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Linkage of Exogenous T-cell Epitopes to the 19-Kilodalton Region of *Plasmodium yoelii* Merozoite Surface Protein 1 ($MSP1_{19}$) Can Enhance Protective Immunity against Malaria and Modulate the Immunoglobulin Subclass Response to $MSP1_{19}$

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The degree of protection against *Plasmodium yoelii* asexual blood stages induced by immunization of mice with the 19-kDa region of merozoite surface protein 1 ($MSP1_{19}$) is H-2 dependent. As a strategy to improve the protection, mouse strains with disparate H-2 haplotypes were immunized with glutathione *S*-transferase (GST)–$MSP1_{19}$ proteins including either a universal T-cell epitope from tetanus toxin (P2) or an I-A^k-restricted T-cell epitope (P8) from *Plasmodium falciparum* Pf332. In H-2^k mice which are poorly protected following immunization with GST-$MSP1_{19}$, GST-P2-$MSP1_{19}$ significantly improved the protection. In mice partially (H-$2^{k/b}$) or well protected by GST-$MSP1_{19}$ (H-2^d and H-2^b), P2 did not further increase the protection. However, the protection of H-$2^{k/b}$ mice and to some extent H-2^k mice was improved by immunization with GST-P8-$MSP1_{19}$. The magnitudes of immunoglobulin G1 (IgG1) and IgG2a responses in mice immunized with the GST-$MSP1_{19}$ variants correlated with low peak parasitemia, indicating a protective capacity of these IgG subclasses. In H-2^k mice immunized with GST-P2-$MSP1_{19}$, both IgG1 and IgG2a responses were significantly enhanced. The epitope P2 appeared to have a general ability to modulate the IgG subclass response since all four mouse strains displayed elevated IgG2a and/or IgG2b levels after immunization with GST-P2-$MSP1_{19}$. In contrast, GST-P8-$MSP1_{19}$ induced a slight enhancement of IgG responses in H-$2^{k/b}$ and H-2^k mice without any major shift in IgG subclass patterns. The ability to improve the protective immunity elicited by *P. yoelii* $MSP1_{19}$ may have implications for improvement of human vaccines based on *P. falciparum* $MSP1_{19}$.

A number of malaria proteins have been proposed as candidates for inclusion in subunit vaccines against asexual blood stages of *Plasmodium falciparum* (30). One of the most promising antigens in this respect is the 19-kDa C-terminal region of merozoite surface protein 1 (MSP1). *P. falciparum* MSP1 is expressed on the surfaces of merozoites and is proteolytically processed in two steps to generate first a complex of polypeptides including a 42-kDa fragment ($MSP1_{42}$) that is subsequently cleaved to a 19-kDa fragment ($MSP1_{19}$) and a 33-kDa fragment ($MSP1_{33}$). The complex containing $MSP1_{33}$ is shed (7), and $MSP1_{19}$ is the only part of MSP1 that remains on the merozoite surface during parasite invasion of red blood cells (RBC) (6). The $MSP1_{19}$ region consists of two epidermal growth factor-like domains (8). A limited number of allelic variants of *P. falciparum* $MSP1_{19}$ occur, with minor amino acid differences between them, making $MSP1_{19}$ one of the most conserved regions of *P. falciparum* MSP1 (48, 57). Importantly, MSP1 is expressed in other *Plasmodium* species and the processing that generates $MSP1_{19}$ appears to be similar (5, 17, 29, 43), enabling the use of various malaria parasites and animal models for the study of $MSP1_{19}$-induced protection.

Both antibodies and effector T cells appear to play important roles in the protection of mice against *Plasmodium yoelii* (4, 23, 28). However, the protective immunity induced by vaccination with recombinant *P. yoelii* $MSP1_{19}$ is mediated largely by antibodies (14, 28, 36). Passive transfer of $MSP1_{19}$-specific antiserum or monoclonal antibodies (MAb) into naive mice suppressed an otherwise lethal *P. yoelii* infection (14, 39, 55), whereas vaccination with $MSP1_{19}$ failed to protect mice deficient in immunoglobulin μ-chains (28). In contrast, immunization with defined $MSP1_{19}$-derived T-cell epitopes does not induce protective immunity and transfer of T-cell lines from $MSP1_{19}$-immunized mice does not confer protection to naive recipients (58). Nevertheless, a functional $CD4^+$ T-cell response is a prerequisite for the generation of potent humoral responses by vaccination with protein antigens.

Several parameters can be modified in order to enhance the protection induced by vaccination with pathogen-derived proteins or protein subunits. Optimized immunization protocols as well as the use of different adjuvants have been shown to have an impact on protective immunity elicited by *P. yoelii* $MSP1_{19}$ (15, 28, 38). Another strategy would be to link additional T-cell epitopes to $MSP1_{19}$ to overcome limited recognition of T-helper-cell epitopes. This procedure may be suitable for improving the H-2-dependent protection against lethal *P. yoelii* infection induced by glutathione *S*-transferase (GST)–$MSP1_{19}$ fusion proteins (59). In inbred mouse strains, GST-$MSP1_{19}$ elicited a higher degree of protection in C57BL/10 (H-2^b) mice than in B10.A(4R) mice (H-$2^{k/b}$), and B10.BR mice (H-2^k) were poorly protected (59). Partial protection against *P. falciparum* and *Plasmodium vivax* has been obtained in monkeys vaccinated with the respective $MSP1_{19}$ proteins linked to T-cell epitopes from tetanus toxoid (TT) (34, 61). However, the impact of the T-cell epitopes on protective immune responses is uncertain, as comparisons with $MSP1_{19}$ alone were not made. Moreover, expression of *P. falciparum*

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TABLE 1. Titers of IgG to $MSP1_{19}$ in mice immunized with GST-$MSP1_{19}$ with or without additional T-cell epitopes, as measured by ELISA

Mouse strain	H-2 haplotype[a]					Mean titer of IgG (10^3) to $MSP1_{19}$[b] ± SD for immunogen		
	K (I)	I-A (II)	I-E (II)	S (III)	D (I)	GST-$MSP1_{19}$	GST-P2-$MSP1_{19}$	GST-P8-$MSP1_{19}$
B10.A(4R)	k	k	(b)	b	b	150 ± 60	330 ± 110	190 ± 80
B10.BR	k	k	k	k	k	115 ± 40	280 ± 55	135 ± 95
C57BL/6	b	b	(b)	b	b	270 ± 100	270 ± 95	nd[c]
B10.D2	d	d	d	d	d	290 ± 115	220 ± 25	nd

[a] Classes (I to III) are in parentheses. (b), the class II molecule I-E^b is not expressed.
[b] Individual sera from five mice per group immunized i.p. were titrated for reactivity with $MSP1_{19}$. The reciprocal titers were estimated as the dilution corresponding to an absorbance value of 0.6. Mean reactivities of serum plus two standard deviations from all groups of mice immunized with GST/MAP-P2 were below 0.3 at dilutions of 1:1,000.
[c] nd, not done.

$MSP1_{19}$ in the yeast *Saccharomyces cerevisiae* resulted, to a large extent, in proteolytic cleavage of the linked T-cell epitopes (34).

To evaluate a strategy to improve the protection induced by *P. yoelii* $MSP1_{19}$, GST fusion proteins containing $MSP1_{19}$ were expressed in bacteria with or without additional T-cell epitopes inserted between the GST and $MSP1_{19}$. The epitopes selected were the universal TT-derived epitope P2 (44) and an I-A^k-restricted epitope (P8) from the *P. falciparum* antigen Pf332 (3). The proteins were used for immunization of four H-2 congenic mouse strains, of haplotypes H-2^b, H-2^d, H-2^k, and H-$2^{k/b}$ (I-A^k/I-E^b). Immune responses after vaccination and protection against parasite challenge were assessed. The results demonstrate that insertion of T-cell epitopes into $MSP1_{19}$-based immunogens can enhance the immunoglobulin G (IgG) responses to $MSP1_{19}$ and can improve the protection against malaria parasite challenge. Additionally, it is shown that the P2 epitope, but not the P8 epitope, modulates the IgG subclass response to $MSP1_{19}$.

MATERIALS AND METHODS

Construction of plasmids encoding variants of recombinant GST-$MSP1_{19}$. A plasmid encoding GST-$MSP1_{19}$ (36), residues 1649 to 1754, of *P. yoelii* YM MSP1 (35) was modified to encode proteins with T-cell epitopes inserted between GST and $MSP1_{19}$. A *Bam*HI site in the plasmid, between the GST and the $MSP1_{19}$ genes, was digested with *Bam*HI (Promega, Madison, Wis.), and the plasmid was dephosphorylated using alkaline phosphatase (Boehringer GmbH, Mannheim, Germany). Complementary oligonucleotides (VH BIO, Newcastle, United Kingdom) encoding the epitope P2 (IQYIKANSKFIGITEL) or P8 (EEGPVDEEIVQEEGTV) were annealed by heating to 95°C followed by slow cooling to 20°C. The duplex oligonucleotide was phosphorylated using T4 polynucleotide kinase (Promega) and ligated into the *Bam*HI site of the plasmid using T4 DNA ligase (Promega). Ligation reactions with a fivefold molar excess of oligonucleotide were used to transform *Escherichia coli* strain DH5α. The oligonucleotides used were 5′-GAT CCA GTA CAT CAA AGC TAA CTC CAA ATT CAT CGG TAT CAC CGA ACT GGG TCA GGT-3′ (P2 sense), 5′-GAT C**A**C CTG ACC CAG TTC GGT GAT ACC GAT GAA TTT GGA GTT AGC TTT GAT GTA CTG-3′ (P2 antisense), 5′-GAT CCT GGA AGA AGG TCC GGT TGA CGA AGA AAT CGT TCA GGA AGA AGG TAC CGT-3′ (P8 sense), and 5′-GAT C**A**C GGT ACC TTC TTC CTG AAC GAT TTC TTC GTC AAC CGG ACC TTC TTC CAG-3′ (P8 antisense) (nucleotides in boldface ensured re-creation of the *Bam*HI site in the 5′ ends of correct inserts and in the 3′ ends of reversed inserts; underlined nucleotides encode a stop codon in reversed inserts). To identify plasmids with inserts, proteins expressed by transformed bacterial clones were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11). Plasmids with correct inserts resulted in the expression of proteins larger than GST-$MSP1_{19}$, and those with reversed inserts resulted in the expression of GST only. The number of inserts was determined by *Bam*HI and *Eco*RI (Promega) restriction of plasmids. The *Eco*RI site is located immediately after the $MSP1_{19}$ sequence, and excised DNA fragments with correct inserts included the $MSP1_{19}$ sequence plus 50 to 60 bp for each insert, unlike the DNA fragments from plasmids with reversed inserts or the parental plasmid. Plasmids encoding GST-P2-$MSP1_{19}$ including two copies of P2 and GST-P8-$MSP1_{19}$ including one copy of P8 were identified, and the inserts were confirmed by DNA sequencing.

Production, purification, and analysis of recombinant proteins. Recombinant proteins were produced as previously described, with modifications (36, 54). Briefly, protein expression in transformed *E. coli* DH5α cells was induced with 1 mM isopropylthio-β-galactosidase (Sigma) for 3 h at 37°C. The cells were harvested and lysed in phosphate-buffered saline (PBS). After centrifugation, the supernatant was incubated with glutathione-agarose (Sigma). Fusion proteins were eluted with 10 mM reduced glutathione (Sigma) and dialyzed against PBS. Solubilization of GST-P2-$MSP1_{19}$ required sonication of bacteria in 2 M urea. The corresponding supernatant was diluted to 0.5 M urea in PBS to enable binding to glutathione-agarose beads. The beads were washed with decreasing concentrations of urea in PBS and eventually with pure PBS before elution and dialysis as described above. To obtain GST-free $MSP1_{19}$, GST-$MSP1_{19}$ was bound to glutathione-agarose and treated for 12 h at 22°C with factor Xa (Sigma), which cleaves a site right after the C terminus of GST. Factor Xa was removed by incubation with *p*-aminobenzamidine-agarose (Sigma). Simultaneous incubation with glutathione-agarose ensured that any contaminating GST was removed before dialysis against PBS. Protein concentrations were determined by multiplying the absorbance at 280 nm with a factor calculated for each protein according to protein size and content of aromatic amino acids (26). The GST-$MSP1_{19}$ variants were further analyzed by Western blotting (11), and GST-free $MSP1_{19}$ was subjected to a dot blot assay (2) using the $MSP1_{19}$-specific MAb B10 and F5 (55).

MAPs. Multiple-antigen peptide (MAP) constructs (56) were made as control immunogens as well as for measurement of specific antibodies. Synthesis and analysis of MAP constructs were performed as described previously (1). The MAP constructs had four identical peptide chains synthesized in parallel on the tetrameric lysine core. Amino acid analysis confirmed the right amino acid composition of each MAP. The sequences of MAP-P2 and MAP-P8 corresponded to those of the P2 and P8 epitopes in the recombinant proteins, respectively.

***P. yoelii* parasites.** Lethal *P. yoelii* YM parasites (63) were kindly provided by D. Walliker, Edinburgh University, and were kept in liquid nitrogen and by passage in mice. All infected mice were given drinking water supplemented with 2.5 g of *p*-aminobenzoic acid/liter (47).

Immunization and parasite challenge of mice. Female B10.A(4R), B10.BR, C57BL/6, and B10.D2 mice (Harlan, Oxford, United Kingdom) (Table 1) were used at 7 to 10 weeks of age. Groups of five mice were injected intraperitoneally (i.p.) with 40 μg of GST-$MSP1_{19}$, GST-P2-$MSP1_{19}$, or a mixture of GST and MAP-P2 emulsified in complete Freund's adjuvant (Sigma). B10.A(4R), B10.BR, and C57BL/6 mice were also immunized with GST-P8-$MSP1_{19}$, GST, or MAP-P8. Booster injections with 40 μg of immunogen in incomplete Freund's adjuvant (Sigma) were given at weeks 3 and 6, and blood to obtain serum was drawn from the tail at week 8. One week later, mice were inoculated intravenously with 10^4 *P. yoelii*-infected RBC. Giemsa-stained blood smears were made daily from day 3 of the infection. Parasitemia was assessed by counting the percentage of infected RBC using light microscopy. Two additional groups of B10.A(4R) mice were immunized subcutaneously (s.c.) in the base of the tail with either GST-$MSP1_{19}$ or GST-free $MSP1_{19}$. Otherwise, the immunizations were made as described above.

Assessment of antibody responses. For the enzyme-linked immunosorbent assay (ELISA), GST-free $MSP1_{19}$, GST, or MAP constructs were adsorbed at 2 μg/ml in PBS to Immulon 4 plates (Dynatech Laboratories, Inc., Billingshurst, United Kingdom) overnight at 8°C. The wells were blocked with 1% bovine serum albumin in PBS for 1 h at room temperature, and then duplicates of serum diluted in PBS with 0.1% bovine serum albumin and 0.05% Tween were added, and the wells were incubated for 1 h at 37°C. Between incubations, wells were washed with PBS containing 0.05% Tween. Specific antibodies were detected by incubation with rabbit anti-mouse IgG (Sigma), goat anti-mouse IgG1, IgG2b, or IgG3 (Southern Biotechnology, Birmingham, Ala.), or sheep anti-mouse IgG2a (The Binding Site, Birmingham, United Kingdom) conjugated to horseradish peroxidase. Absorbance was measured at 492 nm after adding *o*-phenylenediamine (Sigma) as the chromogen and H_2O_2 as the substrate and later 1 M sulfuric acid to stop the reaction. Analyses were standardized by including a pool of mouse antisera to GST-$MSP1_{19}$ in all assays.

For indirect immunofluorescence antibody assay (IFAT), blood was drawn from *P. yoelii* YM-infected mice into heparinized tubes. The blood was washed and diluted 1:200 in phosphate-saline buffer with 0.2% glucose. Ten microliters of cell suspension was added to wells of 15-well multitest slides (ICN Biomedi-

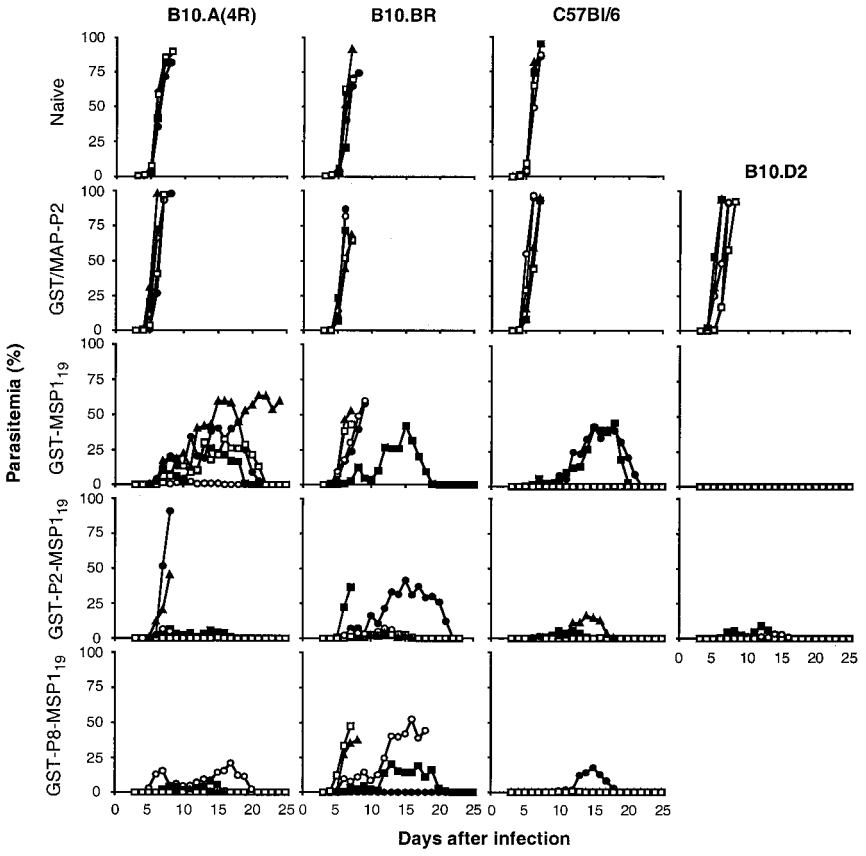

FIG. 1. Parasite challenge of mice immunized with GST-MSP1$_9$ with or without additional T-cell epitopes. Two weeks after the third injection of immunogen, groups of five mice were inoculated intravenously with 10^4 *P. yoelii* YM-infected RBC. Parasitemia was monitored daily from day 3. B10.BR, B10.A(4R), and C57BL/6 mice were either naive or were immunized with GST-MSP1$_{19}$, GST-P2-MSP1$_{19}$, GST-P8-MSP1$_{19}$, or a mixture of GST and MAP-P2; B10.D2 mice were immunized with GST-MSP1$_{19}$, GST-P2-MSP1$_{19}$, or GST/MAP-P2. All groups were analyzed in parallel except for the C57BL/6 mice immunized with GST-P8-MSP1$_{19}$, which were analyzed in a separate experiment where C57BL/6 immunized with GST-MSP1$_{19}$ or GST-P8-MSP1$_{19}$ were found to be similarly protected (maximal parasitemias for the GST-MSP1$_{19}$ group were <0.001 [two mice], 0.22, 0.44, and 1.7% and for the GST-P8-MSP1$_{19}$ group were <0.001, 0.16, 0.18, 0.65, and 17%).

cals, Inc., Aurora, Ohio) that were air dried before storage at −20°C. Prior to the staining, the slides were fixed with acetone for 2 min and blocked in PBS with 1% bovine serum albumin for 30 min at room temperature. The wells were incubated for 1 h at 37°C with serum in consecutive twofold dilutions in PBS. Specific antibodies were detected by incubation for 1 h at 37°C with fluorescein isothiocyanate-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology). Between incubations the slides were washed in PBS. Titers were determined as the reciprocal of the last positive dilution when analyzed by UV microscopy.

Competition ELISA. Wells were adsorbed with MSP1$_{19}$ or 1 μg of native MSP1/ml from *P. yoelii* YM as described above. Native MSP1 was purified as previously described (29). The wells were blocked and incubated with duplicates of serum dilutions, followed by incubation with biotinylated MAb at concentrations established by titration. MAb B10, F5, and D3 (55) were biotinylated by mixing the MAb in PBS with sulfosuccimidyl-6-(biotinamido)-hexanoate (Pierce, Rockford, Ill.) for 1 h at room temperature, followed by dialysis against PBS. Bound MAb was detected by incubation with 1 μg of avidin-peroxidase (Sigma)/ml and addition of the substrate as described above. The level of competition was estimated by comparison with wells incubated with biotinylated MAb only.

Statistical analyses. Statistical analyses were made by linear regression or Student's *t* test (two-tailed, unpaired).

RESULTS

Production and analyses of recombinant proteins. Recombinant GST-MSP1$_{19}$ with or without T-cell epitopes inserted between GST and MSP1$_{19}$ were made. GST-P2-MSP1$_{19}$ included two copies of the universal epitope P2, and GST-P8-MSP1$_{19}$ included one copy of the I-A^k-restricted epitope P8. According to SDS-PAGE, GST-MSP1$_{19}$ and variants thereof were predominantly full-length proteins and MSP1$_{19}$, from which GST had been removed, was free from contamination. Proteins including P2 or P8 displayed increased relative molecular masses compared to GST-MSP1$_{19}$ (42 kDa). Insertion of one P8 epitope resulted in an increase of 5 kDa, which is larger than expected (theoretically 1.5 kDa), most likely because of the highly negative charge of P8 (3). Insertion of two copies of P2 led to an expected increase of 3 kDa. MAb B10 and F5, which recognize parasite-derived MSP1$_{19}$ (55), bound all GST-MSP1$_{19}$ variants in Western blotting, provided the SDS-PAGE separation was performed under nonreducing conditions. MAb B10 and F5 also recognized GST-free MSP1$_{19}$ in a dot blot assay.

***P. yoelii* challenge in mice immunized with GST-MSP1$_{19}$ variants.** B10.A(4R) and B10.BR mice immunized with GST/MAP-P2, GST-MSP1$_{19}$, GST-P2-MSP1$_{19}$, and GST-P8-MSP1$_{19}$ were challenged with 10^4 *P. yoelii* YM-infected RBC (Fig. 1). Naive control mice and the mice immunized with GST/MAP-P2 rapidly developed high parasitemias and succumbed to the infection at days 7 to 9. Similarly, B10.A(4R) and B10.BR mice immunized with GST or MAP-P8 were not protected (data not shown). B10.A(4R) mice immunized with GST-MSP1$_{19}$ quickly developed high levels of parasites, but only one of the mice failed to clear the parasites and died at day 25. The protection of B10.BR mice immunized with GST-MSP1$_{19}$ was poor. Four mice died between days 8 and 10; one mouse survived but had a peak parasitemia of 42%. In B10.A

(4R) mice, immunization with GST-P2-$MSP1_{19}$ resulted in good protection in three mice, which had parasitemias below 7%, but two mice developed high parasitemias and died at day 9. Immunization with GST-P8-$MSP1_{19}$ resulted in the survival of all five B10.A(4R) mice, with significantly lower peak parasitemias than were observed in the GST-$MSP1_{19}$ group ($P = 0.027$). In B10.BR mice, GST-P2-$MSP1_{19}$ induced better protection than GST-$MSP1_{19}$, in terms of increased survival as well as lower peak parasitemias ($P = 0.0074$). Four out of five mice survived, and three of these had parasitemias below 5%. In B10.BR mice, GST-P8-$MSP1_{19}$ did not significantly improve protection although two mice survived and the death of one mouse was delayed (day 19).

Levels of protection induced by GST-$MSP1_{19}$ and GST-P2-$MSP1_{19}$ in C57BL/6 and B10.D2 mice were also compared (Fig. 1). After immunization with GST-$MSP1_{19}$, C57BL/6 mice were reasonably well protected, with peak parasitemias below 0.05% in three mice, although two mice slowly developed high parasitemias, which were cleared by day 23. B10.D2 mice were well protected, and only two mice had detectable parasitemias. There was no significant change in C57BL/6 and B10.D2 mice immunized with GST-P2-$MSP1_{19}$. Immunization of C57BL/6 mice with GST-P8-$MSP1_{19}$ also did not change the level of protection.

IgG responses to $MSP1_{19}$ following immunization with GST-$MSP1_{19}$ constructs. To investigate the relationship between prechallenge IgG levels and protection, antisera from mice immunized with GST-$MSP1_{19}$ variants were analyzed for IgG reactivity with $MSP1_{19}$ by ELISA (Table 1). Following immunization with GST-$MSP1_{19}$, C57BL/6 and B10.D2 mice had approximately two- to threefold-higher $MSP1_{19}$-specific IgG levels than B10.BR mice ($P = 0.0061$ and 0.0052, respectively) and B10.A(4R) mice ($P = 0.032$ and 0.032, respectively). The IgG titers in B10.A(4R) and B10.BR mice were increased two- to threefold after immunization with GST-P2-$MSP1_{19}$ compared to titers for mice immunized with GST-$MSP1_{19}$ ($P = 0.006$ and 0.001, respectively), whereas in C57BL/6 and B10.D2 mice, IgG titers were no different or lower in GST-P2-$MSP1_{19}$ antisera. B10.A(4R) and B10.BR antisera to GST-P8-$MSP1_{19}$ displayed only slightly elevated $MSP1_{19}$-specific IgG levels compared to sera from GST-$MSP1_{19}$-immunized mice.

In all mice taken together, there was a significant inverse correlation of peak parasitemias and IgG titers to $MSP1_{19}$ ($r = 0.53$; $n = 50$; $P \leq 0.0001$).

IgG subclass responses in mice immunized with $MSP1_{19}$-containing immunogens. The IgG subclass distribution of antibodies to $MSP1_{19}$ in prechallenge antisera from mice immunized with GST-$MSP1_{19}$ was examined by ELISA and was found to differ between mouse strains although, in all mice, IgG1 was detected at higher dilutions than other subtypes (IgG2b > IgG2a > IgG3) (Fig. 2). In Fig. 2, the mean serum reactivities for each mouse strain are compared at a serum dilution appropriate for each IgG subclass (IgG1, $1:2^{18}$; IgG2a, $1:2^{12}$; IgG2b, $1:2^{17}$; IgG3, $1:2^{10}$). C57BL/6 and B10.D2 mice displayed significantly higher IgG1 reactivity than both B10.A(4R) ($P = 0.015$ and 0.0007, respectively) and B10.BR mice ($P = 0.0038$ and 0.0005, respectively). B10.D2 mice also had significantly higher levels of IgG2a than B10.A(4R) ($P = 0.028$) and B10.BR mice ($P = 0.0009$). No significant differences between IgG2b and IgG3 levels were observed.

In mice immunized with GST-$MSP1_{19}$, there was an inverse correlation between peak parasitemia and the levels of IgG1 ($r = 0.73$; $n = 20$; $P = 0.0003$) and IgG2a ($r = 0.50$; $n = 20$; $P = 0.026$), but not levels of IgG2b or IgG3.

In mice vaccinated with GST-P2-$MSP1_{19}$, the IgG2a and IgG2b levels were elevated compared to levels in mice immunized with GST-$MSP1_{19}$ (Fig. 2). The increases of both IgG2a and IgG2b levels in B10.BR mice were significant ($P = 0.0008$ and $P \leq 0.0001$, respectively), as was the increase of IgG2a in B10.D2 ($P = 0.013$) mice and the increases of IgG2b in B10.A(4R) ($P = 0.0003$) and C57BL/6 ($P = 0.0073$) mice. IgG1 levels were slightly increased in B10.(4R) and B10.BR mice and significantly decreased in C57BL/6 ($P = 0.027$) and B10.D2 mice ($P = 0.0039$). IgG3 was enhanced in all mice except B10.A(4R) mice after immunization with GST-P2-$MSP1_{19}$, but only significantly so in B10.BR mice ($P = 0.0003$). No significant change of any IgG subclass was observed in antisera to GST-P8-$MSP1_{19}$ (Fig. 4). An analysis of all groups of mice together found a significant association between low maximal parasitemia and high IgG1 ($r = 0.43$; $n = 50$; $P = 0.0019$) or IgG2a levels ($r = 0.49$; $n = 50$; $P = 0.0003$), but not IgG2b or IgG3 levels. Importantly, the levels of IgG1, IgG2a, and IgG2b detected by IFAT correlated with the reactivities of the corresponding IgG subclasses in the ELISA, confirming that the IgG levels determined by ELISA reflected reactivity with parasite-derived $MSP1_{19}$ (Table 2).

In order to determine if the insertion of P2 into GST-$MSP1_{19}$ also affected the IgG subclass response to the GST fusion partner, B10.BR antisera to GST fusion proteins were analyzed by ELISA (Fig. 3). The levels of IgG reactivity with GST in antisera to GST-$MSP1_{19}$ were lower than the levels of $MSP1_{19}$-reactive IgG but displayed a similar IgG subclass distribution. Interestingly, antiserum to GST-P2-$MSP1_{19}$ had increased levels of IgG2a and IgG2b also to GST, in comparison with antisera to GST-$MSP1_{19}$, GST-P8-$MSP1_{19}$, or GST/MAP-

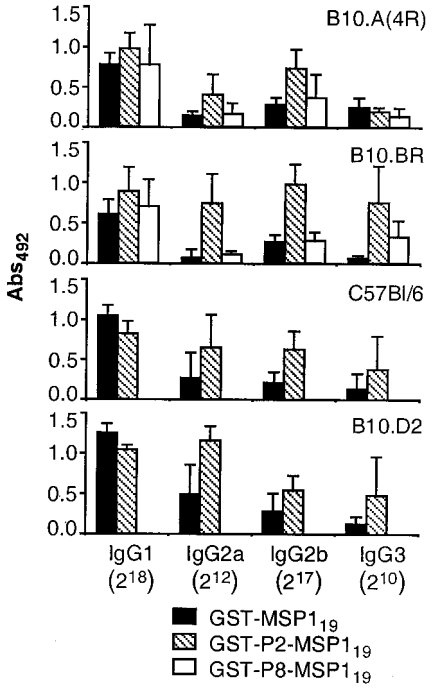

FIG. 2. $MSP1_{19}$-specific IgG subclass responses in mice immunized with GST-$MSP1_{19}$ with or without additional T-cell epitopes as measured by ELISA. Groups of five B10.A(4R), B10.BR, C57BL/6, and B10.D2 mice were immunized with GST-$MSP1_{19}$, GST-P2-$MSP1_{19}$, or GST-P8-$MSP1_{19}$ [B10.A(4R) and B10.BR mice only]. The bars display mean reactivities plus standard deviations of sera analyzed for reactivity with $MSP1_{19}$ at dilutions of $1:2^{18}$ (IgG1), $1:2^{12}$ (IgG2a), $1:2^{17}$ (IgG2b), or $1:2^{10}$ (IgG3). No significant reactivity was displayed by sera from GST-immunized or naive mice at these dilutions (not shown). Abs_{492}, absorbance at 492 nm.

TABLE 2. IgG subclass titers in mice immunized with GST-$MSP1_{19}$ variants as measured by IFAT

Mouse strain	Immunogen	Mean IFAT titer (10^3)[a] ± SD for:			
		IgG1	IgG2a	IgG2b	IgG3
B10.A(4R)	GST-$MSP1_{19}$	4.9 ± 1.8	—[b]	0.4 ± 0.4	—
	GST-P2-$MSP1_{19}$	6.6 ± 2.2	0.4 ± 0.1	1.3 ± 0.7	—
	GST-P8-$MSP1_{19}$	5.7 ± 2.2	—	0.4 ± 0.1	—
B10.BR	GST-$MSP1_{19}$	2.5 ± 0.9	—	0.2 ± 0.1	—
	GST-P2-$MSP1_{19}$	5.7 ± 2.2	0.4 ± 0.4	1.0 ± 0.6	—
	GST-P8-$MSP1_{19}$	3.7 ± 2.9	—	0.3 ± 0.1	—
C57BL/6	GST-$MSP1_{19}$	11.5 ± 4.5	0.2 ± 0.1	0.3 ± 0.1	—
	GST-P2-$MSP1_{19}$	7.4 ± 1.8	0.5 ± 0.4	1.8 ± 0.5	—
B10.D2	GST-$MSP1_{19}$	19.7 ± 7.3	0.3 ± 0.2	0.5 ± 0.4	—
	GST-P2-$MSP1_{19}$	10.7 ± 5.5	0.7 ± 0.3	1.2 ± 0.8	—

[a] Sera from five mice per group were titrated for IgG subclass reactivity in IFAT. The titers were estimated as the reciprocal of the last positive dilution. Sera from mice immunized with GST/MAP-P2 were negative for all subclasses at the lowest dilution tested (1:64).

[b] —, no reactivity at the lowest dilution tested.

P2, suggesting that P2 influenced the IgG subclass response to GST as well as $MSP1_{19}$.

Moreover, B10.A(4R) antisera to GST-$MSP1_{19}$ and GST-free $MSP1_{19}$ were compared for reactivity with $MSP1_{19}$ (Fig. 4). Notably, these two groups of mice were immunized by s.c. injections, which resulted in slightly higher IgG responses to $MSP1_{19}$ than those in mice immunized i.p. (data not shown). Irrespective of that finding, the levels of IgG subclasses in the two groups immunized s.c. with GST-$MSP1_{19}$ and $MSP1_{19}$ were similar, indicating that the presence of GST did not influence the IgG subclass response to $MSP1_{19}$ (Fig. 4).

Analysis of the epitope specificity induced by the GST-$MSP1_{19}$ variants. A competitive ELISA was used to analyze whether antisera from the different immunization groups differed not only in levels of $MSP1_{19}$-specific IgG but also in their specificities for known, protective epitopes. Sera from mice immunized with GST-$MSP1_{19}$ variants were tested for the capacity to inhibit the binding of MAb F5 and B10 to $MSP1_{19}$ or MAb D3 to purified native MSP1 protein. All three MAb confer partial protection when passively transferred to mice (55). MAb F5 recognizes an epitope in the first domain of

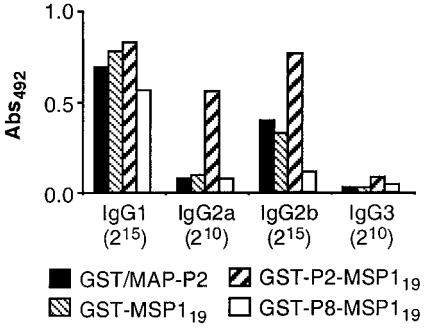

FIG. 3. GST-specific IgG subclass responses in B10.BR mice immunized with GST fusion proteins, as determined by ELISA. Groups of five mice were immunized with GST/MAP-P2, GST-$MSP1_{19}$, GST-P2-$MSP1_{19}$, or GST-P8-$MSP1_{19}$. The bars display reactivities of pooled sera analyzed for reactivity with GST at dilutions of $1:2^{15}$ (IgG1 and IgG2b) or $1:2^{10}$ (IgG2a and IgG3). Sera from naive mice did not react significantly at these dilutions (not shown). Abs_{492}, absorbance at 492 nm.

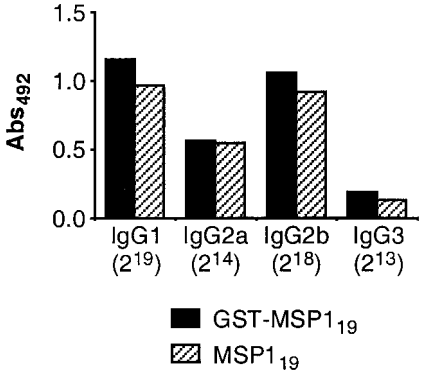

FIG. 4. Comparison of $MSP1_{19}$-specific IgG subclass responses in B10.A(4R) mice immunized with GST-$MSP1_{19}$ or $MSP1_{19}$ as measured by ELISA. Groups of five mice were immunized s.c. with either GST-$MSP1_{19}$ or GST-free $MSP1_{19}$. The bars display mean reactivities of sera analyzed for reactivity with $MSP1_{19}$ at dilutions of $1:2^{19}$ (IgG1), $1:2^{14}$ (IgG2a), $1:2^{18}$ (IgG2b), or $1:2^{13}$ (IgG3). Abs_{492}, absorbance at 492 nm.

$MSP1_{19}$; B10 requires both domains together for binding, and D3 binds *P. yoelii* $MSP1_{42}$ but not $MSP1_{19}$ or $MSP1_{33}$ separately (55). All sera inhibited, to a variable extent, the binding of MAb F5 or B10 to $MSP1_{19}$ (Fig. 5), whereas no inhibition of MAb D3 binding to MSP1 was observed. The inhibition of both MAb F5 and B10 was strongly associated with the levels of $MSP1_{19}$-reactive IgG in the sera (Fig. 5). Thus, although

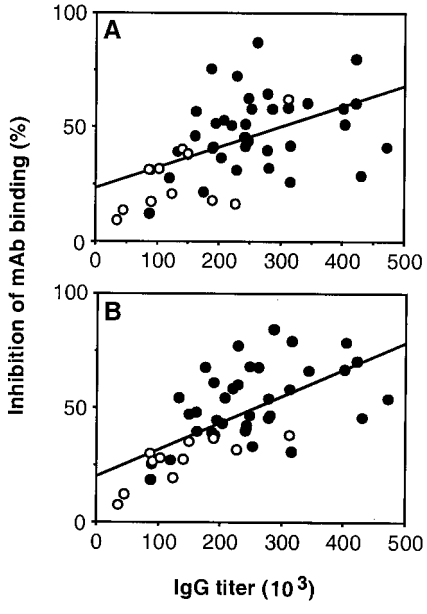

FIG. 5. Relationship between the $MSP1_{19}$-specific IgG titers of antisera to GST-$MSP1_{19}$ variants and the capacity to inhibit the binding of MAb F5 or MAb B10 to $MSP1_{19}$. The $MSP1_{19}$-specific titers of sera from B10.A(4R), B10.BR, C57BL/6, and B10.D2 mice immunized with GST-$MSP1_{19}$, GST-P2-$MSP1_{19}$, or GST-P8-$MSP1_{19}$ [B10.A(4R) and B10.BR mice]) were determined by ELISA. To establish the capacity of the sera to inhibit the binding of MAb to $MSP1_{19}$, ELISA plates were incubated first with serum diluted 1:600 and subsequently with biotinylated MAb F5 (A) or MAb B10 (B). The IgG titers of individual sera are plotted against the level of competition obtained with the corresponding sera. Open circles, mice that died following challenge infection. The lines display the association between IgG titer and inhibition of MAb F5 ($r = 0.52$; $n = 50$; $P \leq 0.0001$) (A) and MAb B10 ($r = 0.66$; $n = 50$; $P \leq 0.0001$) (B). The binding of biotinylated MAb could be inhibited by adding an excess of nonlabeled homologous but not heterologous MAb (not shown).

sera from well-protected mice displayed higher competition, the results do not suggest that the competitive capacity varied between groups due to major differences in epitope specificity. Competition with both MAb B10 and F5 was also inversely associated with peak parasitemia in the corresponding mice ($r = 0.49$, $n = 50$, $P \leq 0.0001$, and $r = 0.58$, $n = 50$, $P \leq 0.0001$, respectively).

DISCUSSION

Numerous studies have indicated the protective potential of the malaria antigen $MSP1_{19}$. The levels of antibodies in malaria-exposed humans to *P. falciparum* $MSP1_{19}$, or epitopes within $MSP1_{19}$, are associated with clinical immunity (20, 51, 52). Moreover, immunization of monkeys with antigens that include either *P. falciparum* or *Plasmodium cynomolgi* $MSP1_{19}$ confers protection (12, 34, 46), although protection has not always been achieved or has been insufficient (10, 12, 34). Similarly, inbred mouse strains can be protected against *P. yoelii* by immunization with GST linked to *P. yoelii* $MSP1_{19}$ (16, 36) but are differently protected in an H-2-dependent manner; C57BL/10 ($H\text{-}2^b$) mice are better protected than B10.A(4R) ($H\text{-}2^{k/b}$) and B10.BR ($H\text{-}2^k$) mice (59). The genetically restricted protection induced by GST-$MSP1_{19}$ was previously assessed using residues 1619 to 1754 of *P. yoelii* MSP1 (59). We found that using a shorter fragment of MSP1 (residues 1649 to 1754), still including both domains of $MSP1_{19}$ that are essential for induction of protection (37), did not change the relative protection observed in C57BL/6 ($H\text{-}2^b$), B10.A(4R), and B10.BR mice. We also found that B10.D2 ($H\text{-}2^d$) mice were similarly or slightly better protected than $H\text{-}2^b$ mice.

As a strategy to improve the protection induced by *P. yoelii* $MSP1_{19}$, we inserted additional T-cell epitopes into GST-$MSP1_{19}$ and compared the protective capacities of these immunogens with GST-$MSP1_{19}$. Insertion of the TT-derived T-cell epitope P2 in GST-$MSP1_{19}$ led to increased survival and protection against *P. yoelii* challenge infection in B10.BR mice, a mouse strain otherwise not protected by immunization with GST-$MSP1_{19}$. Inclusion of the $I\text{-}A^k$-restricted epitope P8 markedly improved the ability of B10.A(4R) mice to suppress parasitemia but had a lesser effect on B10.BR mice, suggesting that differences at the I-E locus or a minor histocompatibility locus may affect presentation of the P8 epitope. In mice well protected by GST-$MSP1_{19}$ (C57BL/6 and B10.D2 mice), insertion of P2 in the immunogen had no significant effect on protection. The course of infection in mice immunized with MAP constructs containing P2 or P8 was similar to that in GST-immunized or naive mice, demonstrating that immune responses to these epitopes were not protective.

The P2 epitope has been described as a universal T-cell epitope, and T cells from most TT-vaccinated humans recognize it (44). When tested with B10.A(4R), B10.BR, C57BL/6, and DBA/2 ($H\text{-}2^d$) mice, incorporation of P2 in a peptide immunogen enhanced the total IgG responses to otherwise poorly immunogenic B-cell epitopes in all mouse strains (33). We have shown here that insertion of P2 into GST-$MSP1_{19}$ led to enhanced total IgG responses to $MSP1_{19}$ in B10.A(4R) and B10.BR mice but not in C57BL/6 and B10.D2 mice. However, in all mouse strains, GST-P2-$MSP1_{19}$ induced significantly increased levels of $MSP1_{19}$-reactive IgG2a and/or IgG2b. The ability to modulate the IgG subclass response may be a general feature of P2, as IgG2a and IgG2b responses to the GST moiety were also enhanced. In contrast, P8 did not significantly change the IgG subclass pattern induced.

In mice, IgG1 responses are usually associated with Th2 responses whereas high levels of IgG2a, sometimes associated with IgG2b and IgG3, are thought to reflect Th1 responses (18, 22, 25, 27, 42). Several parameters influence the IgG subclass responses to proteins, including the use of adjuvants or delivery systems as well as the intrinsic immunogenicity of the protein itself (13, 31, 53, 64). The response elicited by GST-$MSP1_{19}$, with high levels of multiple IgG subclasses, is thus likely to be a combined effect of several parameters. Nevertheless, Freund's adjuvant appears to be a strong regulator of immune responses and usually induces high IgG1 as well as considerable IgG2a and IgG2b levels, reflecting an adjuvant-dependent mixed Th1/Th2 response (13, 15, 31, 62). The modulation of IgG subclass responses induced by P2 suggests that T-cell responses to P2 can, to some extent, override other factors determining the IgG subclass response, possibly reflecting a polarization towards Th1, although studies on P2-induced cytokine responses are required to confirm this. The decreased IgG1 levels in $H\text{-}2^b$ and $H\text{-}2^d$ mice support this, but, in contrast, the IgG1 levels were slightly increased in $H\text{-}2^k$ and $H\text{-}2^{k/b}$ mice. The decrease of IgG1 in $H\text{-}2^b$ and $H\text{-}2^d$ mice may explain why no improvement of the protection was observed in these mice following immunization with GST-P2-$MSP1_{19}$. Moreover, the differential effect of P2 on the IgG response in mice with disparate H-2 haplotypes suggests that this epitope, despite being universally recognized, has a different impact on the IgG subclass response in a major histocompatibility complex-dependent manner.

The prechallenge levels of IgG to $MSP1_{19}$, as well as the levels of specific IgG1 and IgG2a, in mice immunized with GST-$MSP1_{19}$ variants were inversely correlated with peak parasitemia. This is in agreement with previous studies that have implicated antibodies to *P. yoelii* $MSP1_{19}$ as the main effector mechanism for protection (14, 28, 36). Specific IgG1 and IgG2a were found at higher levels in C57BL/6 and B10.D2 mice than in B10.BR or B10.A(4R) mice after immunization with GST-$MSP1_{19}$, providing a plausible explanation for the differential protection in these mouse strains. Moreover, the enhanced levels of these IgG subclasses may be responsible for the improved protection of B10.BR mice immunized with GST-P2-$MSP1_{19}$. Surprisingly, in the B10.A(4R) mice vaccinated with GST-P2-$MSP1_{19}$, a similar improvement of the protection was not observed despite increased IgG levels. However, three mice in this group had peak parasitemias comparable to that of the most protected B10.A(4R) mouse in the GST-$MSP1_{19}$ group.

Several pieces of evidence indicate an important role for IgG2a in protection against *P. yoelii*. Only the IgG2a fraction of isotype- and subclass-fractionated polyclonal antibodies to whole *P. yoelii* blood stage parasites was protective (60). Also, mice inoculated with recombinant *Mycobacterium bovis* BCG bacteria expressing $MSP1_{19}$ were partially protected against *P. yoelii*; this was accompanied by rapid production of $MSP1_{19}$-specific IgG2a (but not IgG1, IgG2b, or IgG3) during challenge infection (41). In contrast, in a study by Tian et al. (59), protection following immunization with recombinant GST-$MSP1_{19}$ was associated with high levels of $MSP1_{19}$-specific IgG1 and Hirunpetcharat et al. (28) found associations with IgG1 and IgG2b. The association between levels of both IgG1 and IgG2a to $MSP1_{19}$ and low peak parasitemia found in this study suggests that both of these IgG subclasses mediate protection against *P. yoelii*. Differences in immunization protocols and use of different mouse strains may to some extent underlie the different associations observed in these studies, but the choice of detection reagents can also affect measurement of IgG subclasses, especially IgG2a (40).

The protective capacity of the IgG subclasses could relate to their differential recognition by Fcγ receptors and thus their

ability to trigger cell-mediated parasite clearance (19, 24, 49). However, a recent study showed that Fcγ-receptor γ-chain knockout mice were protected by passive transfer of antiserum to $MSP1_{19}$ (50). Since IgG1, IgG2a, and IgG2b cannot mediate antibody-dependent cell-mediated cytotoxicity or phagocytosis in such mice and since antisera with low levels of IgG3 were used, the observed protection suggests that IgG specific for $MSP1_{19}$ can exert a direct inhibitory effect on the parasite (50). However, it does not follow that the subclass of the IgG response to $MSP1_{19}$ is irrelevant to its protective capacity. IgG Fc regions vary in size, shape, and mobility and can thus affect the ability of Fab regions to gain access to, and bind to, their binding site. Also, complement-mediated mechanisms relying on specific IgG subclasses may also contribute to protective immunity.

In mice immunized with GST-P8-$MSP1_{19}$, protection was improved despite a relatively small increase of IgG levels compared to those in mice immunized with GST-$MSP1_{19}$. Similarly, improved protection was also observed in B10.A(4R) mice immunized with GST-free P8-$MSP1_{19}$ despite a modest increase of IgG levels in comparison with those in $MSP1_{19}$-immunized mice (data not shown). Although even a slight increase in IgG is likely to have a positive effect on protection, other explanations could be sought as well. For example, antibodies with different fine specificities for *P. falciparum* $MSP1_{19}$ exert variable effects on the processing of $MSP1_{42}$. Some antibodies inhibit the proteolytic processing, whereas others do not and may even block the effect of the inhibitory antibodies (9, 45). Whether or not IgG antibodies with such distinct activities are induced by *P. yoelii* $MSP1_{19}$ is not known. To examine if the groups of mice in the present study displayed different epitope specificities, antisera were analyzed for the capacity to inhibit the binding of MAb to *P. yoelii* $MSP1_{19}$. Antisera from well-protected mice generally displayed better inhibition, but the inhibition appeared to be related to the total level of $MSP1_{19}$-reactive IgG rather than reflecting drastically different epitope specificities between groups. A recent study indicated that the avidity of IgG elicited by GST-$MSP1_{19}$ is of importance for protection against *P. yoelii*. Prechallenge sera from protected mice vaccinated with GST-$MSP1_{19}$ protein not only had higher levels of multiple IgG subclasses but also displayed a higher avidity for $MSP1_{19}$ than sera from nonprotected mice immunized by DNA vaccination (32). It was also observed that B10.BR mice immunized with GST-$MSP1_{19}$ in Freund's adjuvant responded with lower avidity than C57BL/10 mice (32). Thus, since high-avidity responses depend on somatic mutation in B cells, which is a T-cell-dependent process, inclusion of additional T-cell epitopes in GST-$MSP1_{19}$ may improve the protection by enhancing IgG avidity as well as antibody titers.

In summary, the genetically restricted protection against *P. yoelii* induced by GST-$MSP1_{19}$ in mice can be overcome by inserting additional T-cell epitopes into the immunogen. Importantly, both T-cell epitopes used in the present study could enhance the IgG responses to $MSP1_{19}$ but one of them (P2) also modulated the IgG subclass response. The enhanced IgG responses induced by the modified GST-$MSP1_{19}$ variants are likely to explain the improved protection, as the prechallenge levels of both IgG1 and IgG2 were inversely correlated with peak parasitemia after infection. Whether or not inclusion of additional T-cell epitopes in a *P. falciparum* $MSP1_{19}$ vaccine will overcome problems of low immunogenicity and poor T-cell activation associated with immune responses to *P. falciparum* $MSP1_{19}$ in humans (21) remains to be investigated.

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