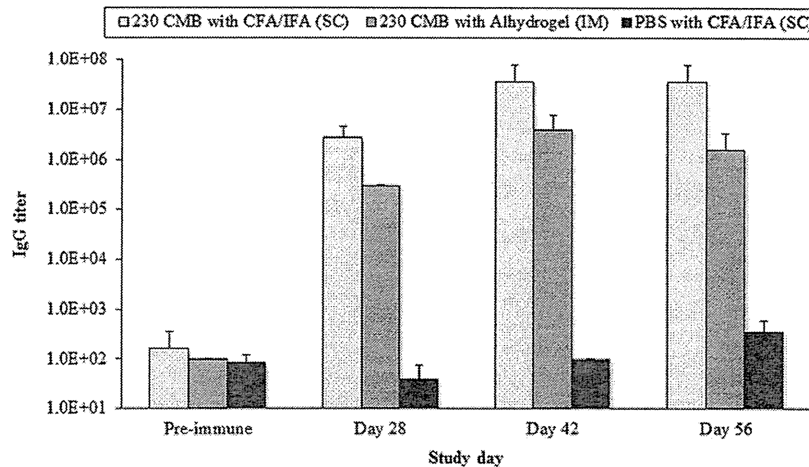

FIG. 3. IgG responses elicited by the 230CMB vaccine in rabbits using either complete Freund's adjuvant (CFA) followed by incomplete Freund's adjuvant (IFA) subcutaneously (SC) or Alhydrogel intramuscularly (IM). Serum 230CMB-specific IgG titers were measured by ELISA using samples collected before (Preimmune) antigen administration, and on days 28, 42, and 56 postvaccination. Reciprocal serum dilutions that gave a mean OD value greater than 4-fold over the background were determined as the endpoint titers. Data are shown as mean serum IgG endpoint titers ± standard deviations.

hibiting a 55% reduction, while the group receiving antigen adsorbed on Alhydrogel showed an 80% reduction. These findings were confirmed by the data from an independent laboratory using the same rabbit sera (see Table S1 in the supplemental material). The experimental sera were compared to known TB MAb 63F2A2 (IgG2a isotype) (37) and were shown to perform as well as the control, which achieved 99% TB in the presence of complement (Table 1).

DISCUSSION

TBVs represent a unique opportunity to limit the spread of the malaria parasite and are viewed as critical for the successful eradication of the parasite. Multiple targets present on the sexual stages of *Plasmodium* parasites show initial promise in producing antibodies capable of disrupting development of the parasite in the mosquito. Pfs230, a large and complex protein found on the surface of the gametocytes, which has an important function during fertilization (1, 9), is among these targets. Recombinant expression of full-length Pfs230 for TBV development has not been successful due to the size and complexity of the protein. Additionally, production of subdomains of Pfs230 in early studies using both *E. coli* (48) and *Saccharomyces cerevisiae* (44) has not been successful in forming a correctly folded molecule capable of consistently eliciting a high level of TB antibodies. However, expression of recombinant Pfs230C with TB activity has been achieved by Tachibana et al. (40a), who demonstrated TB activity of Pfs230C produced in the wheat germ cell-free expression system. The wheat germ cell-free expression system, however, presents a challenge for large-scale manufacturing.

In this study, we have successfully used a plant-based transient-expression system (28) to produce a number of target proteins with desired biological activity and with levels of tar-

TABLE 1. Evaluation of sera from rabbits immunized with 230CMB in the standard membrane feeding assay

Vaccine candidate, adjuvant, and sample	With complement				Without complement			
	No. inf./diss.[d]	Median (IQR)[e]	% Reduction[b]	P value[b]	No. inf./diss.	Median (IQR)	% Reduction[b]	P value[b]
230CMB, Freund's[a]								
Pre	20/20	60.5 (31.5–80.0)			19/20	52.0 (22.0–59.0)		
Final	5/50	0.0 (0.0–0.5)	99	<0.001	19/20	16.5 (6.5–31.5)	55	>0.05
230CMB, Alhydrogel								
Pre	20/20	67.5 (39.0–75.5)			19/20	49.0 (16.0–62.5)		
Final	3/20	0.0 (0.0–0.0)	100	<0.001	16/20	8.0 (3.5–13.5)	80	<0.05
NA,[f] NA, 63F2A2[c]	7/20	0.0 (0.0–1.0)	99	<0.001	19/19	44.0 (33.0–64.0)	0	>0.05

[a] The immunization strategy consisted of complete Freund's adjuvant (prime) followed by incomplete Freund's adjuvant (boost).
[b] Percent reductions in oocyst counts and *P* values were obtained by comparing the day 42 immune pooled rabbit sera (Final) with the preimmune pooled rabbit sera (Pre).
[c] 63F2A2 (IgG2a isotype) is a MAb (37) that was used as a positive control.
[d] No. inf./diss., numbers of infected/dissected mosquitoes.
[e] IQR, interquartile range.
[f] NA, not applicable.

get expression ranging from 100 mg to over 1 g/kg of fresh whole leaf tissue (7, 19, 23, 26, 40). Due to the complexity of the Pfs230 protein, we endeavored to use our transient-expression system in *N. benthamiana* to express the portion of Pfs230C (230CMB) that was shown to have TB activity using an *in vitro* translation system (40a). In this study, we expressed, purified, and characterized 230CMB (aa 444 to 730). The 230CMB antigen described here is similar to r230/MBP.C5 (aa 443 to 791) produced by Bustamante et al. (2), which comprises the region of Pfs230 positioned just after the N-terminal cleaved domain until midway through the first cysteine motif domain and contains four cysteine residues. However, unlike the MBP fusion, 230CMB is a stand-alone soluble antigen which is recognized in both Western blot assays and ELISA by two different anti-Pfs230 MAbs possessing TB activity. SEC-MALLS data indicated that the purified protein was in a monomeric state and had a molecular mass of 48 kDa. When administered to rabbits in the presence of adjuvant, plant-produced 230CMB elicited high titers of anti-Pfs230 antibodies. Differences between the antibody titers elicited by the two adjuvants tested were detected, with the complete/incomplete Freund's adjuvant combination eliciting higher antibody titers than Alhydrogel. This enhanced immunogenicity is likely to be a result of the presence of inactivated mycobacteria in the complete Freund's adjuvant used for the primary immunization. However, antibodies generated using either adjuvant were shown to bind specifically to the native protein of *P. falciparum* on the surfaces of gametes/zygotes, resulting in significant (>99%) TB activity. This, in turn, indicates that plant-produced 230CMB is correctly folded and antibodies generated against it are able to fix complement, resulting in destruction of the parasite in the mosquito.

The capacity of our subdomain, 230CMB, to produce effective TB antibodies indicates that targeting of this complex protein domain to the ER of a plant cell is sufficient to confer proper folding and results in an antigen that reflects the native conformation. Additional process development and formulation studies are under way to improve target yields and establish methodologies for cGMP production, as well as examine the potential for enhancement of immunogenicity. The demonstrated success of this potential TBV consisting of a target protein from the gametocyte stage of the parasite also lends itself to the investigation of a combination vaccine consisting of an additional target from a later sexual stage of the parasite, such as Pfs25, which is present on gametes/zygotes, retorts, and ookinetes. Combining Pfs230 and Pfs25 targets in a single vaccine may lead to a highly effective TBV. Also, combining Pfs230 and Pfs48/45 (involved in the fertilization process) can lead to a more effective TBV.

The efficacy of plant-produced 230CMB in eliciting TB antibodies not only shows the potential of this vaccination approach as an essential part of a malaria eradication program but also further supports the potential of expressing complex recombinant proteins in plants. The transient-expression technology used here is both time efficient and easily scalable (6, 39).

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METHODOLOGY Open Access

Plasmodium vivax: comparison of immunogenicity among proteins expressed in the cell-free systems of *Escherichia coli* and wheat germ by suspension array assays

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Abstract

Background: In vitro cell-free systems for protein expression with extracts from prokaryotic (*Escherichia coli*) or eukaryotic (wheat germ) cells coupled to solid matrices have offered a valid approach for antigen discovery in malaria research. However, no comparative analysis of both systems is presently available nor the usage of suspension array technologies, which offer nearly solution phase kinetics.

Methods: Five *Plasmodium vivax* antigens representing leading vaccine candidates were expressed in the *E. coli* and wheat germ cell-free systems at a 50 μl scale. Products were affinity purified in a single-step and coupled to luminex beads to measure antibody reactivity of human immune sera.

Results: Both systems readily produced detectable proteins; proteins produced in wheat germ, however, were mostly soluble and intact as opposed to proteins produced in *E. coli*, which remained mostly insoluble and highly degraded. Noticeably, wheat germ proteins were recognized in significantly higher numbers by sera of *P. vivax* patients than identical proteins produced in *E. coli*.

Conclusions: The wheat germ cell-free system offers the possibility of expressing soluble *P. vivax* proteins in a small-scale for antigen discovery and immuno-epidemiological studies using suspension array technology.

Background

The recent call for malaria eradication has re-emphasized the importance of bringing *Plasmodium vivax* into the research agenda [1]. *Plasmodium vivax* remains the most widely distributed human malaria parasite with 2.85 billion people living at risk of infection [2]. Noticeably, the number of yearly clinical cases seems to be increasing from 70-80 million [3] to 300 million cases [4] and these include cases of severe disease and death exclusively associated with *P. vivax* [5,6]. Moreover, experts agree that present tools against *Plasmodium falciparum* will not be effective against *P. vivax*, reinforcing the development of control measurements for this species [7]. Among these tools, vaccines continue to represent the most cost-effective control measurement but unfortunately vaccine development in *P. vivax* lags well behind that of *P. falciparum* [8].

The genomes of human malaria parasites encode approximately 5,400 coding genes opening an avenue for antigen discovery in this species [9]. Unfortunately, cell-based expression systems have met limited success to obtain soluble proteins largely attributed to the high AT-content, the existence of long stretches of repeated amino acid sequences and much larger proteins than their homologues in other eukaryotes [10]. In contrast to cell-based systems, cell-free expression systems for protein synthesis with extracts from prokaryotic or eukaryotic cells has offered a valid alternative to express soluble proteins [11]. In the case of malaria, using the *Escherichia coli* cell-free system, Doolan and co-workers

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first reported on the expression of 250 *P. falciparum* proteins subsequently coupled to solid arrays and analysed with immune sera discovering putative new antigens [12]. Using this same approach, expression of 1,204 *P. falciparum* proteins later expanded these analysis and predicted new antigens [13]. Parallel efforts were reported on the use of cell-free extracts from wheat germ to similarly produce hundreds of *P. falciparum* proteins [14,15]. More recently, the wheat germ expression system has been used for antigen discovery in *P. vivax* [16]. Thus, 89 different soluble proteins were expressed and shown to be immunogenic on analyses of protein arrays and immune sera. Together, this data demonstrates that cell-free expression systems coupled to protein arrays offer a scalable platform for antigen discovery in malaria.

Suspension array technologies with high-throughput capacity to simultaneously analyse several proteins with minimal amount of immune sera have also been developed and used in analysis of multiple malaria vaccine candidates as well as in developing functional assays [17-20]. Suspension arrays offer several advantages as compared to flat protein arrays including nearly solution phase kinetics and total assay sensitivity [21]. The aim of this study was to develop a small-scale method for soluble expression of *P. vivax* proteins using the *E. coli* and wheat germ cell-free systems and to compare their usage by multiplexing assays.

Methods

Human samples

Human plasma samples were obtained from endemic areas of Brazil and from a non-endemic region. The first group comprised immune sera from adults living in the Brazilian Amazon [22]. The other group comprised sera from four healthy adult volunteers living in the city of Barcelona (Spain) that have never been exposed to malaria or visited malaria endemic regions. These studies received the ethical approval of Local Institutional Reviewing Boards.

Construction of plasmids

Plasmid pIVEX1.4d for expression in wheat germ and pIVEX2.4d for expression in *E. coli* were purchased from Roche and modified by inserting GST after the 6xHis tag sequence. Modified plasmids were termed pIVEXGST1.4d and pIVEXGST2.4d (Figure 1). Both vectors carry the same T7-DNA promoter elements, the ampicillin selectable marker and identical His-GST tags in the same positions. The following proteins were engineered into these vectors: PvMSP1-19 (1590-1699 aa, id PVX_099980) and PvMSP1-Nter (170-675 aa, id PVX_099980); PvDBP-RII (196-521 aa, id PVX_110810); PvCSP-S (51-319 aa, id PVX_119355); PvMSP5 (full length, id PVX_003770);

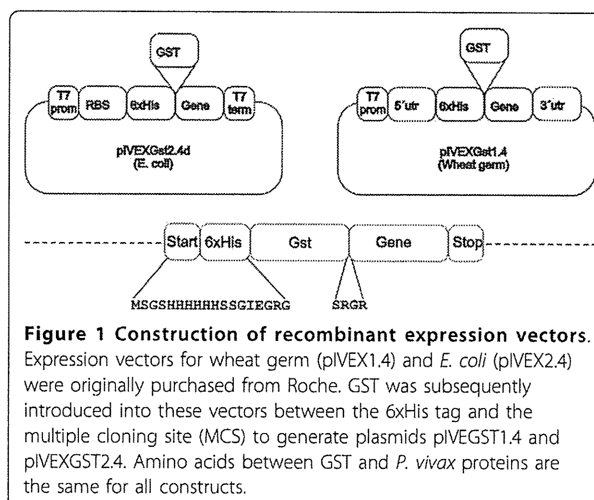

Figure 1 Construction of recombinant expression vectors. Expression vectors for wheat germ (pIVEX1.4) and *E. coli* (pIVEX2.4) were originally purchased from Roche. GST was subsequently introduced into these vectors between the 6xHis tag and the multiple cloning site (MCS) to generate plasmids pIVEGST1.4 and pIVEXGST2.4. Amino acids between GST and *P. vivax* proteins are the same for all constructs.

PvMSP7 (full length, id PVX_082695 (Figure 2). Further information on these proteins and primers used for amplifications can be obtained as supplementary information (Additional file 1).

The circumsporozoite antigen of *P. vivax* is dimorphic based on the central repeat region and the two alleles, VK210- and VK247-type, share no immunological cross-reactivity [23]. Therefore, a recombinant chimeric PvCSP protein containing VK210-(PVX_119355) and VK247-type (GenBank#M69059, *P. vivax* PNG strain) amino acid repeat sequences (PvCSP-c) which may cover the vivax parasite population globally was developed (Figure 3). The PvCSP-c was constructed and expressed in a large-scale wheat germ cell-free system

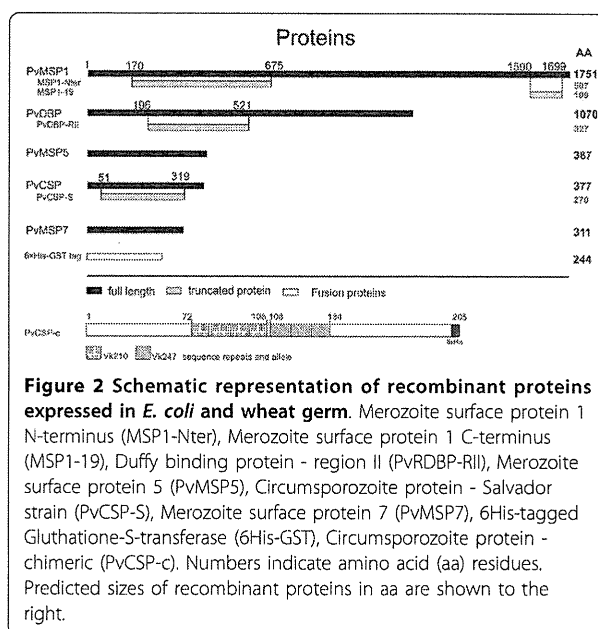

Figure 2 Schematic representation of recombinant proteins expressed in *E. coli* and wheat germ. Merozoite surface protein 1 N-terminus (MSP1-Nter), Merozoite surface protein 1 C-terminus (MSP1-19), Duffy binding protein - region II (PvRDBP-RII), Merozoite surface protein 5 (PvMSP5), Circumsporozoite protein - Salvador strain (PvCSP-S), Merozoite surface protein 7 (PvMSP7), 6His-tagged Gluthatione-S-transferase (6His-GST), Circumsporozoite protein - chimeric (PvCSP-c). Numbers indicate amino acid (aa) residues. Predicted sizes of recombinant proteins in aa are shown to the right.

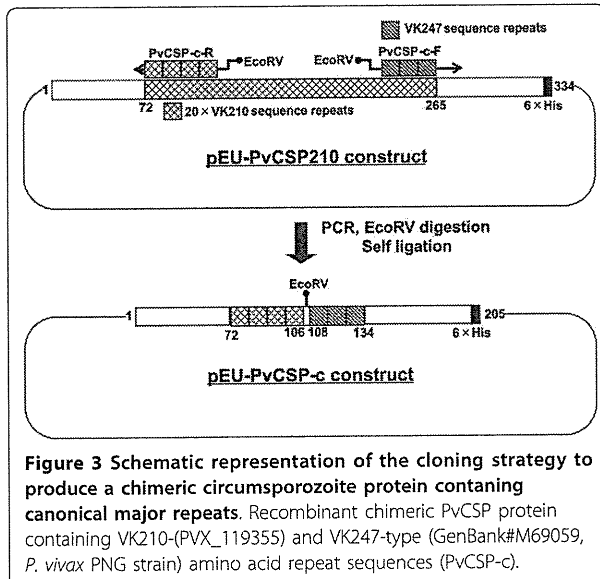

Figure 3 Schematic representation of the cloning strategy to produce a chimeric circumsporozoite protein contaning canonical major repeats. Recombinant chimeric PvCSP protein containing VK210-(PVX_119355) and VK247-type (GenBank#M69059, *P. vivax* PNG strain) amino acid repeat sequences (PvCSP-c).

(CellFree Sciences, Matsuyama, Japan). Briefly, the nucleotide sequences of PvCSP (SalI strain, VK210 type: PVX_119355) excluding the signal peptide and the GPI anchor signal, with addition of penta-His-tag sequence at the C-terminus, was amplified from SalI gDNA by PCR using VK210-F and VK210-R primers, and was cloned at the EcoRV site into the pEU-E01-MCS plasmid (CellFree Sciences) in the presence of both EcoRV restriction enzyme and T4 DNA ligase generating the pEU-PvCSP210 construct without original EcoRV site. The pEU-PvCSP210 was then inversely amplified by PCR using antisense-primer encoding the four times of the VK210-repeat amino acid sequence "GQPAGDRAD" at the 5' end with EcoRV site (PvCSP-c-R) (Additional file 1) and sense-primer encoding the three times of the VK247-repeat amino acid sequence "GANGAGNQP" at the 5' end with EcoRV site (PvCSP-c-F) (Additional file 1). Then the PCR product was digested with EcoRV, and self ligated after the gel-purification of the restricted DNA fragment. Finally, the presence of tetra-VK210-type sequence was confirmed followed by tri-VK247-type repeat amino acid sequences after the nucleotide sequencing of the final pEU-PvCSP-c plasmid (Figure 3). Deduced amino acid sequences, Gly_2 to Asp_{106} and Asn_{134} to Cys_{199} in PvCSP-c was identical to Gly_{23} to Asp_{127} and Asn_{286} to Cys_{347} based on the *SalI* sequence, PVX_ 119355, and Gly_{108} to Pro_{134} in PvCSP-c was identical to Gly_{248} to Pro_{274} based on the deduced amino acid sequence from *P. vivax* PNG strain, M69059.

In vitro protein synthesis

In vitro protein synthesis followed the original manufacturers' instructions (Roche) and was done on a 50 µl scale, excepting for PvCSP-c (see below). Expressed proteins were purified on GST SpinTrap purification columns (GE Healthcare). Briefly, soluble fractions from cell-free system extracts were applied to a Glutathione Sepharose® 4B column that had been equilibrated with PBS. The column was washed with PBS and the bound GST-HBx fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Eluted proteins were extensively dialyzed in PBS to remove glutathione. Proteins were analysed by SDS-page and Western blot and quantified as described else where [24]

Larger scale wheat germ cell-free protein synthesis

The recombinant PvCSP-c protein was synthesized with the wheat germ cell-free protein expression system using the bilayer translation reaction method on a 30 ml scale as manufacturer's recommendation (CellFree Sciences) [14]. The PvCSP-c protein was affinity purified by Ni-affinity chromatography as described previously [25]. Briefly, add imidazole (pH 8.0) in the translation reaction mixture (final concentration, 20 mM) and then add Ni-NTA beads (QIAGEN, Valencia, CA). Incubate the tube for 16 h on a continuous rotator, at 4°C, for the binding of proteins on to the beads. Transfer the solution with the beads into a Poly-Prep chromatography column (Bio-Rad, Hercules, CA). Wash the beads by five bed-volumes of PBS containing 30 mM imidazole three times and then elute the recombinant protein with one bed-volume of PBS containing 500 mM imidazole.

Covalent coupling of recombinant proteins to beads

BioPlex carboxylated beads (Bio-Rad) were covalently coated with the different recombinant proteins following the manufacturer's instructions (BioPlex Amine Coupling Kit). Briefly, activated beads (1.25×10^6 beads) were resuspended in 100 µl of PBS and 1 µg of each recombinant protein used per coupling reaction. Incubation under rotation was done at 4°C overnight and coupled beads were washed with 500 µl of PBS pH 7.4. After re-suspending coupling beads in 250 µl of blocking buffer and further incubation under rotation at room temperature for 30 min, beads were washed with 500 µl of storage buffer and centrifuged for six minutes at 14,000 × g. Pellets were resuspended into 125 µl of the same buffer and stored at 4°C protected from light until use.

Analysis of coupled beads on the BioPlex system

Coupled beads were analysed in the Bioplex system as previously described [20] with slight modifications. Briefly, circa 3,000 coated beads were used for each assay. Frozen plasma samples were thawed at room temperature, diluted 1:50 in assay buffer and 50 µl aliquots

added to the beads (final plasma dilution 1:100). Aliquots of 50 μl of Biotinylated human IgG antibody (Sigma) diluted 1:10,000 and of phycoerythrin conjugated streptavidin diluted to 1 μg/ml were used in subsequent incubations. Beads were re-suspended in 125 μl of assay buffer (BioRad) and analysed on the BioPlex100 system and results were expressed as median fluorescent intensity (MFI).

Statistical analysis

T-test and chi-square or fisher exact test were used to compare mean levels for prevalence, respectively, between groups. Averages were expressed as geometric mean (GM) plus 95% confidence intervals (CI). To evaluate the statistical measure of agreement between two independent proteins the index Kappa was calculated.

Results

Cloning and expression of *Plasmodium vivax* proteins

Expression of genes encoding five *P. vivax* proteins: PvMsp1-19, PvMsp1-Nter, PvMsp5, PvMsp7, PvDBP-RII, PvCsp-S and GST as control was initially attempted in *E. coli* and wheat germ cell-free expression systems using commercially available vectors (Roche). Yields, however, were very low and highly degraded as detected by Western blot analysis. It was thus decided to incorporate a GST tag into these vectors (Figure 1) as GST increased the solubility and yields of different recombinant proteins [26]. Noticeably, when cloned into these vectors, both expression systems produced readily detectable proteins by Western blot analysis under reducing condition (Figure 4). Proteins expressed by the cell-free *E. coli* system, however, were mostly degraded and showed low amounts of intact proteins with predicted sizes (Figure 4A). In contrast, soluble proteins expressed in wheat germ cell-free system were of predicted sizes and had much less degradation products (Figure 4B). All proteins produced by wheat germ system were affinity-purified to 60-85% and yielded 1-10ug/50ul (Additional file 2). Soluble purified proteins were coupled to individual bioplex beads and coupling efficiency was verified prior to multiplexing using an anti-GST or anti-his antibody (Additional file 3).

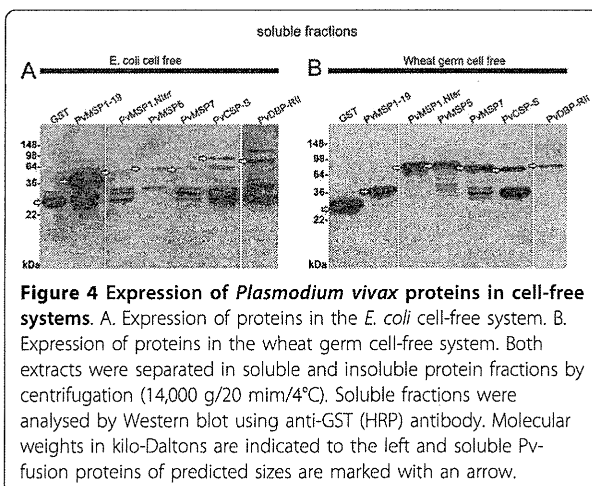

Figure 4 Expression of *Plasmodium vivax* proteins in cell-free systems. A. Expression of proteins in the *E. coli* cell-free system. B. Expression of proteins in the wheat germ cell-free system. Both extracts were separated in soluble and insoluble protein fractions by centrifugation (14,000 g/20 mim/4°C). Soluble fractions were analysed by Western blot using anti-GST (HRP) antibody. Molecular weights in kilo-Daltons are indicated to the left and soluble Pv-fusion proteins of predicted sizes are marked with an arrow.

Proteins produced by wheat germ system are recognized by significantly higher number of immune sera than those produced by *E. coli*

Only three soluble proteins produced in the 50 μl scale in *E. coli* could be purified in a single-step and coupled to Bioplex beads using exactly the same methodology as those produced and purified by wheat germ system. A comparison of naturally acquired humoral IgG responses against these proteins was thus made using immune sera of 40 malaria patients from Brazil known to have large reactivity against PvMSP1 [22]. GST values were subtracted from MFI values obtained against individual recombinant proteins and the cut-off defined as the mean value of control sera +3 standard deviations. Noticeably, proteins produced in the wheat germ system were recognized in significantly higher numbers than those produced in the *E. coli* system (MSP1-19 wheat germ 37/40 (92.5%) vs MSP1-19 *E. coli* 19/40 (47.5%), p = 0.000; MSP1-N wheat germ 26/40 (65%) vs MSP1-N *E. coli* 8/40 (20%), p = 0.000; MSP5 wheat germ 34/40 (85%) vs MSP5 *E. coli* 23/40 (57.5%) (p = 0.001) (Figure 5). Moreover, values of geometric means of all proteins produced in wheat germ system were significantly higher than those produced in *E. coli* system and 95% confidence intervals reinforced such differences. This data demonstrates that identical soluble proteins expressed in wheat germ system and coupled to bioplex beads are better recognized by the same immune sera than those expressed by *E. coli* system.

Multiplex assays with proteins produced in wheat germ system as an alternative platform for antigen discovery

To illustrate the use of soluble proteins produced by wheat germ system in a 50 μl scale and multiplexing assays for immuno-epidemiological studies, the responses of other proteins also considered important targets for *P. vivax* vaccine development were determined. These include (besides MSP1-19, MSP1-N, and MSP5), MSP7 [27], PvDBP-RII [28], and CSP [29]. Moreover, a chimeric CSP protein produced in large-scale in wheat germ and containing the two major allele repeats of PvCSP was also included (Figure 3 and Additional file 1). Of note, for this analysis a different group of 40 sera pertaining to other individuals with no particular strong reactivity against PvMSP1 was used [22].

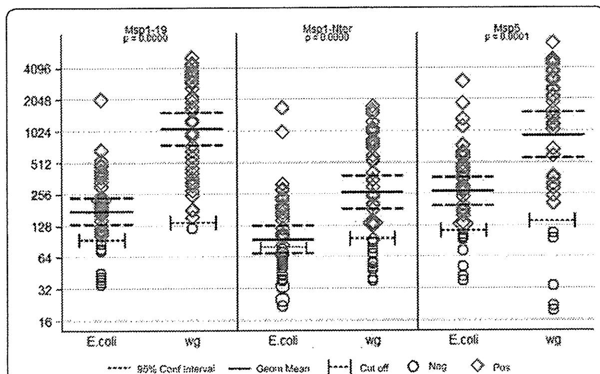

Figure 5 Comparative analysis of immune responses to *P. vivax* proteins expressed in the *E. coli* and wheat germ cell-free systems by Bioplex. One μg of each affinity-purified protein was individually coupled to beads and analysed by multiplex assays using immune sera (1:100 dilution) pertaining to 40 different *P. vivax* patients. Fluorescence was determined as the mean fluorescence intensity (MFI). GST values were subtracted from MFI values obtained against individual recombinant proteins and the cut-off defined as the mean value of control sera + 3 standard deviations. Circles represent samples which MFI values were below the cut-off and were considered negative whereas squares represent samples which MFI values were above the cut-off and were considered positive. Geometric means and 95% confidence intervals are shown.

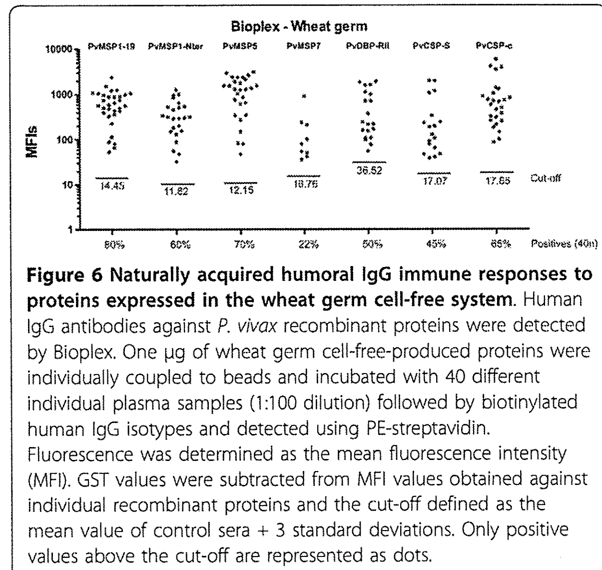

Figure 6 Naturally acquired humoral IgG immune responses to proteins expressed in the wheat germ cell-free system. Human IgG antibodies against *P. vivax* recombinant proteins were detected by Bioplex. One μg of wheat germ cell-free-produced proteins were individually coupled to beads and incubated with 40 different individual plasma samples (1:100 dilution) followed by biotinylated human IgG isotypes and detected using PE-streptavidin. Fluorescence was determined as the mean fluorescence intensity (MFI). GST values were subtracted from MFI values obtained against individual recombinant proteins and the cut-off defined as the mean value of control sera + 3 standard deviations. Only positive values above the cut-off are represented as dots.

All proteins were first analysed individually using 1 μl of serum diluted 1:100 and then simultaneously using the same quantity and the same dilution. At this dilution, the same sera reacting against PvCSP-S reacted against PvCSP-c even though a subtraction effect was detected in singleplex vs multiplex (Additional file 4). Thus, dilutions of sera in these assays must be taken into consideration to avoid missing immune responders to different alleles of the same protein. The data corroborated the immunogenicity of all these proteins albeit, as expected, to different levels (MSP1-19 80%, MSP1-Nter 60%, MSP5 70%, MSP7 22.5%, PvDBP-RII 50%, PvCSP-S 45% and PvCSP-c 65%) (Figure 6). Moreover, a cross-comparison between responses to the different proteins revealed, for instance, that sera that reacted against PvMSP1-19 also reacted against PvMSP1-N (58.06%), PvMSP5 (64.52%), PvMSP7 (9.68%), PvDBP-RII (45.16%), PvCSP-S (45.16%) and PvCSC-c (74%) (Figure 7). Values for all other cross-comparisons showed similar results with varying percentages of recognition by immune sera against any one particular protein and comparisons with the others (Additional file 5). Of note, there were a significant larger percentage of immune sera reacting against the chimeric CSP (PvCSP-c) as opposed to the one expressing only one allele (PvCSP-S). Moreover, cross-comparison of responses against PvCSP-S and PvCSP-c demonstrated that 92.86% of sera reacted against these two proteins.

Discussion

Protein arrays containing hundreds to thousands of malarial proteins have been recently reported for antigen discovery [13,15,16]. In these experiments, in vitro transcribed/translated products are directly spotted into solid matrices for analysis and reactivity against human sera. The goal here was developing an alternative simple small-scale method for soluble expression and single-step affinity purification of proteins to be analysed by suspension array technology. To this end, vectors expressing GST fused to the protein of interest were constructed to facilitate soluble expression of *P. vivax* proteins in a 50 μl scale in the cell-free systems of *E. coli* and wheat germ. Soluble proteins were affinity-purified in a single-step, coupled to luminex beads and analysed against immune sera from *P. vivax* patients.

Variable		PvMsp1-19		
		neg	pos	Total
PvMsp1-Nter	neg	6 (66.67%)	13 (41.94%)	19 (47.50%)
	pos	3 (33.33%)	18 (58.06%)	21 (52.50%)
PvMsp5	neg	1 (11.11%)	11 (35.48%)	12 (30.00%)
	pos	8 (88.89%)	20 (64.52%)	28 (70.00%)
PvMsp7	neg	8 (88.89%)	28 (90.32%)	36 (90.00%)
	pos	1 (11.11%)	3 (9.68%)	4 (10.00%)
PvDBP-RII	neg	7 (77.78%)	17 (54.84%)	24 (60.00%)
	pos	2 (22.22%)	14 (45.16%)	16 (40.00%)
PvCSP-S	neg	9 (100.00%)	17 (54.84%)	26 (65.00%)
	pos	0 (0.00%)	14 (45.16%)	14 (35.00%)
PvCSP-c	neg	6 (66.67%)	8 (25.81%)	14 (35.00%)
	pos	3 (33.33%)	23 (74.19%)	26 (65.00%

Figure 7 Cross comparisons of immune responses to PvMSP1-19. Immune responses to different proteins in the same serum using a 2 × 2 Table on the response distribution over proteins pairs.

Significantly higher number of immune sera reacted against proteins expressed in wheat germ system and multiplexing of five leading vaccine candidates illustrated the use of this method for immuno-epidemiological studies in *P. vivax*.

A major bottle-neck in antigen discovery for vaccine development in malaria is the little success achieved in producing soluble proteins in different cell-based or viral systems. Thus, cell-based *E. coli* and baculo-virus systems have reported expression of soluble malaria proteins anywhere from 6.3-30% [10,30]. In these reports, modifications involving codon optimization, construction of synthetic genes, extensive manipulations of culture conditions, different temperatures, and large culture volumes were needed to achieve solubilisation of proteins [10]. While these methods and expression systems remain highly valuable tools for structural and functional studies, they are difficult to implement on large-scale analysis of malarial proteins for antigen discovery. Noticeably, the development of cell-free expression systems offered a valid and efficient alternative to this objective. In fact, using malarial proteins expressed in cell-free extracts of either *E. coli* or wheat germ and analysed on flat solid arrays with immune sera, recent reports have paved the way for genome-wide antigen discovery of the two major human malaria parasites [13,16]. In these systems, proteins are directly spotted on linear flat surfaces with no formal demonstration of solubility or purity of expressed products. As the goal of these studies is the screening of thousand of antigens in combination with powerful statistical analyses, the presence of false-positives have been considered negligible. Increasing evidence, however, indicates that proteins expressed in wheat germ cell-free system are more suitable for these analyses as they are mostly soluble and retained enzymatic activity [15,31]. Moreover, suspension arrays offer major advantages when compared to protein arrays including nearly solution phase kinetics and total assay sensitivity [21].

The methodology reported here largely facilitates the production of soluble proteins in a small-scale compatible with automation and in quantities allowing analysis of hundreds of sera (roughly 1 μg of soluble/affinity-puried protein can be used to screen approximately 250 sera) using suspension arrays. To illustrate this, we expressed five leading vaccine candidates against two different life stages, the pre-erythrocytic stages (CSP) and asexual blood stages (MSPs and DBP). CSP is considered a leading vaccine candidate in *P. falciparum* [32] and the homologous protein has entered clinical trials in *P. vivax* [8]. PvCSP contains two major allele forms, PvCSP-VK210 [29] and PvCSP-VK247 [23]. We expressed PvCSP-VK210 in the 50 μl scale and also tested a chimerical protein composed of both major alleles (PvCSP-c) produced in large-scale. Both proteins were readily recognized by immune sera even though significantly larger number of sera reacted against the PvCSP-c protein representing these two major alleles. The fact that lower number of sera reacted against PvCSP-S could be due to lower amounts of full CSP coupled to the beads as there was a major degradation product detected by SDS-PAGE (Additional file 2). Alternatively, these results are due to the presence of both major alleles in this chimerical protein as both readily circulate in the Brazilian Amazon [33]. In the absence of further evidence, this remains to be investigated.

Proteins expressed during the asexual blood stages are responsible for pathology associated with malaria and are, therefore, the target of intense efforts to discover antigens for vaccination. Naturally acquired humoral immune responses against merozoite surface proteins were thus initially analysed as they are involved in invasion to red blood cells and are considered candidates to develop sub-unit vaccines against malaria [27]. In particular, MSP1, MSP5 and MSP7 were studied as different reports from these proteins indicate their potential in vaccine development [8]. MSP1 and MSP5 are encoded by single gene whereas MSP7 pertains to a highly variant multi-allelic family [9]. As expected, results demonstrated that MSP proteins are immunogenic in natural infections. Moreover, results confirmed that MSP1-19 is more immunogenic than MSP1-N [20] and that in spite of MSP5 being highly polymorphic [34], it is also highly immunogenic. Furthermore, in line with being a multigene family differentially expressed during blood stages [35], reactivity against MSP7 was lower than MSP1 or MSP5. In addition to MSPs, the response against the Duffy binding protein region II (PvDBP-II) a leading vaccine candidate against *P. vivax*, was also analysed. PvDBP-II is cysteine-rich and requires a complex series of steps to fold it correctly [28]. Results confirmed the immunogenicity of PvDBP-II in natural infections as previously reported using sera from adult patients in Brazil [33]. Whether these antibody responses against different asexual blood stages are inhibitory as shown for the PvDBP-II [36] awaits the development of functional assays.

In summary, expression of soluble proteins from *P. vivax* for analysis in multiplexing assays using the wheat germ cell-free system in a 50 μl scale has been achieved. In addition to the five leading vaccine candidates illustrating here this methodology, several other proteins including subtelomeric variant Vir and PfamD proteins, Pvs48/45, and several hypothetical antigenic proteins, have been solubly expressed at this scale. Up to 100 proteins can be presently coupled to different beads and analysed simultaneously with as little as one microliter of immune sera. Prospective longitudinal studies from

endemic regions with different degrees of transmission and clinical immunity using this methodology will complement studies using protein arrays and will accelerate antigen discovery and vaccine development in *P. vivax*.

Additional material

Additional file 1: Proteins and primers used in this study. ID, identification. AA, amino acids. MW, molecular weight. IP, isoelectric point. Columns to the right represente GST-fusion proteins. Sequence of primers.

Additional file 2: Purification of proteins from wheat germ lysates. Soluble fractions from wheat germ extracts were applied to a Glutathione Sepharose®® 4B column equilibrated with PBS. Columns were washed with PBS and bound GST-fusion proteins eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Collected fractions were analysed by SDS-PAGE. Molecular weights of standard control proteins are indicated and soluble GST-fusion proteins are marked with an arrow.

Additional file 3: Coupling efficiency of proteins to activated beads. Specific detection of 8 tagged Pv-protein on beads. Protein were expressed in wheat germ cell free system, purified and 1 ug bound to the beads. Prior to multiplexing, protein coupling was verified by incubating coupled beads with mouse anti-Gst or anti-his (for PvCSP-c) antibody followed by biotinylated anti-mouse IgG. The biotinylated antibodies were detected using PE-streptavidin with the Luminex analyzer beads, and fluorescence was determined in the mean fluorescence intensity (MFI).

Additional file 4: Comparative analysis of immune responses to PvCSP-S and PvCSP-c by singleplex and multiplex. Immune sera were analysed in a single-vs multiplex assay. Values above 1 indicates increased response as multiplex assay. Values below 1 indicates that there was a decrease of the response as multiplex assay.

Additional file 5: Comparative analysis between responses to different proteins in the same serum using 2 × 2 tables.

List of abbreviations
GST: Glutathione S-Transferase; MFI: Median fluorescent intensity; PE: phycoerythrin

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Authors' contributions
ER contributed to write the manuscript, to design and to conduct the experiments. ST and TT made substantial constructive advice in the initial design of the project and constructed as well as expressed the PvCSP chimerical protein. SS performed statistical analyses. MVGL made advice in the last design of the project and critically read the manuscript. CFB and HAP conceived this study and contributed to write the manuscript and to design experiments. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Tricomponent Immunopotentiating System as a Novel Molecular Design Strategy for Malaria Vaccine Development[▽]

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The creation of subunit vaccines to prevent malaria infection has been hampered by the intrinsically weak immunogenicity of the recombinant antigens. We have developed a novel strategy to increase immune responses by creating genetic fusion proteins to target specific antigen-presenting cells (APCs). The fusion complex was composed of three physically linked molecular entities: (i) a vaccine antigen, (ii) a multimeric α-helical coiled-coil core, and (iii) an APC-targeting ligand linked to the core via a flexible linker. The vaccine efficacy of the tricomponent complex was evaluated using an ookinete surface protein of *Plasmodium vivax*, Pvs25, and merozoite surface protein-1 of *Plasmodium yoelii*. Immunization of mice with the tricomponent complex induced a robust antibody response and conferred substantial levels of *P. vivax* transmission blockade as evaluated by a membrane feed assay, as well as protection from lethal *P. yoelii* infection. The observed effect was strongly dependent on the presence of all three components physically integrated as a fusion complex. This system, designated the tricomponent immunopotentiating system (TIPS), onto which any recombinant protein antigens or nonproteinaceous substances could be loaded, may be a promising strategy for devising subunit vaccines or adjuvants against various infectious diseases, including malaria.

Adjuvants are defined as any substances that enhance immune responses to vaccine antigens; however, they can be categorized into targeting molecules or systems that facilitate the delivery of antigens to antigen-presenting cells (APCs) and substances that help elicit their activation (2, 11, 14, 25, 27). Adjuvants are essential components of recombinant protein-based subunit vaccines, because nonreplicating inert antigens are often weakly immunogenic when administered in the absence of extraneous adjuvants (25). Aluminum hydroxide (Alum) is the only adjuvant that has been used clinically over the past several decades (11), but recently a number of APC-activating adjuvants have been developed, and some of them have already been released to the international market (14, 27). However, no targeting molecules or systems are licensed for clinical use yet.

Dendritic cells (DCs) are considered the most effective APCs. They have highly efficient and specialized functions in the uptake and presentation of foreign antigens to T and B lymphocytes, allowing them to mount appropriate immune responses (5, 8). DCs are important initiators and modulators of immune responses, and hence, in the field of vaccine research, much attention has been focused on the APC function of DCs. However, B lymphocytes also take up foreign antigens via surface immunoglobulin (Ig) (B cell receptors [BCRs]) and present protein epitopes through the major histocompatibility complex (MHC) class II–T cell receptor interaction for antibody production (9, 10, 17, 23, 24, 33, 34). Thus, B lymphocytes are unique in that they are APCs as well as effector cells. An additional unique feature of B lymphocytes, which distinguishes them from DCs, is that they can recognize conformational epitopes (for example, those present on the surfaces of protein antigens) in addition to linear epitopes. They capture cognate antigens via specific BCRs, present linear epitopes within the captured antigen on the MHC class II molecules, and subsequently receive cognate help from T lymphocytes in the secondary lymphoid organs, such as local draining lymph nodes (10). This T-B lymphocyte interaction does not require DC involvement (26, 33). Therefore, it is theoretically plausible that targeting of the BCRs present on the surfaces of the B lymphocytes in the follicles of the draining lymph nodes by exploiting the Ig binding ligands would increase the chance that antigens would encounter cognate B lymphocytes in the follicles and would be captured and presented to T lymphocytes for efficient antibody production. By exploiting this immunological mechanism, it may be possible to augment immune responses to otherwise weakly immunogenic recombinant antigens (2).

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TABLE 1. Sequences of oligonucleotides and a synthetic gene encoding the COMP(Gly26–Gly80) coiled-coil domain used in this study

Oligonucleotide	Sequence[a]
#1	5′-CCATGGGTAGCATTATCAACGAAACCGCCGATGATATTGTGTATCGCTTGACCGTGATCATTGATGATCGCTATGAAAGCCTGAAAAATCTGATTACCTTACGTGCCGACCGCCTGGAAATGATTATTAATG-3′
#2	5′-CTCGAGCGGGCCCGGGCCGCTACCGCCACCGCCGTGATGATGGTGATGATGGCTACCGCCACCGCCCGGGCCCGGGCCGATGCTCGCCAAGATGGTCGAAACATTGTCATTAATAATCATTTCCAGGCGGTC-3′
#3	5′-GTCGACGTGGATAACAAATTTAATAAAGAACAGCAGAACGCCTTCTATGAAATTCTGCATCTGCCGAACCTGAACGAAGAACAGCGTAACGCCTTTATTCAGAGCCT-3′
#4	5′-CTCGAGTTATTTCGGGGCCTGTGCATCGTTCAGTTTTTGGCTTCTGCCAGCAGATTGGCGCTCTGGCTCGGATCATCTTTCAGGCTCTGAATAAAGGCGTTACGCT-3′
#5	5′-TCGACCATCTTGGCGTGCATCGGCCCGGGCCCG-3′
#6	5′-CGGGCCCGGGCCGATGCACGCCAAGATGGTCG-3′
#7	5′-CCGGGCCCGGGCGGTTGCGGCGGTAGCCATCATCAC-3′
#8	5′-ATGATGGCTACCGCCGCAACCGCCCGGGCCCGG-3′
#9	5′-GCGCCATGGGTGATCTGGCGCCGCAGATG-3′
#10	5′-GGCTCGAGGCCACACGCATCGCATTCCATAAC-3′
#11	5′-TCGACGGCCCGGGCCCGGGCGGTGGCGGTAGCCATCATCACCATCATCACGGCGGTGGCGGTAGCGGCCCGGGCCCGC-3′
#12	5′-TCGAGCGGGCCCGGGCCGCTACCGCCACCGCCGTGATGATGGTGATGATGGCTACCGCCACCGCCCGGGCCCGGGCCG-3′
COMP(Gly26–Gly80)	5′-GGCGGTGATCTGGCGCCGCAGATGCTGCGCGAACTGCAGGAAACCAACGCGGCCCTGCAAGATGTGCGTGAACTGCTGCGCCAGCAAGTGAAAGAAATTACCTTTCTGAAAAATACCGTTATGGAATGCGATGCGTGTGGCATGCAGCCGGCCCGTACCCCGGGC-3′

[a] Underlined sequences represent restriction enzyme recognition sites.

It should be noted that certain anti-infectious vaccines need to rely on recombinant subunit proteins, because some infectious diseases, including malaria and other parasitic diseases, defy conventional methods of pathogen inactivation or attenuation for vaccine production; therefore, antigens derived from these pathogens need to be transformed into efficacious vaccines with the help of adjuvants (2).

In this study, we devised a new immune enhancing system for the development of malaria vaccines, and to demonstrate its efficacy, we exploited two malaria parasite antigens, the *Plasmodium vivax* ookinete surface protein (OSP) Pvs25 and *Plasmodium yoelii* merozoite surface protein-1 (MSP1), which are known to require native conformational epitopes in order to function as effective vaccines (7, 16, 31, 32). We also exploited the Z domain, a derivative of the B domain of the Ig-binding domains (IBDs) of *Staphylococcus aureus* protein A (SpA), as a targeting ligand for B lymphocytes (19). The Z domain was genetically conjugated to an α-helical coiled-coil multimer-forming domain (20) of tetrabrachion (TB) (29) or cartilage oligomeric matrix protein (COMP) (12) both to increase its structural stability and binding avidity and to facilitate receptor cross-linking. We demonstrated that antigens loaded onto these multimeric delivery complexes targeted B lymphocytes and robustly enhanced antiparasitic immunity when administered to mice through the subcutaneous (s.c.) or intranasal (i.n.) route. Furthermore, not only are all three of these components (i.e., the antigen, the core motif, and the ligand) essential, but they must also be integrated into the fusion complex for the efficient induction of an immune response.

MATERIALS AND METHODS

Construction of the TB- and COMP-based delivery molecule expression plasmids. An *Escherichia coli* codon-optimized synthetic gene encoding the TB coiled-coil domain (Gly1 to Ile52; Protein Data Bank [PDB] accession no. 1YBK) fused to a spacer sequence (see Fig. 1a, region b) was constructed by annealing two overlapping oligonucleotides (oligonucleotides #1 [sense] and #2 [antisense], containing an NcoI and an XhoI site, respectively), followed by PCR amplification using *Vent* DNA polymerase (New England BioLabs, Beverly, MA). The amplified fragment was cloned into pCR2.1 (Life Technologies, Carlsbad, CA), and then, after digestion with NcoI and XhoI, the fragment generated was subcloned into the corresponding sites in pET-21d (Merck KGaA, Darmstadt, Germany) to construct the TB-spacer (TB coiled-coil domain fused to a spacer) expression plasmid (see Fig. 1a). Similarly, a synthetic gene encoding the Z domain (Val1 to Lys58; PDB accession no. 2SPZ), a derivative of the B domain of SpA, was PCR amplified by annealing two overlapping oligonucleotides (oligonucleotides #3 [sense] and #4 [antisense], containing a SalI and an XhoI site, respectively). The amplified fragment was cloned into pCR2.1, which was then digested with SalI and XhoI, and the fragment generated was subcloned into the XhoI site on the pET-21d-TB-spacer expression plasmid to construct the TB-Z expression plasmid (Fig. 1a).

Next, to introduce a cysteine (Cys) residue into the TB-Z to construct TB-(Cys)-Z, site-directed mutagenesis was performed (QuikChange II site-directed mutagenesis kit; Agilent Technologies Inc., Wilmington, DE). TB(S52C)-Z was constructed by substituting Cys for serine 52 (Ser52) within the TB coding region (see Fig. 1a, region b) by PCR using primer set #5 (sense)–#6 (antisense) with the TB-Z expression plasmid as template DNA. Similarly, TB(C60)-Z was constructed by PCR using primer set #7 (sense)–#8 (antisense) to introduce a Cys residue immediately after Gly59 (see Fig. 1a, region b).

A gene encoding the COMP coiled-coil domain (Gly27 to Gly72; PDB accession no. 1VDF) was PCR amplified by using primer set #9 (sense)–#10 (antisense), containing an NcoI and an XhoI site, respectively, and plasmid DNA containing an *E. coli* codon-optimized synthetic gene encoding COMP(Gly26–Gly80) as the template (12). The amplified fragment was digested with NcoI and XhoI and was subcloned into the corresponding sites in pET-22b (Merck KGaA) to construct the COMP expression plasmid (see Fig. 2a). To fuse a spacer sequence to the COMP, two oligonucleotides (oligonucleotides #11 [sense] and #12 [antisense]) were annealed and inserted into a unique XhoI site in the COMP expression plasmid, constructing the COMP-spacer expression plasmid. For construction of the COMP-spacer fused to the Z domain (COMP-Z), oligonucleotides #3 and #4 were used as described above for the construction of the TB-Z expression plasmid (Fig. 2a). Table 1 lists the sequences of all the oligonucleotides and of a synthetic gene encoding the COMP(Gly26–Gly80) coiled-coil domain (12) used in this study.

Expression and purification analysis of the delivery molecules. *E. coli* BL21(DE3) was transformed with each engineered expression plasmid and was cultured in LB broth with ampicillin; then protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a 16-h

induction, the supernatant was collected by centrifugation (9,600 × *g*, 20 min), followed by filtration (FastCap filter; pore size, 0.2 mm; Nalgene Nunc International Inc., Rochester, NY). The supernatant was subjected to Ni-nitrilotriacetic acid (NTA) affinity chromatography (HisTrap FF [Fast Flow] columns prepacked with Ni Sepharose 6; GE Healthcare, Little Chalfont, United Kingdom).

Affinity-purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 10% skim milk in phosphate-buffered saline (PBS), and washed with PBS-T (PBS with 0.05% Tween 20). Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat IgG (1:4,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescence was detected using the Western Lightning kit (Perkin-Elmer, Inc., Waltham, MA). Purified proteins were also analyzed by size exclusion chromatography (flow rate, 0.8 ml/min; HiLoad 16/60 Superdex 75 pg column; GE Healthcare).

Chemical conjugation of antigens to delivery molecules. Recombinant Pvs25H-A was expressed and purified as described previously (22) and was chemically conjugated to the affinity-purified TB(Cys)-Z [TB(S52C)-Z or TB(C60)-Z] or COMP-Z using the heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Thermo Scientific, Inc., Rockford, IL). Six milligrams of Pvs25H-A (2 mg/ml in PBS-EDTA) was incubated with SPDP (final concentration, 0.6 mM) for 1 h at room temperature (designated Pvs25H-A^{pyr} in Fig. 3a). The reaction mixture was buffer-exchanged with PBS (Amicon Ultra-15 centrifugal filter unit; molecular weight cutoff [MWCO], 10,000; Millipore, Billerica, MA) to remove excess reagents and by-products. Concomitantly, 5 mg of the TB(Cys)-Z or COMP-Z (2 mg/ml in PBS-EDTA) was treated with dithiothreitol (DTT; 50 mM) for 30 min at 37°C and was buffer-exchanged with PBS [designated TB(Cys)-Z^{red} or COMP-Z^{red} in Fig. 3a]. Three milligrams of Pvs25H-A^{pyr} and 1 mg of TB(Cys)-Z^{red} or COMP-Z^{red} were mixed and incubated at room temperature overnight for conjugation. The conjugated sample was buffer-exchanged with PBS as before.

A 19-kDa C-terminal fragment of MSP1 (MSP1-19) of the rodent malaria parasite *P. yoelii* was loaded onto the delivery molecules using essentially the same conjugation method as that described above for the Pvs25H-A antigen.

The endotoxin levels of all of the conjugated proteins were measured (Pyrogen Single Test Vials; Cambrex, East Rutherford, NJ) prior to administration to mice, and we found that they were less than 15 pg endotoxin/μg of protein.

Ig-ELISA. A human IgG–enzyme-linked immunosorbent assay (ELISA) wasconducted to analyze the tricomponent complexes. Briefly, 5 μg/ml of human IgG (Sigma-Aldrich, St. Louis, MO) diluted with bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) (50 μl/well) was used as a capture antigen for the Z domain-containing proteins; it was applied to a 96-well microtiter plate (Sumilon; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and was incubated at 4°C overnight. The plate was blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at 37°C. Samples (2 μg of total protein/well) were applied and incubated for 2 h at 37°C, followed by incubation with 5 μg/ml of human IgG for 2 h at 37°C to mask unbound free Z domains. A mouse anti-His tag antibody (1:4,000; GE Healthcare), a mouse anti-Pvs25 antiserum (1:500), or a mouse anti-MSP1-19 antiserum (1:100) was applied and incubated for 2 h at 37°C. Anti-mouse IgG conjugated to alkaline phosphatase (AP) (1:4,000; Sigma-Aldrich), followed by *p*-nitrophenylphosphate (Bio-Rad Laboratories Inc., Redmond, WA), was added and incubated for 20 min at 37°C. The optical density at 415 nm (OD_{415}) was measured using a microplate reader (Bio-Rad).

Using essentially the same human IgG-ELISA protocol as that described above, the affinity of the COMP-Z delivery molecule for various human or mouse Igs was evaluated. The Igs used as capture antigens were 5 μg/ml of human or mouse IgG (Sigma-Aldrich), 10 μg/ml of human (Sigma-Aldrich) or mouse (Bethyl Laboratories Inc., Montgomery, TX) IgM, 10 μg/ml of human or mouse IgA (Sigma-Aldrich), 10 μg/ml of human IgG1 Fab lambda or kappa (Bethyl Laboratories Inc.), or 10 μg/ml of mouse IgG Fab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After the plate was blocked with 1% BSA in PBS, the COMP-Z or COMP (2 μg/well each) was applied and incubated for 2 h at 37°C, followed by incubation with 5 μg/ml of human IgG for 2 h at 37°C to mask unbound free Z domains. A mouse (1:4,000; GE Healthcare) or rabbit (1:4,000; Rockland Immunochemicals, Inc., Boyertown, PA) anti-His tag antibody was applied for detection of the human or mouse Ig-bound delivery molecules, respectively. Then anti-mouse or anti-rabbit IgG conjugated to AP (1: 4,000; Sigma-Aldrich), followed by its substrate, was applied for analysis.

Analysis of the target immune cells of the COMP-Z by flow cytometry. The COMP (2 mg; 62.5 nmol) or COMP-Z (2 mg; 27.6 nmol) was treated with DTT (50 mM) for 30 min at 37°C, and the buffer was replaced with PBS (Amicon Ultra-15 centrifugal filter unit; MWCO, 10,000; Millipore). The DTT-treated COMP or COMP-Z (2 mg/ml in PBS) was incubated with maleimide-polyethylene oxide (PEO_2)-biotin (final concentration, 20 mM; Thermo Scientific Inc.) for 16 h at room temperature, and the buffer was replaced with PBS as before.

Splenocytes isolated from naïve BALB/c mice were suspended in RPMI medium (Gibco Inc., Grand Island, NY) and were collected by centrifugation (377 × *g*, 10 min). Cells were incubated in red blood cell (RBC) lysis buffer (17 mM Tris buffer [pH 7.6], 140 mM NH_4Cl) for 5 min at room temperature, washed, and resuspended in RPMI medium. Cells were counted (KOVA Glasstic slide 10; Hycor Biomedical Inc., Garden Grove, CA), and the concentration was adjusted to 5 × 10^6 cells/ml with cell suspension buffer (PBS containing 2% newborn calf serum and 0.03% NaN_3). Fluorescein isothiocyanate (FITC)-conjugated antibodies to CD19 (clone 1D3), CD3e (clone 145-2C11), CD11b (clone M1/70), or CD11c (clone HL3), along with R-phycoerythrin (PE)-conjugated antibodies to Ly-6G and Ly-6C (Gr-1, clone RB6-8C5) (BD Biosciences, Sparks, MD), allophycocyanin-conjugated anti-mouse MHC class II (I-A/I-E) (eBioscience, San Diego, CA), and isotype control antibodies (BD Biosciences), were used for analysis. All antibodies were used at the concentrations recommended by the manufacturers.

Spleen cells (5 × 10^5) were first treated with a monoclonal antibody (clone 2.4G2) to block Fc receptors and were then washed, and biotinylated COMP or COMP-Z (0.31 pmol) was added to the cells and incubated. Then PE-conjugated streptavidin (BD Biosciences) was added to the cells and incubated. Cells were washed and adjusted to 1 ml with cell suspension buffer. The incubation conditions for each step were 20 min at 4°C. Data were acquired using a FACSCalibur flow cytometer and were analyzed with CellQuest software (BD Biosciences).

Immunization of mice. Seven-week-old female BALB/c or C57BL/6 mice (Japan SLC, Shizuoka, Japan), 4 to 10 per group, were immunized at weeks 0, 2, and 4 via the s.c. or i.n. route with various immunization samples. For all mouse immunization studies, 30 μg of the vaccine antigen (i.e., Pvs25H-A or MSP1-19) was administered as a conjugated or unconjugated protein. Incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI), Alum (Imject Alum adjuvant; Thermo Scientific, Inc.), or cholera toxin (CT; List Biological Laboratories, Campbell, CA) was used as an s.c. or i.n. adjuvant where indicated. The endotoxin levels of all of the immunization samples were measured (Cambrex) prior to administration to mice, and we found that they were less than 15 pg endotoxin/μg of protein.

Animal experimental protocols were approved by the University of the Ryukyus Animal Care and Use Committee, and the experiments were conducted according to the institutional ethical guidelines for animal experiments.

Determination of antigen-specific serum IgG titers by ELISA. Mice were anesthetized 2 weeks after the third immunization (week 6) by intraperitoneal (i.p.) injection of pentobarbital sodium salt (Nacalai Tesque Inc., Kyoto, Japan) and were euthanized by exsanguination for the collection of antisera. For analysis of the long-term serum IgG response to the Pvs25H-A antigen, blood was collected at days 0, 14, 42, 91, 126, 196, and 273.

Antigen-specific serum antibodies were analyzed by ELISA as described previously (3, 4, 21, 22). Briefly, the ELISA plate was coated with the Pvs25H-A (5 μg/ml) or MSP1-19 (5 μg/ml) protein in bicarbonate buffer at 4°C overnight and was then blocked with 1% BSA in PBS for 2 h at 37°C. Twofold serial dilutions of the antisera, starting with a 50-fold dilution in PBS with 0.5% BSA, were applied and incubated for 2 h at 37°C. AP-conjugated anti-mouse IgG (1:4,000; Sigma-Aldrich), IgG1 (1:4,000; MP Biomedicals, Solon, OH), or IgG2a (1:4,000; MP Biomedicals) was applied and incubated for 2 h at 37°C. Then *p*-nitrophenylphosphate (Bio-Rad) was applied, and the OD_{415} was measured after 20 min of incubation at 37°C by using a microplate reader (Bio-Rad). The antibody titer was defined either as the serum dilution that gave an OD_{415} value equal to 0.1 or as the serum dilution for which a 1-magnitude-higher dilution gave an OD_{415} value less than 0.1.

Mosquito membrane feed assay and detection of native Pvs25 protein by antisera derived from immunized mice. The mosquito membrane feed assay was conducted by a method described previously (3, 4, 21, 22). Single-species infection with *P. vivax* was confirmed by Giemsa staining of thick and thin blood smears. The levels of parasitemia and gametocytemia were 0.03% and 0.002% for donor 1, 0.23% and 0.01% for donor 2, and 0.21% and 0.01% for donor 3, respectively. Native Pvs25 protein on the surfaces of *P. vivax* ookinetes was detected by immunofluorescence (21, 22) using antisera obtained from mice immunized with the tricomponent complex.

All human subject research conducted in this study was reviewed and approved by the Ethics Committee of the Thai Ministry of Public Health and the Institutional Review Board of the Walter Reed Army Institute of Research.

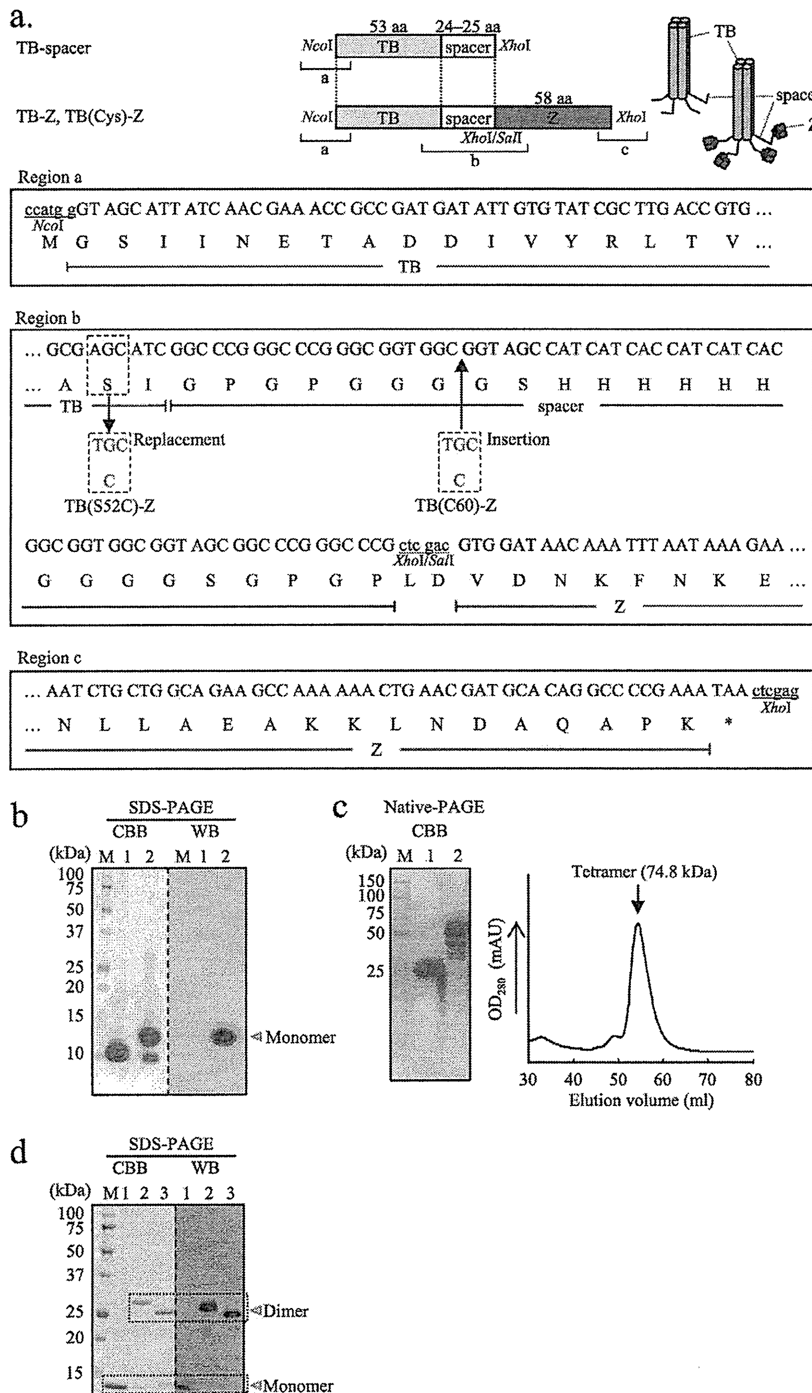


FIG. 1. Expression of the TB-based delivery molecules. (a) Schematic drawing of the tetrabrachion (TB)-derived coiled-coil domain-based constructs: TB-spacer, the TB coiled-coil domain fused to a spacer sequence; TB-Z, TB-spacer fused to the Z domain; TB(Cys)-Z, TB-Z into which a cysteine (Cys) residue has been introduced. The nucleotide and predicted amino acid sequences of the 5′-terminal, junction, and the 3′-terminal regions are shown as regions a, b, and c, respectively. All constructs were cloned between the NcoI and XhoI sites of pET-21d. TB(S52C)-Z is a TB(Cys)-Z in which a Cys was substituted for Ser52 within the coiled-coil domain to introduce a sulfhydryl group for chemical conjugation (as indicated in region b). TB(C60)-Z is another TB(Cys)-Z with a Cys insertion at amino acid position 60 within the hinge region (as indicated in region b). (b and c) SDS-PAGE (b) and native PAGE (c) (left) of the affinity-purified TB-spacer (lanes 1) and TB-Z (lanes 2) and size exclusion chromatography of the TB-Z (c) (right). The protein bands were either stained with Coomassie brilliant blue (CBB) or subjected to Western blotting (WB). (d) SDS-PAGE of the affinity-purified TB-Z (lanes 1), TB(S52C)-Z (lanes 2), and TB(C60)-Z (lanes 3). M, molecular mass marker. The protein bands were either stained with CBB or subjected to WB. For WB (panels b and d), HRP-conjugated goat IgG was applied directly to the blotted membrane for detection of the Z domain-containing proteins.

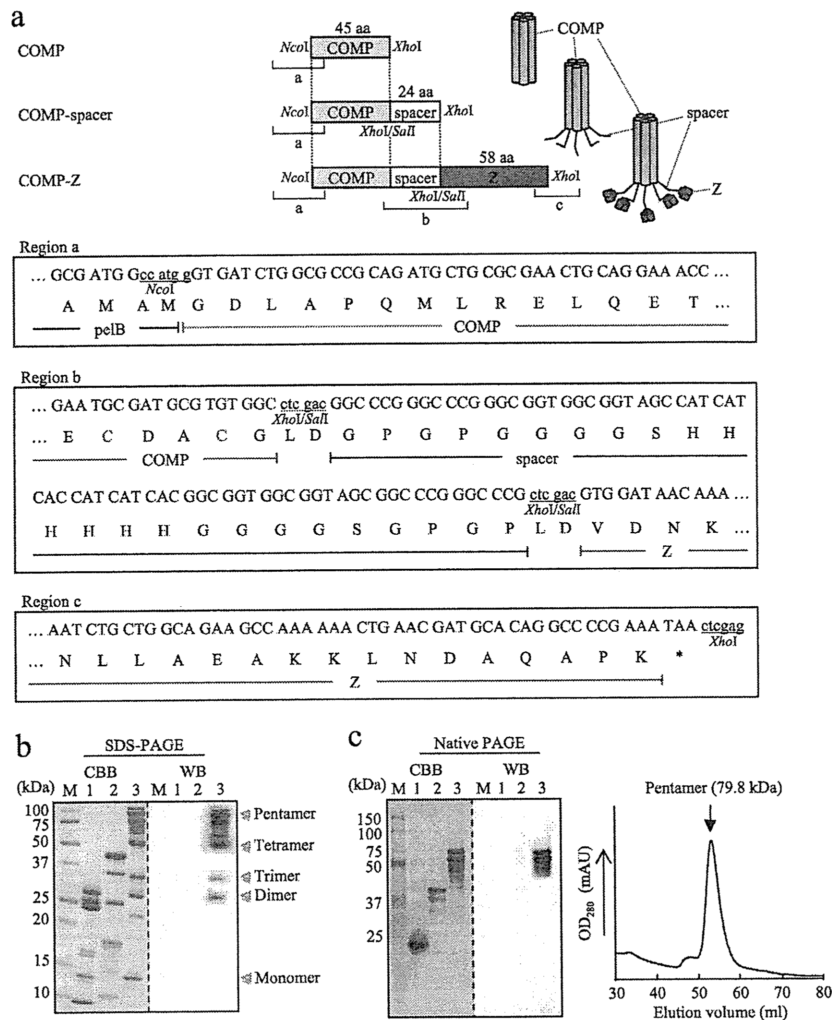


FIG. 2. Expression of the COMP-based delivery molecules. (a) Schematic drawing of the rat cartilage oligomeric matrix protein (COMP)-derived coiled-coil domain-based constructs: COMP, COMP coiled-coil domain; COMP-spacer, COMP fused to a spacer sequence; COMP-Z, COMP-spacer fused to the Z domain. All constructs were cloned between the NcoI and XhoI sites of pET-22b and were expressed as *pelB* fusion proteins. The nucleotide and predicted amino acid sequences of the 5′-terminal, junction, and the 3′-terminal regions are shown as regions a, b, and c, respectively. Two inherent Cys residues within the COMP coiled-coil domain (as indicated in region b) are specific sites used for chemical conjugation. (b and c) SDS-PAGE (b) and native PAGE (c) (left) of the affinity-purified COMP (lanes 1), COMP-spacer (lanes 2), and COMP-Z (lanes 3) and size exclusion chromatography of the COMP-Z (c) (right). M, molecular mass marker. The protein bands were either stained with Coomassie brilliant blue (CBB) or subjected to Western blotting (WB). For WB, HRP-conjugated goat IgG was applied directly to the blotted membrane for detection of the Z domain-containing proteins.

Rodent malaria parasite infection and parasitemia determination. C57BL/6 mouse erythrocytes parasitized by the lethal *P. yoelii* 17XL strain were inoculated i.p. into female C57BL/6 mice (1×10^4 parasitized RBCs/mouse). At various times after inoculation, parasitemia in peripheral blood was monitored by microscopy on Giemsa-stained thin blood smears.

Statistical analysis. The Wilcoxon-Mann-Whitney test was performed to compare antibody titers, or the number of oocysts per mosquito, between the nonimmune control group and a particular immunization group, or between two immunization groups. The Kruskal-Wallis test was performed to compare antibody titers, or the number of oocysts per mosquito, among particular groups. The chi-square test was performed to analyze the difference in the proportion of parasite-free mosquitoes among the total number of mosquitoes examined between the nonimmune control group and a particular immunization group, or between two immunization groups. Kaplan-Meier analyses with the log rank test were performed to compare the survival rates for *P. yoelii*-infected mice between the PBS control group and a particular immunization group. All statistical analyses were conducted with JMP software, version 8.0 (SAS Institute Inc., Cary, NC).

RESULTS

Expression of delivery molecules. The expression of the TB-based constructs, i.e., the TB-spacer and the TB-Z (Fig. 1a), was analyzed (Fig. 1b to d). *E. coli* cultures, including culture supernatants and cell extracts, were subjected to SDS-PAGE. The proteins were detected mainly in the culture supernatants, even though they lacked the leader peptide. Interestingly, the TB constructs fused to the *pelB* signal sequence were not expressed, for unknown reasons. The secreted proteins were

conveniently purified by Ni-NTA chromatography. The affinity-purified TB-spacer and the TB-Z appeared on SDS-PAGE as protein bands with molecular masses of approximately 10 kDa and 12 kDa, respectively (Fig. 1b, CBB). Since the molecular masses predicted from the primary structures of the TB-spacer and TB-Z were 9.2 kDa and 14.9 kDa, respectively, the bands we observed presumably represented their monomers. In contrast, protein bands that were predicted tetramers of the TB-spacer and TB-Z were observed by native PAGE (Fig. 1c, left). In addition, size exclusion chromatography of the TB-Z showed a single chromatographic peak with an estimated molecular mass of 74.8 kDa (Fig. 1c, right), which appeared substantially higher than its calculated mass (i.e., 59.6 kDa for the tetramer). This discrepancy may be attributed to the long rod-like structure of the TB coiled-coil domain, because such molecules generally appear higher in molecular mass than globular proteins of the same mass. These results indicated that the TB-based constructs were secreted from recombinant *E. coli* predominantly as tetramers but were disassembled into monomers in the presence of SDS. The TB-Z, but not the TB-spacer, was shown to bind to the Ig molecule (Fig. 1b, WB).

Next, Cys residues were introduced into the wild-type sequence of the TB-Z for use as a specific antigen-coupling site, because no Cys residues existed within the TB and Z protein moieties. Three candidate sites were selected for Cys insertion. Two of these sites represented Ser substitutions (Ser3 and Ser52), which were chosen out of the four possible Ser residues (Ser3, Ser26, Ser47, and Ser52) because of their presumed degree of side chain exposure to the surface of the coiled-coil domain, based on the 3-dimensional (3D) crystal structure (PDB accession no. 1YBK), and one site represented *de novo* Cys insertion based on the presumed molecular flexibility within the spacer region. Thus, three TB-Z constructs containing Cys residues [TB(Cys)-Z] were engineered: TB(S3C)-Z, TB(S52C)-Z, and TB(C60)-Z. The TB(S3C)-Z construct failed to be expressed in any cellular compartment or in the culture supernatant, but the TB(S52C)-Z and TB(C60)-Z constructs (Fig. 1a, region b) were fully expressed in the culture supernatant, as observed for parental TB-Z, and were purified by Ni-NTA chromatography. For the affinity-purified TB(Cys)-Z [TB(S52C)-Z and TB(C60)-Z], but not for the TB-Z, dimers were observed as predominant molecular species by SDS-PAGE analysis (Fig. 1d, compare lanes 1 with lanes 2 and 3), indicating that disulfide bonds formed between the two subunit pairs within the tetramer. The TB(Cys)-Z could also bind to the Ig molecule, as seen for the TB-Z (Fig. 1d, WB).

COMP-based constructs, i.e., the COMP, COMP-spacer, and COMP-Z, were also engineered (Fig. 2a) and analyzed for their expression in *E. coli*. All three constructs were expressed in culture supernatants similarly to the TB-based constructs. They were purified by Ni-NTA chromatography and were subjected to SDS-PAGE and native PAGE (Fig. 2b and c). Unlike the TB-based proteins, the COMP-based proteins appeared as several bands of various molecular masses under denaturing conditions (Fig. 2b). However, by native PAGE, they appeared predominantly as pentameric forms (Fig. 2c, left). Only the COMP-Z bound to the Ig molecule (Fig. 2b and c, WB). A single chromatographic peak was observed for the COMP-Z by size exclusion chromatography (Fig. 2c, right); its estimated

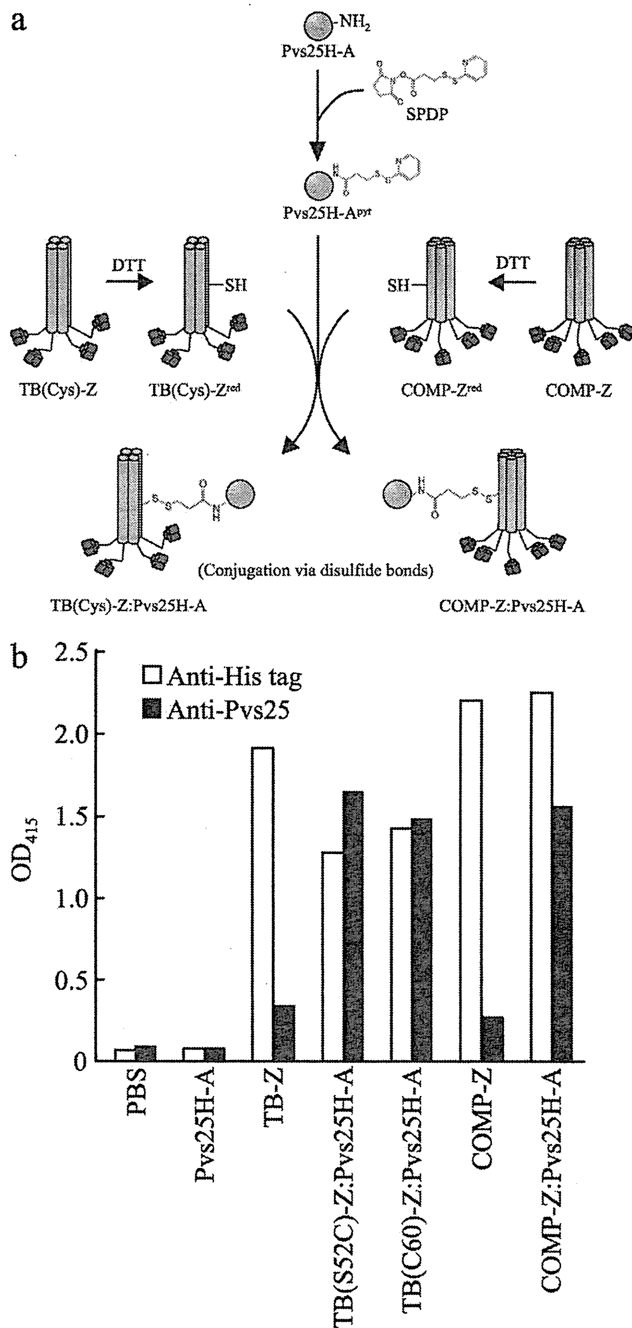


FIG. 3. Chemical conjugation of Pvs25H-A to TB(Cys)-Z [TB(S52C)-Z or TB(C60)-Z] or COMP-Z. (a) Chemical conjugation scheme for the construction of the TB(Cys)-Z:Pvs25H-A and COMP-Z:Pvs25H-A tricomponent complexes. The heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was used to link the Pvs25H-A antigen (22) to the TB(Cys)-Z or COMP-Z delivery molecule via disulfide bonds. The delivery molecules were first treated with dithiothreitol (DTT) to expose free sulfhydryls [designated TB(Cys)-Z^{red} or COMP-Z^{red}], and then pyridyldithiol-activated Pvs25H-A (Pvs25H-A^{pyr}) was reacted with the delivery molecules to generate the tricomponent complexes. (b) The complexes generated were analyzed by a human IgG-ELISA using an anti-His (open bars) or anti-Pvs25 (filled bars) antiserum.

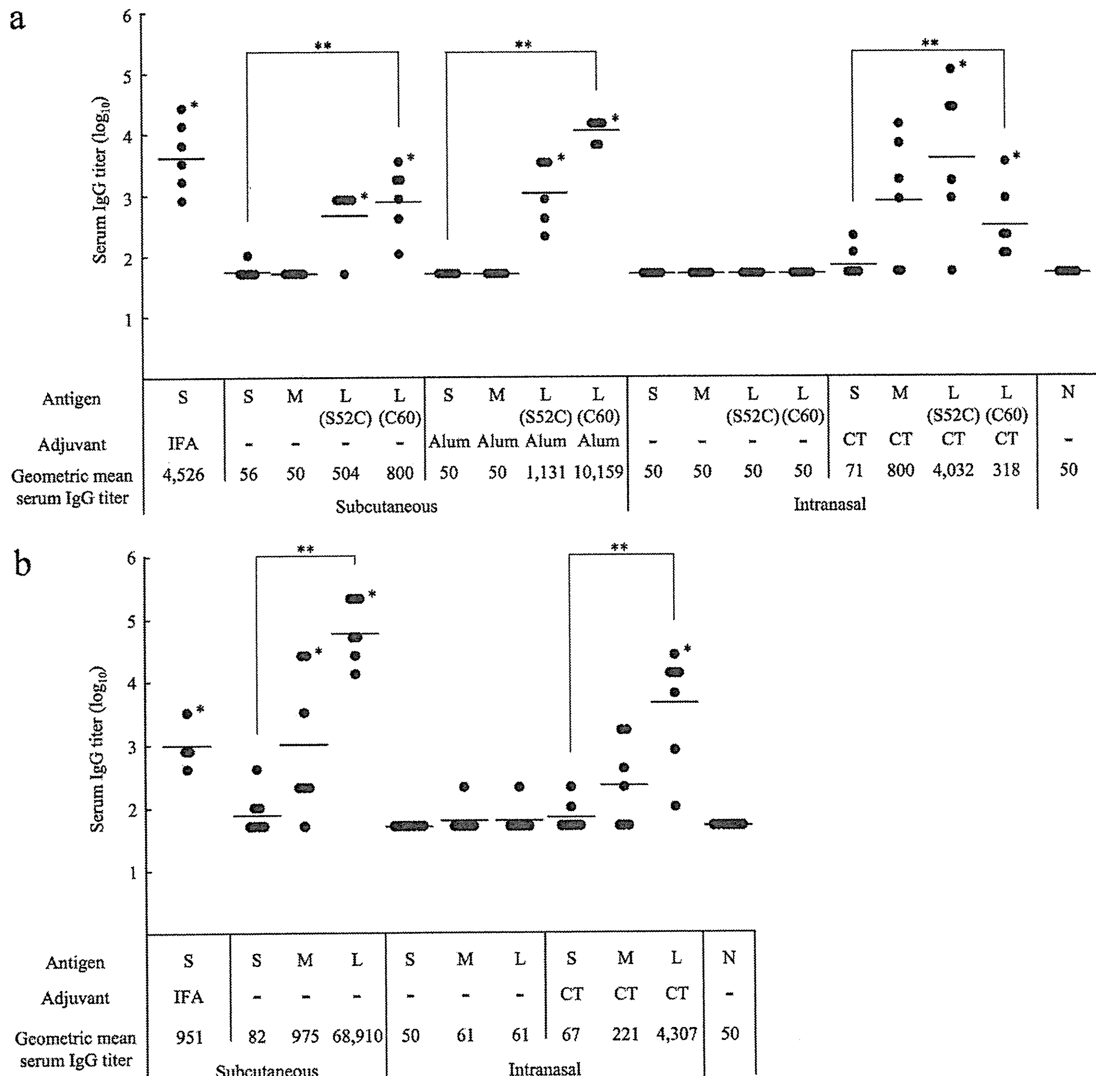


FIG. 4. Immunogenicity of the tricomponent complex. Mice were immunized by the subcutaneous or intranasal route three times, at weeks 0, 2, and 4, and antisera were collected 2 weeks after the third immunization to evaluate the Pvs25-specific IgG titers. All mice received 30 μg of the Pvs25H-A antigen as a conjugated or unconjugated protein. Incomplete Freund's adjuvant (IFA), aluminum hydroxide (Alum), or cholera toxin (CT) (1 μg) was used as the subcutaneous or intranasal adjuvant, as indicated. N, nonimmune sera from unimmunized control mice. Antibody titers were defined either as the serum dilution that resulted in an OD_{415} of 0.1 or as the serum dilution for which a 1-point-higher dilution (2-fold) resulted in an OD_{415} of <0.1. (a) Female BALB/c mice (six per group) were immunized with either the Pvs25H-A antigen alone (30 μg) (S), a mixture of the antigen (30 μg) and the TB-Z (21.4 μg) (M), the TB(S52C)-Z:Pvs25H-A tricomponent complex (51.4 μg) [L(S52C)], or the TB(C60)-Z:Pvs25H-A tricomponent complex (51.4 μg) [L(C60)]. (b) Female BALB/c mice (four or seven per group) were immunized with either the Pvs25H-A antigen alone (30 μg) (S), a mixture of the antigen (30 μg) and the COMP-Z (10.8 μg) (M), or the COMP-Z:Pvs25H-A tricomponent complex (40.8 μg) (L). Asterisks indicate significant differences from the unimmunized control group by the Wilcoxon-Mann-Whitney test (*, $P < 0.05$) or among the three groups indicated [S, M, and L(S52C) or L(C60) for the TB-based constructs, or S, M, and L for the COMP-based constructs] by the Kruskal-Wallis test (**, $P < 0.001$).

molecular mass was 79.8 kDa, higher than its calculated mass (i.e., 72.5 kDa for the pentamer). This difference may be attributed to the rod-like structure of the COMP coiled-coil domain, as observed for the TB-Z.

Taking these findings together, we concluded that the TB-Z and COMP-Z were secreted as tetrameric and pentameric forms, respectively, retaining their binding affinities for the Ig molecule. Their expression levels reached 30 mg/liter of bacterial culture. Multimerization of the Z domain mediated by the coiled-coil domain assembly significantly enhanced the avidity of the delivery molecules for the Ig molecule, as evidenced by the fact that the disassembled monomeric Z domain exhibited reduced affinity for the Ig molecule (Fig. 1d and 2b). This was also true when the Z domain was expressed as a single independent monomeric protein (data not shown).

Chemical conjugation of the Pvs25H-A antigen to the TB(Cys)-Z or COMP-Z delivery molecule to generate tricomponent complexes. The TB(Cys)-Z [TB(S52C)-Z and TB(C60)-Z] or COMP-Z constructs contained one artificially introduced (Fig. 1a, region b) or two inherent (Fig. 2a, region

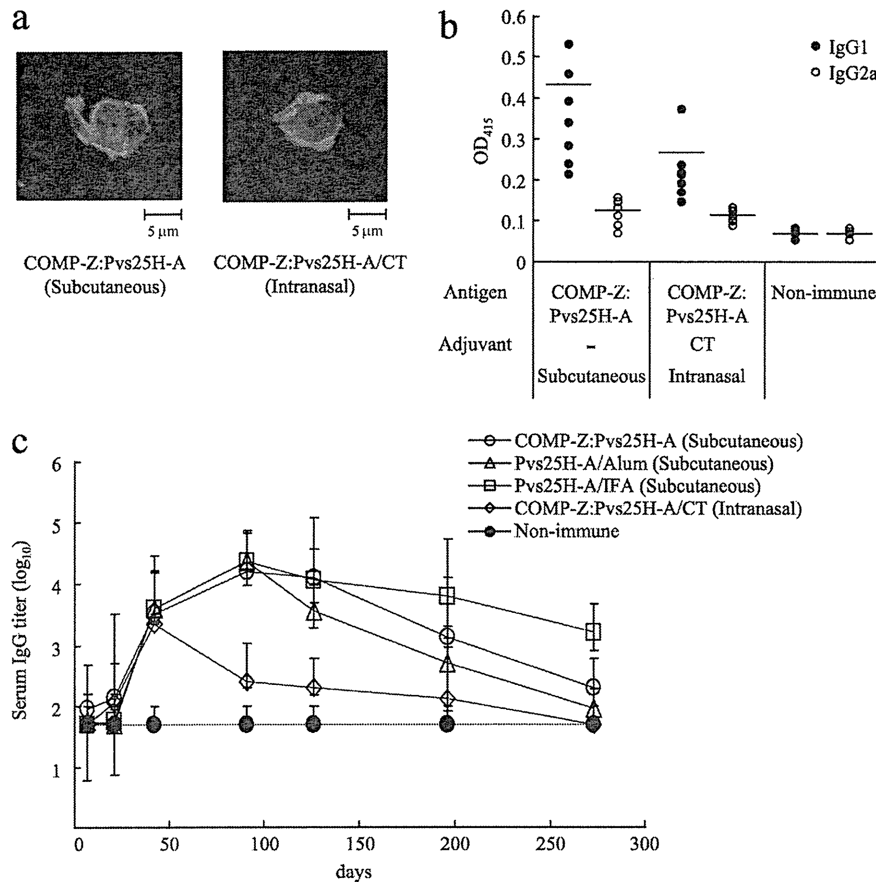
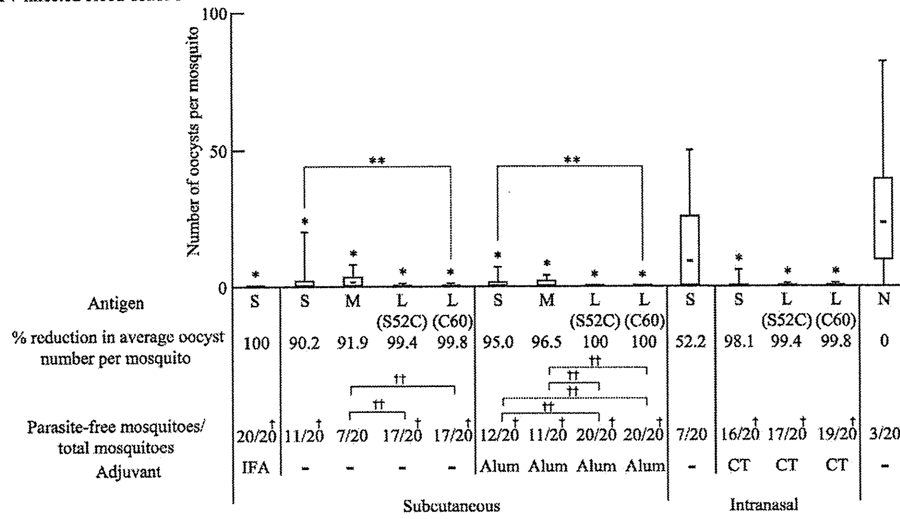


FIG. 5. Parasite recognition, IgG subclasses, and maintenance of the antisera induced by the COMP-Z-based tricomponent complex. The antisera obtained from the immunized mice in the experiments described in the legend to Fig. 4 were analyzed for parasite recognition (a) and IgG subclasses (b). (a) The ookinete-specific reactivities of the antisera induced by subcutaneous or intranasal immunization with the COMP-Z: Pvs25H-A tricomponent complex were determined by immunofluorescence analysis. The antisera specifically recognized native Pvs25 protein expressed on the surfaces of immature *Plasmodium vivax* ookinetes. (b) Pvs25H-A-specific IgG1 and IgG2a analysis of the antisera induced by the COMP-Z:Pvs25H-A tricomponent complex. (c) Mice were immunized as described in the legend to Fig. 4, and the Pvs25H-A-specific serum IgG responses over a prolonged period were evaluated. Antibody titers were defined as described in the legend to Fig. 4.

b) Cys residues per subunit, respectively. The recombinant Pvs25H-A protein expressed in the yeast *Pichia pastoris* (22) was chemically conjugated to the delivery molecules via the sulfhydryl groups of the Cys residues by a heterobifunctional cross-linker, SPDP (Fig. 3a). Since disulfide bonds in the Pvs25 protein are known to be important for vaccine function (16, 31, 32), the delivery molecules, but not the Pvs25H-A antigen, were treated with a reducing agent to expose free sulfhydryls, making them reactive with the pyridyldithiol groups added to Pvs25H-A (Fig. 3a). The human IgG-ELISA indicated that all complexes, but not the delivery molecules alone, reacted strongly with an anti-Pvs25 antiserum (Fig. 3b). In contrast, all proteins, except for Pvs25H-A, which could not be captured by the human IgG, reacted to the anti-His antibody, since each of them contained a hexahistidine (6×His) tag (Fig. 3b). These results indicated that delivery molecules that retained affinity for the Ig molecule were loaded with the Pvs25H-A antigen to generate the tricomponent complexes.

Immunogenicity of the tricomponent complexes. Female BALB/c mice (4 to 7 per group) were immunized with the Pvs25H-A antigen alone (designated S), a mixture of the antigen and the delivery molecules (designated M), or the antigen ligated to the delivery molecules (the tricomponent complexes, designated L), by the s.c. or i.n. route, with or without the indicated adjuvants, at weeks 0, 2, and 4, and antisera collected at week 6 were analyzed for the antigen-specific IgG (Fig. 4a and b [TB-based and COMP-based proteins, respectively]). We found that for s.c. immunization, (i) the tricomponent complexes consistently induced higher IgG responses than the antigen alone or the mixture of proteins, regardless of the adjuvant present; (ii) the mixture of the antigen with the COMP-Z, but not with the TB-Z, augmented the response; (iii) the COMP-based tricomponent complex induced higher responses than the TB-based tricomponent complexes; (iv) the COMP-based tricomponent complex without the addition of an extraneous adjuvant induced a greater response than that induced by the antigen emulsified with IFA. We also found that for i.n. immunization, the general trends were similar to those observed for s.c. immunization, but supplementation with CT was essential for induction of the response. We tested the i.p. and intravenous immunization routes and found no immune-enhancing effects (data not shown).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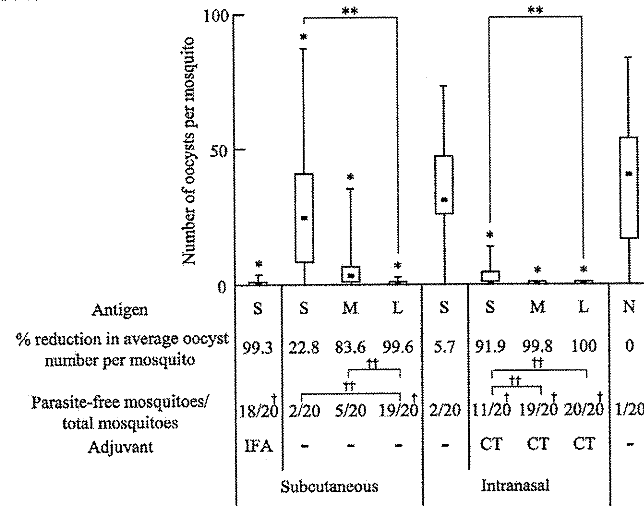
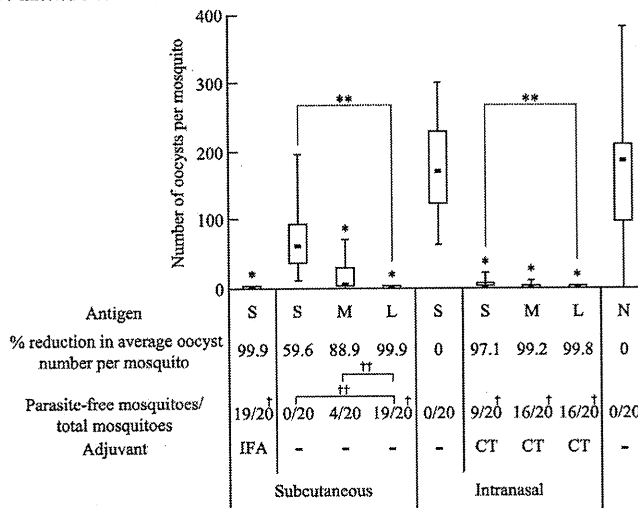
Number of oocysts per mosquito
100
50
0
Antigen
% reduction in average oocyst number per mosquito
Parasite-free mosquitoes/ total mosquitoes
Adjuvant
S S M L L S M L L S S L L N
(S52C) (C60) (S52C) (C60) (S52C) (C60)
100 90.2 91.9 99.4 99.8 95.0 96.5 100 100 52.2 98.1 99.4 99.8 0
20/20 11/20 7/20 17/20 17/20 12/20 11/20 20/20 20/20 7/20 16/20 17/20 19/20 3/20
IFA - - - - Alum Alum Alum Alum - CT CT CT -
Subcutaneous
Intranasal


b Pv-infected blood donor 2
Number of oocysts per mosquito
100
50
0
Antigen
% reduction in average oocyst number per mosquito
Parasite-free mosquitoes/ total mosquitoes
Adjuvant
S S M L S S M L N
99.3 22.8 83.6 99.6 5.7 91.9 99.8 100 0
18/20 2/20 5/20 19/20 2/20 11/20 19/20 20/20 1/20
IFA - - - - CT CT CT -
Subcutaneous
Intranasal


c Pv-infected blood donor 3
Number of oocysts per mosquito
400
300
200
100
0
Antigen
% reduction in average oocyst number per mosquito
Parasite-free mosquitoes/ total mosquitoes
Adjuvant
S S M L S S M L N
99.9 59.6 88.9 99.9 0 97.1 99.2 99.8 0
19/20 0/20 4/20 19/20 0/20 9/20 16/20 16/20 0/20
IFA - - - - CT CT CT -
Subcutaneous
Intranasal