overnight on LB agar plate containing 30 μg/ml kanamycin. Recombinant plasmids were extracted from random colonies and used to transform *E. coli* BL21-(DE3), and heterologous expression of recombinant (r)YIR1 and rYIR4 were induced with IPTG. Recombinant YIR1 and rYIR4 were purified from the bacterial lysates using an anti-His-tag column.

Antigenicity of rYIR1 and rYIR4

The antigenic properties of rYIR1 and rYIR4 were assessed by their reactivities to FKPy-immune and to hyperimmune (HIS) mouse sera. Western blotting revealed that rYIR1 (22 kDa) and rYIR4 (31 kDa) were weakly antigenic to FKPy IgG and IgG2a, but stronger to HIS IgG and IgG2a (Fig 1). This was consistent with the low antigenicity of *P. yoelii* crude soluble extract to FKPy IgG and IgG2a compared to the more marked antigenicity to HIS IgG and IgG2a (Fig 1).

Response of mice immunized with rYIR1 and rYIR4 against *P. yoelii* challenge

Six groups of female BALB/c mice (eight per group) were immunized as follows: Group I was unimmunized; Group II immunized with FKPy-pRBC ($3x10^7$); Group III was immunized by infection with *P. yoelii*-pRBC ($1×10^4$) and cured with pyrimethamine (200 μg in saline/mouse after 5 days for three consecutive days), the protocol being repeated three times; Group IV with rYIR1 (10 μg); Group V with rYIR4 (10 μg); and Group VI with a mixture of rYIR1 (10 μg) and rYIR4 (10 μg). All immunization contained adjuvants CpG ODN1826 (50 μg each) in Montanide ISA51 except in Group III. Sera from five random immunized mice from each group were prepared one day before initiation of immunization and before challenge infection for ELISA antibody assay. Fourteen days after the last immunization, mice were challenged by intravenous injection of $1×10^4$ *P. yoelii* YM-pRBC in PBS. Parasitemia and mouse survival were monitored every two days from day 5 to day 23 post-challenge.

Unimmunized negative control mice (group I) showed rising parasitemia (up to 60.8 % on day 21) and all five mice died by day 23 post-challenge (Table 2). Group II mice showed a slower rise in parasitemia (30 % on day 19) but all five mice died by day 21. Group III mice showed protection with a peak parasitemia of 1.4 % at day 9, then parasitemia was cleared and the five mice survived to day 23 of experiment. Groups IV, V

and VI mice showed no protection, with rises in parasitemia similar to that of Group I and all five mice in the three groups died by day 23: in Group IV highest level of parasitemia (47.3 %) was reached on day 17, in Group V 62.1 % parasitemia was observed on day 21 and in Group VI 38.5 % parasitemia on day 11. Interestingly, in the Group VI (immunization with a mixture of rYIR1 and rYIR4), parasitemia

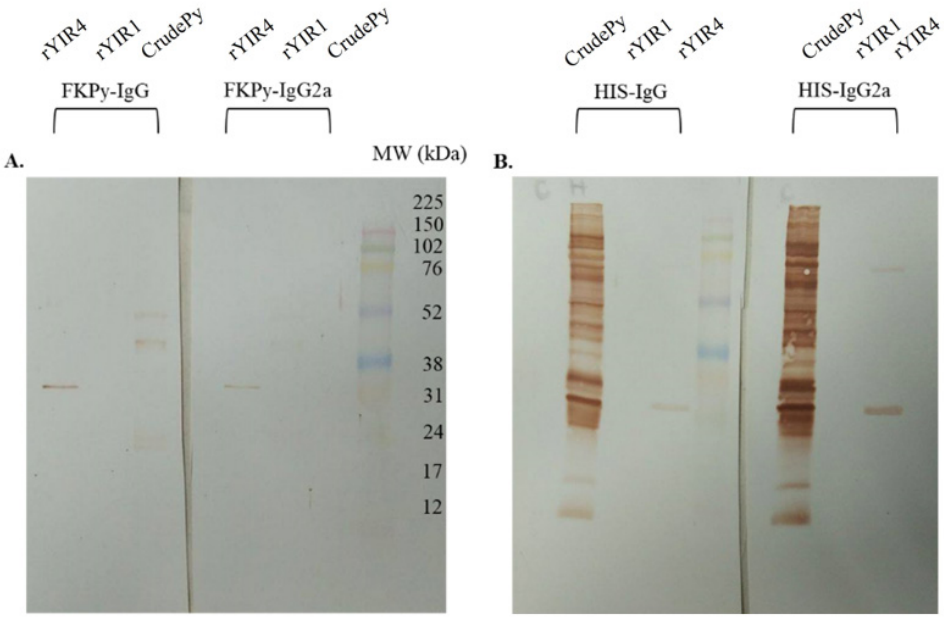

Fig 1 - Western blot analysis of recombinant (r)YIR1 and rYIR4 antigenicity

Recombinant (r)YIR1 and rYIR4 were separated by 12% SDS-PAGE, then transferred onto PVDF membrane, treated with serum from FKPy-immunized mice (A) and hyperimmune mouse serum (HIS) (B) followed by HRP-conjugated goat anti-mouse IgG or IgG2a. Immunoreactive protein bands were visualized using diaminobenzidine (DAB) solution.

CrudePy: *Plasmodium yoelii* YM crude extract; FKPy: formalin-treated *P. yoelii* YM parasites; FKPy-IgG: treated with FKPy-immune mouse serum, HRP-conjugated goat anti-mouse IgG; FKPy-IgG2a: treated with FKPy-immune mouse serum, HRP-conjugated goat anti-mouse IgG2a and DAB; HIS-IgG: treated with mouse HIS, HRP-conjugated goat anti-mouse IgG and DAB; HIS-IgG2a: treated with mouse HIS, HRP-conjugated goat anti-mouse IgG2a and DAB; HRP: horseradish peroxidase; MW: molecular weight markers

Table 2

Parasitemia and survival of immunized mice after challenge infection

Mouse group	Average percent parasitemia on observed day (number of mice died)									
	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17	Day 19	Day 21	Day 23
Group I	1.1 (0)	9.7 (0)	24.8 (0)	29.0 (0)	46.0 (0)	45.2 (1)	40.8 (0)	58.8 (1)	60.8 (1)	NA (2)
Group II	0.3 (0)	2.4 (0)	14.2 (0)	8.6 (2)	3.0 (1)	14.4 (0)	26.3 (0)	30.0 (1)	NA (1)	*
Group III	0.0 (0)	0.1 (0)	1.4 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Group IV	0.7 (0)	12.3 (0)	17.6 (0)	30.7 (0)	42.8 (1)	43.2 (0)	47.3 (1)	37.3 (1)	NA (2)	*
Group V	0.8 (0)	16.3 (0)	17.2 (0)	37.4 (0)	38.5 (0)	44.7 (1)	47.5 (1)	53.4 (1)	62.1 (1)	NA (1)
Group VI	0.7 (0)	14.0 (0)	16.8 (0)	38.5 (1)	38.2 (0)	25.0 (1)	NA (3)	*	*	*

Five mice of each group immunized with different antigens were monitored for parasitemia and survival after challenge infection with 1×10^4 *Plasmodium yoelii* YM-parasitized red blood cells. Group I: unimmunized; Group II: immunized with 3×10^7 FKPy-pRBC; Group III: with 1×10^4 live *P. yoelii* -infected RBCs and cured with 200 μg of pyrimethamine in saline/mouse after 3 days for three consecutive days, and this protocol repeated three times; Group IV: immunized with 10 μg of recombinant YIR1; Group V: immunized with 10 μg of recombinant YIR4; Group VI: immunized with a mixture of 10 μg recombinant YIR1 and 10 μg of recombinant YIR4. The adjuvants used for immunization in Groups II, IV, V, and VI were 50 μg of CpG-ODN 1826 (Ward Medic Ltd Part, Bangkok, Thailand) and Montanide ISA51 (Seppic Chem Co, Courbevoie, France).

FKPy-pRBC: formalin-killed *P. yoelii* YM-infected red blood cells; NA: parasitemia is not applicable because of all remaining mice found dead on the observed day; *: no mice left

dropped after the peak on day 11, but by day 17 all five mice had died. Taken altogether, complete protection against lethal *P. yoelii* YM challenge was only obtained by immunization with *P. yoelii* infection and drug cured (Group III).

Antibody response of immunized mice

IgG and IgG2a antibody responses of Groups I-VI immunized mice to *P. yoelii* YM crude soluble extract were determined by ELISA. Sera from mice immunized with rYIR1 (Group IV), rYIR4 (Group V) or a mixture of rYIR1 and rYIR4 (Group VI) showed similarly low levels of IgG and IgG2a antibodies, which increased 5 folds from day 0 to day 69 of blood collection (Fig 2). However, it was noted that IgG and IgG2a antibody responses of sera from Group VI

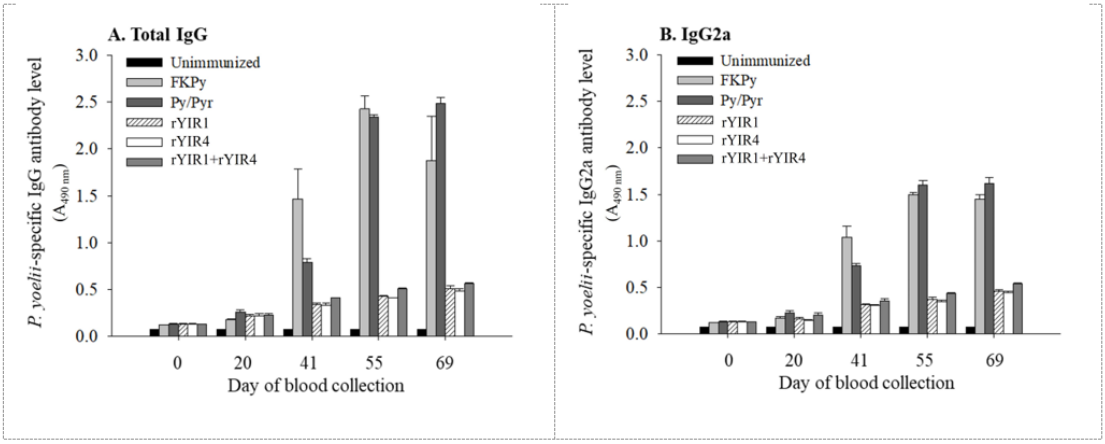

Fig 2 - Antibody response of immunized mice

Sera were collected on day indicated from mice (3 per group) immunized with FKPy (Group II), *Plasmodium yoelii* YM-infected RBCs and pyrimethamine cured (Group III), recombinant (r)YIR1 (Group IV), rYIR4 (Group V), and mixture of rYIR1 and rYIR4 (Group VI), in comparison with sera from unimmunized mice (Group I) as described in footnote of Table 2. Sera were assayed for total IgG (A) and IgG2a (B) antibodies by ELISA using *P. yoelii* YM crude soluble extract. Data are reported as mean ± SEM.

A490nm: Absorbance read at wavelength of 490 nanometers; FKPy: formalin-killed *P. yoelii* parasites; Py/Pyr: immunized by infection with *P. yoelii* YM-infected RBCs and cured with pyrimethamine, protocol repeated three times; SEM: standard error of mean

mice showed a bit higher than those from Groups IV and V mice. IgG and IgG2a levels in Groups II and III became prominent on day 41 and plateaued on day 55 at equal concentrations, although on day 41 levels of IgG and IgG2a in Group III are significantly lower than those of Group II mice. It is worth noting that IgG2a levels were ~60 % of IgG in both groups, and that IgG and IgG2a levels on day 69 in Groups II and III were 5- and 3-fold higher respectively than those of Groups IV, V and VI mice. As expected, IgG and IgG2a levels remained low and unchanged in unimmunized mice (Group 1) throughout the experimental period.

Cell-mediated immune response in immunized mice

Splenocytes harvested from the three unchallenged mice in all six Groups proliferated [reported as stimulation index (SI)] when incubated with FKPy, rYIR1, rYIR4, or ConA (positive control) (Fig 3). SIs of ConA-treated splenocytes were similar in Groups I, II, III, and VI, but were reduced ~50% in Groups IV and V. As expected, SIs of splenocytes from immunized mice incubated with cognate antigen were higher than that of ConA stimulation, namely, Groups IV (Fig 3D) and V (Fig 3E); however, this was not observed in Groups II (Fig 3B) and VI (Fig 3F). In all Groups, SI of splenocytes induced with FKPy was ~1, irrespective of the control ConA SI value. As for Group VI, mice were immunized with a mixture of rYIR1 and rYIR4, but their splenocytes were treated with rYIR1 and rYIR4 individually.

Interferon-γ (IFN-γ) (Fig 4) and interleukin-4 (IL-4) (Fig 5) concentrations in culture supernatants of splenocytes described above were measured using ELISA methods. As expected, the levels of IFN-γ and IL-4 were lowest in unstimulated and highest in ConA- stimulated samples from all six groups, although the actual concentrations varied among samples from the six groups, with the levels of IL-4 being significantly higher than those of IFN-γ. For both IFN-γ and IL-4, there were no obvious changes in concentrations between those from splenocytes treated with the same antigen used for immunization and those of splenocytes treated with an antigen different from that used for immunization. These findings indicated that cell-mediated immune response played a minimal role in protecting mice immunized with formalin-killed whole blood-stage *P. yoelii* (FKPy), or with rYIR1 or rYIR4.

DISCUSSION

Due to the complexity of the malaria parasite life cycle, a vaccine

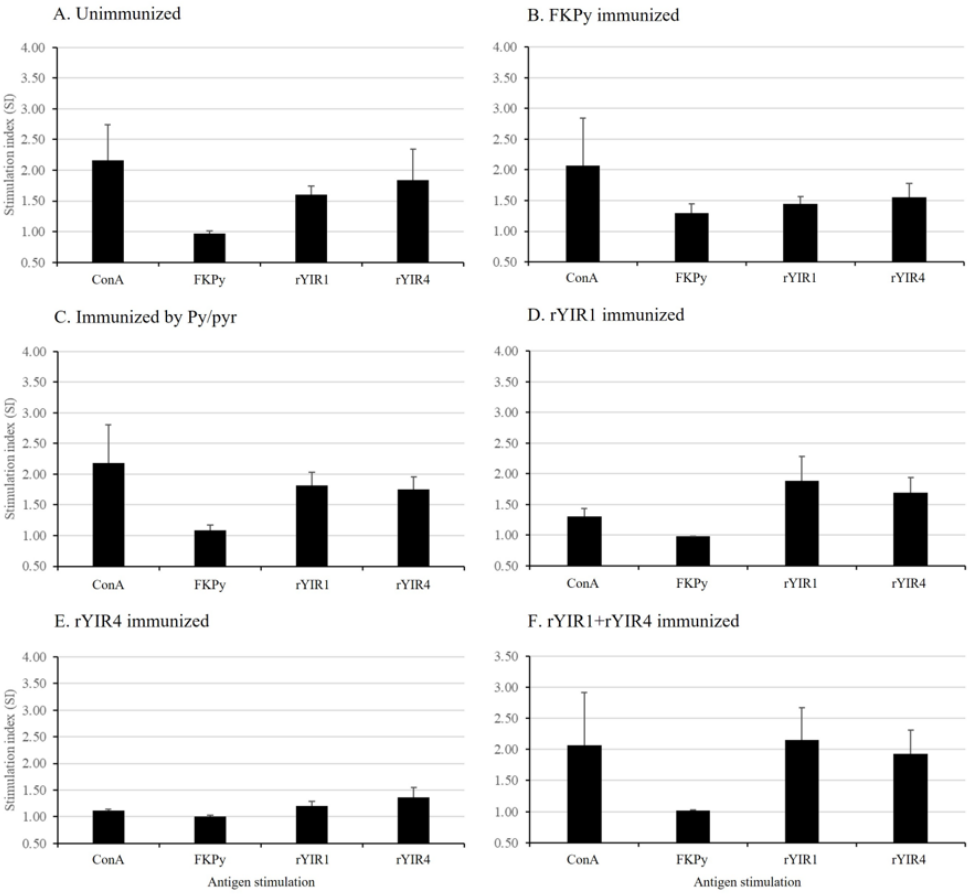

Fig 3 - Lymphoproliferation of spleen cells from immunized mice following antigen stimulation

Mice (3 per group) were (A) unimmunized (Group I); (B) immunized with FKPy (Group II); (C) immunized by infection with *Plasmodium yoelii* and cured with pyrimethamine (Py/pyr; Group III); (D) recombinant (r)YIR1 (Group IV); (E) immunized with rYIR4 (Group V); (F) immunized with a mixture of rYIR1 and rYIR4 (Group VI) as described in footnote to Table 2. Spleen from each mouse was collected on day 14 post-immunization, and splenocytes treated with the indicated antigen and assayed for lymphoproliferative response, reported as stimulation index (SI), using a cell proliferation reagent WST-1. ConA was used as positive control. Data are reported as mean ± SEM.

ConA: Concanavalin A; FKPy: formalin-killed *P. yoelii* YM-infected mouse red blood cells; SEM: standard error of mean; WST-1: water-soluble tetrazolium salt-1

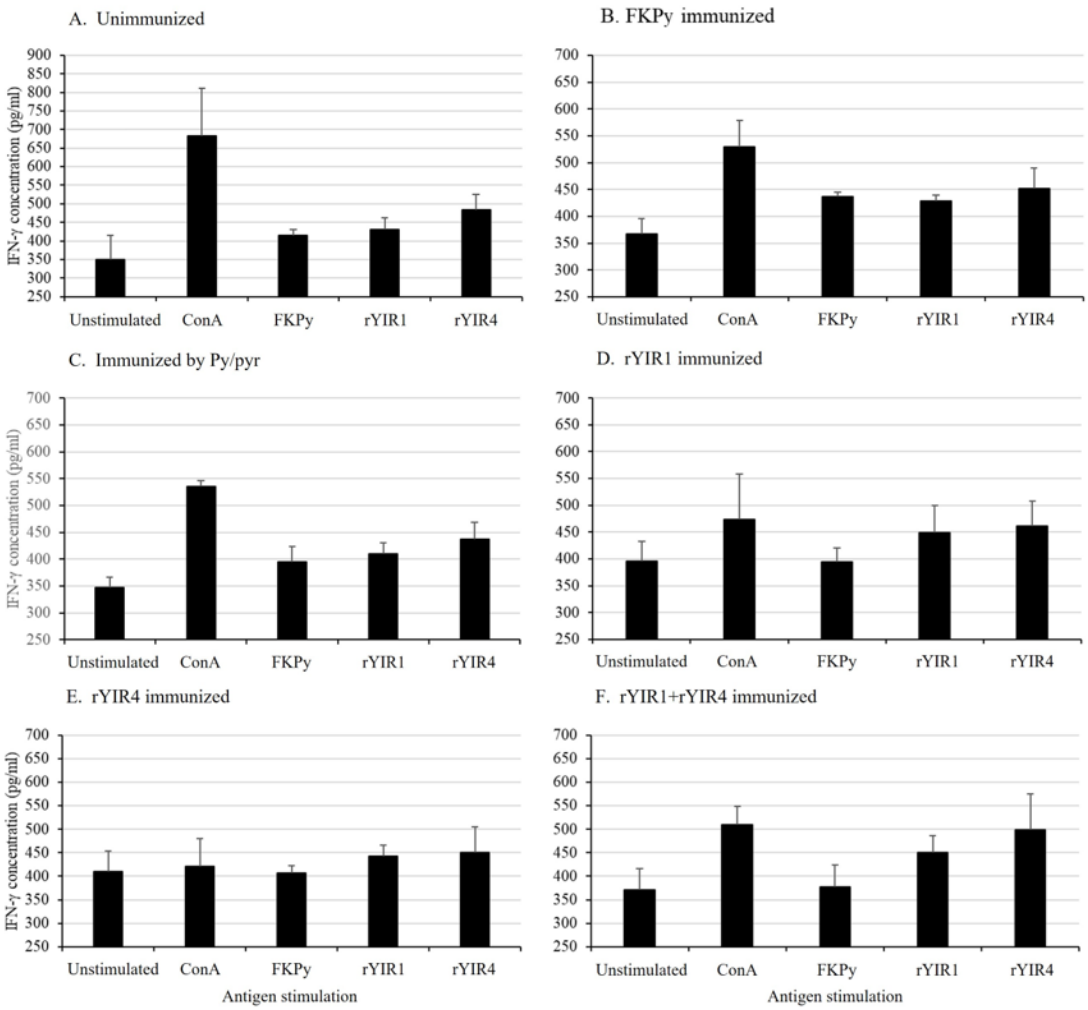

Fig 4 - Interferon-γ production of spleen cells from immunized mice after *in vitro* stimulation with antigen

Mice (3 per group) were treated as described in legend to Fig 3. Spleen from each mouse was collected on day 14 post-immunization and splenocytes treated with the indicated antigen. Supernatants were collected and assayed for IFN-γ by ELISA. The concentration of IFN-γ was determined using a standard curve. Data are reported as mean ± SEM.

ConA: Concanavalin A; ELISA: enzyme-linked immunosorbent assay; FKPy: formalin-killed *Plasmodium yoelii* YM-infected mouse red blood cells; IFN-γ: interferon-γ; SEM: standard error of mean

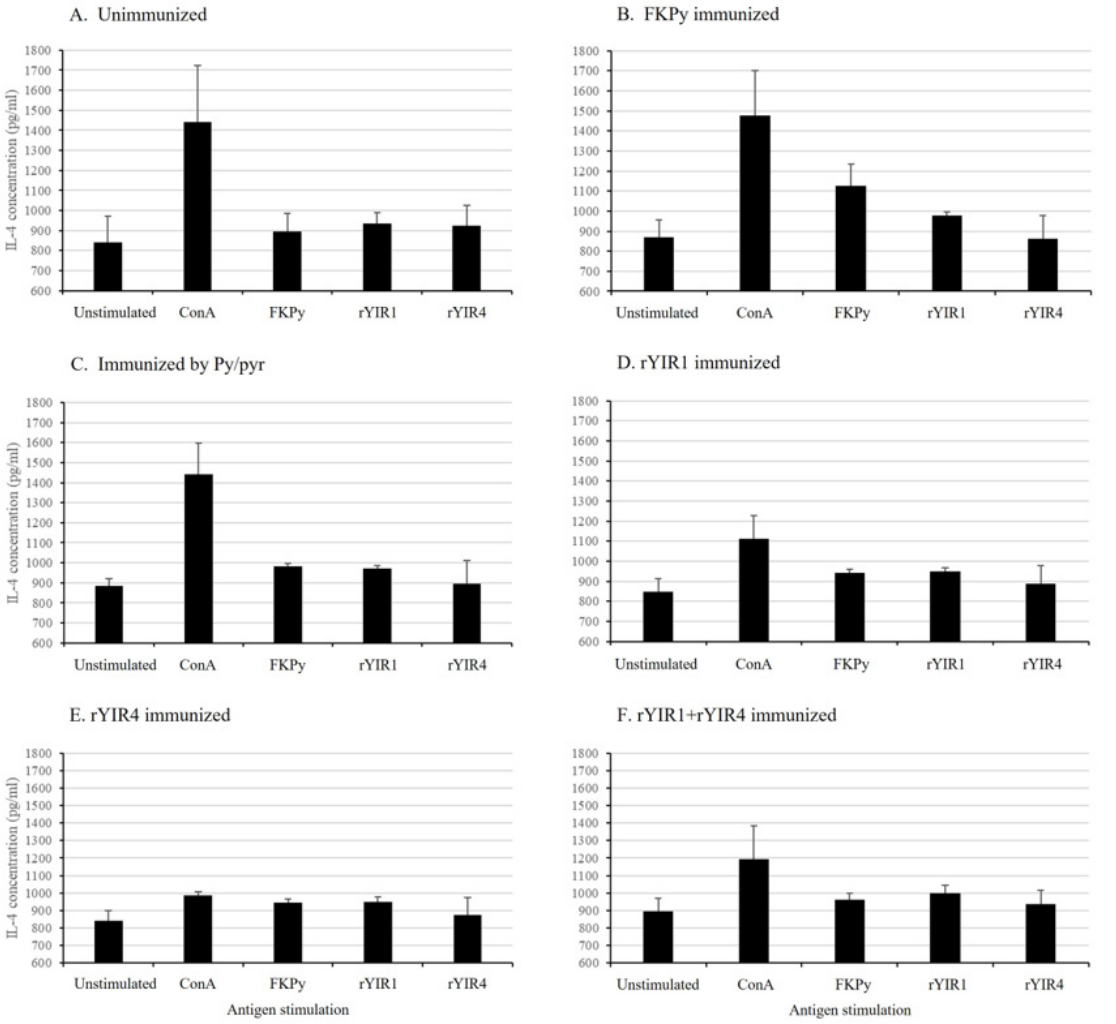

Fig 5 - Interleukin-4 production of spleen cells from immunized mice after *in vitro* stimulation with antigen

Mice (3 per group) were treated as described in legend to Fig 3. Spleen from each mouse was collected on day 14 post-immunization and splenocytes treated with the indicated antigen. Supernatants were collected and assayed for IL-4 by ELISA. The concentration of IL-4 was determined using a standard curve. Data are reported as mean ± SEM.

ConA: Concanavalin A; ELISA: enzyme-linked immunosorbent assay; FKPy: formalin-killed *Plasmodium yoelii* YM-infected mouse red blood cells; IL-4: interleukin-4; SEM: standard error of mean

developed from antigen(s) from only a particular growth stage of the parasite may not be sufficient to provide complete protection (Wipasa and Riley, 2007). The sporozoite subunit vaccine RTS,S, which consists of a part (RTS) of *P. falciparum* circumsporozoite protein fused with hepatitis B virus surface antigen (S), has been recently licensed (Mosquirix) and approved by the World Health Organization in 2021 for vaccination in children under the age of two residing in regions of moderate to high malaria transmission (Stanisic and Good, 2023). However, the RTS,S vaccine is far from satisfactory with lower efficacy in cohorts with high malaria parasite exposure (39.5%) compared with low exposure (57.5%) and wanes more rapidly in participants with high exposure. Thus, there is still room for improvements in malaria vaccine development, in particular incorporating an mRNA vaccine strategy (Tsoumani *et al*, 2023).

Mice immunized with killed blood-stage parasite lysate (by freeze-thawing of parasitized RBCs) formulated with CpG-ODN adjuvant in alum can generate vigorous T-cell response, especially Th1 type and that the immunized mice are protected against homologous and heterologous infections (Pinzon-Charry *et al*, 2010). Choolue (2011) has shown that immunization of mice with high doses of FKPy formulated with CpG ODN and Montanide adjuvants can protect against homologous challenge infection and induce IgG1, IgG2a and IgG2b antibodies response.

Choolue (2011) also identified a *P. yoelii* protein of 39.6 kDa recognized by IgG2a of FKPy-immune serum. Srimark (2016) subsequently demonstrated that the 39.6 kDa antigen is composed of two proteins of nearly identical size. Here, we have identified using LC-MS/MS the antigens as YIR1 and YIR4. YIRs constitute a group of surface antigens encoded by a *yir* gene family consisting of 838 genes (693 complete and 145 partial) (Carlton *et al*, 2002). YIRs are expressed during the erythrocytic, gametocyte and sporozoite stages, similarly to the expression patterns of *P. falciparum var* and *rif* (Florens *et al*, 2002).

In this study, we have heterologously expressed in *E. coli* BL21(DE3) cells soluble rYIR1 (22 kDa) and rYIR4 (31 kDa) containing only the extracellular sequences as heterologous expression of the whole proteins (36.2 and 35.3 kDa, respectively) (Table 1). Antigenicity of rYIR1 and rYIR4 was demonstrated

by their (weak) reactivities with FKPy-immune serum and HIS using western blotting (Fig 1). Immunization of female BALB/c mice with rYIR1 and rYIR4 (individually or combined) formulated with CpG ODN and Montanide adjuvants failed to protect against challenge with live virulent *P. yoelii* YM-infected RBCs. Interestingly, mice infected with live *P. yoelii* followed by antimalarial (pyrimethamine) cure (protocol repeated three times) also succumbed to challenge. Only mice immunized with FKPy-pRBC formulated with the same adjuvants afforded complete protection against challenge. Immunity was apparently antibody- and not cell-mediated (Figs 2-5), consistent with the previous report (Choolue, 2011). The lower parasitemia on days 13-15 post-challenge of mice immunized with a mixture of rYIR1 and rYIR4 compared to mice immunized with individual recombinant proteins may reflect the activation of Th1 type response, possibly together with TNF-α and nitric oxide synthase (NOS) secretion (Hirunpetcharat *et al*, 1999); however, the Group VI immunized mice had a significantly shorter period of protection than those of Groups IV and V) (p=0.032, and 0.008, respectively (Table 2).

The observation that immunization with a mixture of rYIR1 and rYIR4 produced higher antibodies response compared to immunization with individual recombinant proteins (p=0.013 and 0.002, respectively) (Fig 2) suggests a synergistic effect in the induction of antibody response.

On the other hand, human volunteers immunized with ultra-low doses of *P. falciparum* parasites and then drug cured can be protected from a subsequent homologous parasite challenge (Pombo *et al*, 2002). The immunized volunteers' immunity shows an absence of detectable antibodies against the parasite or infected red cells, but their PBMCs showed proliferative T-cell response, involving $CD4^+$ and $CD8^+$ T-cells, IFN-γ but not IL-4 or IL-10, and high concentrations of nitric oxide synthase activity. Similarly, immunization of mice with killed whole *P. yoelii* parasites using CpG ODN plus alum or Montanide ISA as adjuvant also supports the role of T-cell induction in protective immunity (Hirunpetcharat *et al*, 2003; Pinzon-Charry *et al*, 2010). The discrepancies between the results of the earlier mouse studies with those of the current study warrant further investigation.

In conclusion, our data show that recombinant YIR1 and YIR4 were weakly antigenic when tested against mouse anti-*P. yoelii* immune sera but were unable to provide sterile protection against a challenge by live virulent *P. yoelii* YM parasites in BALB/c mice immunized with adjuvant formulated individual recombinant protein or an equal mixture of the two proteins. As both YIR1 and YIR4 are present in formalin-killed intact *P. yoelii* parasites that provide sterile immunity to mice against challenge by live *P. yoelii* YM parasites, these two proteins may prove useful as components in the development of a protein-based malaria subunit vaccine.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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