Electronic Supplementary Material (ESI) for Nanoscale.

Electronic Supporting Information

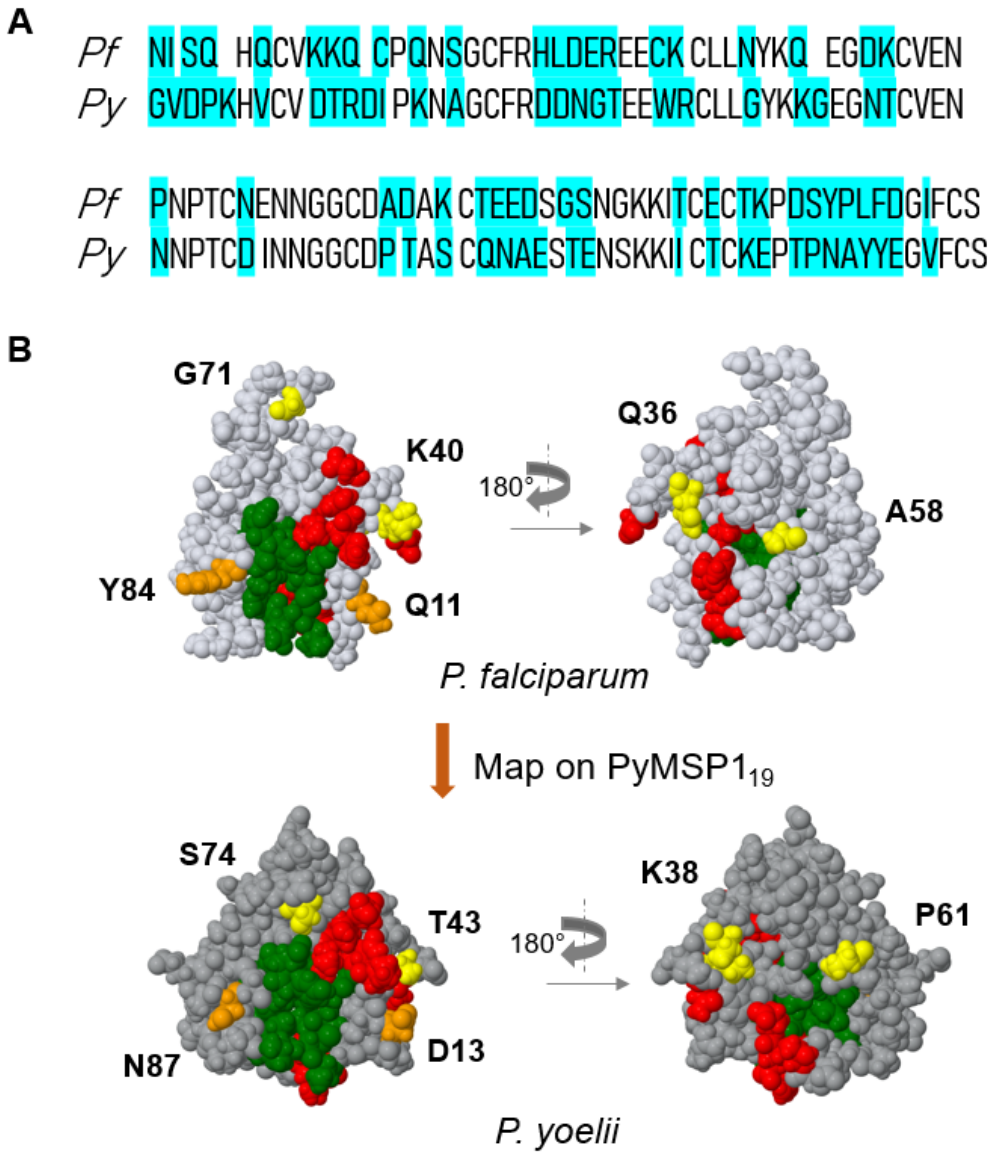

Figure S1. Designing mutant proteins for *P. yoelii* MSP1$_{19}$. (A) Alignment and comparison of the protein sequences for *P. falciparum* (*Pf*) and *P. yoelii* (*Py*) MSP1$_{19}$. Residues that differ between the two sequences are highlighted in cyan. Despite being structurally similar, MSP1$_{19}$ from these two species only has ~50% homology. (B) *Top Panel*: Structure of *Pf*MSP1$_{19}$ (PDB ID: 1CEJ), with residues involved in binding the inhibitory antibodies shown in green and residues involved in the binding of blocking antibodies shown in red. Residues mutated to F* to allow site-specific PEGylation to shield the inhibitory antibody epitope are colored orange. Residues mutated to F* to enable shielding of the blocking antibody epitopes are colored yellow. *Bottom Panel:* Analogous regions (inhibitory epitope, blocking epitopes, and residues mutated to F*) on *Py*MSP1$_{19}$ (PDB ID: 2MGP) based on sequence homology.

Experimental Section

DNA design and Protein Expression and Purification

DNA encoding superfolder green fluorescent protein (PDB ID: 2B3P), SpyCatcher (PDB ID: 4MLI)[1], *Pf*MSP1$_{19}$ (*Wellcome allele*, PDB ID: 1CEJ)[2] and *Py*MSP1$_{19}$ (*P. yoelii yoelii*, isolate 17XNL, PDB ID: 2MGP)[3] was optimized for expression in *E. coli* and synthesized by Integrated DNA Technologies, Inc. with C-terminal hexahistidine tags. SpyCatcher had an additional N-terminal Strep-tag® II. GFP had an additional N-terminal SpyTag[4, 5]. The genes were then cloned in a modified pET28b vector using Gibson cloning (E5520S, New England Biolabs, Ipswich, MA) using primers designed by the NEBuilder tool and following manufacturer's instructions. A version of *Py*MSP1$_{19}$ with a StreptagII replacing the hexahistidine tag was designed by Site-directed mutagenesis (SDM), performed using the Q5-SDM kit (E0554S, New England Biolabs) following manufacturer's instructions. Primers for SDM were designed using the NEBase changer tool. DNA for GFP Nanobody (NB) (PDB: 3OGO) and *Py*MSP1$_{19}$ (*P. yoelii nigeriensis*, isolate N67)[6] was optimized for expression in *E. coli* and synthesized and cloned in a modified pET28b vector between NdeI/XhoI sites by Gene Universal Inc., Newark, DE. GFP NB had a C-terminal c-myc tag followed by a hexahistidine tag for purification and *Py*MSP1$_{19}$ (N67 isolate) had a C-terminal Strep-tag® II for purification.

For protein expression, the plasmids encoding GFP, SpyCatcher and NB were transformed into BL21 (DE3) chemically competent *E. coli* cells (C2527H, New England Biolabs) and all plasmids for MSP1$_{19}$ were transformed into Origami™ 2(DE3) competent *E. coli* cells (71408-3, MilliporeSigma) and grown at 37°C in 2xYT media with 35 µg/mL of Kanamycin overnight. The starter cultures were scaled up to the appropriate culture volume and grown at 37°C. Expression was induced with 1 mM Isopropyl-beta-D-thiogalactoside (IPTG) at OD ~ 0.8-0.9. After induction

the temperature was lowered to 16°C and expression continued overnight. For the expression of proteins containing the non-canonical amino acid (ncAA) p-azidophenyl alanine (F*), cells were co-transformed with pEvol-pAcFRS.2.t1 (Addgene plasmid # 73544)[7] to add the machinery to allow incorporation of the non-canonical amino acid at the amber codon (UAG). Cell cultures in this case were additionally supplemented with 35 µg/mL of Chloramphenicol. At the time of induction at OD ~ 0.8-0.9, 1 mM IPTG, 0.02% L-Arabinose and 1 mM F* (06162, Chem-Impex International Inc.) were added and expression continued overnight at 16°C.

After expression, for hexahistidine tagged proteins, the cells were pelleted at 6,000-8,000xg for 15 min and gently resuspended in binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol, pH 8.0) supplemented with a protease inhibitor tablet (S8830, Sigma-Aldrich), lysozyme (0.5 mg/mL; J60701, Alfa Aesar), and benzonase (0.5 µL; E1014, Sigma-Aldrich). The cells were then incubated on ice for 10-15 min with intermittent mixing. Sodium deoxycholate (302-95-4, Alfa Aesar) was added to achieve a final concentration of 0.1% (w/v) before sonication for a total of 6 min (3 s on, 3 s off and 25% amplitude; Sonifier S-450, Branson Ultrasonics). The cell debris was then sedimented at 25,000xg (30 min). The supernatant was filtered (0.45 µm filter; 6869-2504, GE Healthcare) before purification of the hexahistidine-tagged proteins via Immobilized Metal Affinity Chromatography (IMAC) at 4 °C. The supernatant was passed twice over a bed of 2 mL Ni-NTA resin (88222, Thermo Fisher Scientific) in a drip column (786-197, G-Biosciences) that was pre-equilibrated in the binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 5% v/v glycerol, pH 8.0). The resin was then washed with 25 column volumes of the binding buffer. For elution, one column volume of elution buffer (20 mM Tris, 500 mM NaCl, 400 mM imidazole, 5% v/v glycerol, pH 8.0) was incubated with the resin for 4-5 min. This elution step was repeated four additional times to obtain five column volumes of the eluted protein.

Finally, the protein was concentrated to 1 mL using spin concentrators (Amicon Ultra-15, 10 kDa MWCO, Millipore Sigma) and further purified via size-exclusion chromatography using a Superdex 75 10/300 GL column (GE Healthcare) on an AKTA Pure purification system (29018224, GE Healthcare) with a PBS running buffer (0.5 mL/min).

For purification of proteins with a Strep-tag® II, StrepTrap chromatography was performed. Cells were harvested as described above except the binding buffer used was 100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0. After harvesting, the clarified lysate was run through a 5 mL StrepTrap HP column (28-9075-47, GE Healthcare) on an AKTA start purification system (29022094, GE Healthcare) at a flow rate of 0.5 mL/min. The column was next washed with the binding buffer (8-10 column volumes) followed by elution with 2-3 column volumes of the elution buffer (100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM desthiobiotin). All proteins were finally purified using size exclusion chromatography (SEC) on a Superdex 75 Increase 10/300 GL column (GE Healthcare, 29-1487-21) in PBS buffer. The SEC refining step removes aggregates if any. The proteins were concentrated and stored at 4°C in the dark until further use.

PEGylation and purification

The unnatural amino acid F* was incorporated into GFP at residues 175 (S175F*) and 204 (Q204F*) for Mut1-NB* and at residues 111 (E111F*) and 190 (D190F*) for Mut2*. Similary F* was incorporated into *Pf*MSP1$_{19}$ at residues 11 (Q11F*) and 84 (Y84F*) for *Pf*MutI*; 36 (Q36F*) and 58 (A58F*) for *Pf*MutB1*; 40 (K40F*) and 71 (G71F*) for *Pf*MutB2*; residues13 (N13F*) and 87 (N87F*) for *Py*MutI*; and 38 (K38F*), 43 (T43F*), 61 (P61F*), and 74 (S74F*) for *Py*MutB*. The purified mutants incorporating the non-canonical amino acids were then allowed to react with 4-5 fold stoichiometric excess (per F*) of mPEG-DBCO (MW 5,000 Da; Nanocs

Inc.). The reaction mixture was left at 4°C in the dark overnight and then run on SDS-PAGE to determine the extent of PEGylation. The completely PEGylated mutant proteins (doubly or quadruply PEGylated) were purified by SEC using a HiLoad 16/600 Superdex 200 column (GE Healthcare) on an AKTA Pure purification system (GE Healthcare) in PBS. The purified PEGylated proteins were concentrated and quantified using a Bicinchoninic acid assay (BCA) (#23227, Thermo Scientific) and stored at 4°C until further use.

Multivalent display of GFP antigens

All GFP antigens were expressed with an N-terminal SpyTag. A SpyCatcher mutant with F* incorporated at residue 56 (T56F*) was expressed in BL21 (DE3) *E. coli* cells and purified by IMAC followed by StrepTrap chromatography and then allowed to react with a 4-arm branched polyethylene glycol scaffold (5 kDa PEG arms with terminal DBCO groups) (Creative PEGworks, Chapel Hill, NC). The protein was added at a 4-fold stoichiometric excess (per DBCO group) and the reaction was allowed to proceed at 4°C in the dark overnight. The reaction mixture was characterized by SDS-PAGE to confirm the formation of tetrameric product, which was separated from the incompletely reacted products by SEC using a HiLoad 16/600 Superdex 200 column (GE Healthcare) on an AKTA Pure purification system (GE Healthcare) in PBS. The tetrameric scaffold was concentrated and quantified using the BCA protein assay.

The GFP proteins with an N-terminal SpyTag were then allowed to react with the tetrameric SpyCatcher scaffold overnight at 4°C. The SpyTag-SpyCatcher pair spontaneously forms an isopeptide bond[5], thereby immobilizing the antigens on the tetrameric scaffold, and enabling its multivalent display. The final tetrameric-GFP mutants were purified using SEC and characterized

by SDS-PAGE. The amount of GFP in the final tetrameric form was quantified using a fluorescence-based assay (λ_{ex}=400 nm; λ_{em}=509 nm).

ELISA characterization

Anti-*Pf*MSP1$_{19}$ antibodies 12.8, 12.10, 7.5 and 2.2 were procured from The European Malaria Reagent Repository. Anti-His antibody (MA1-21315, Invitrogen), anti-GFP antibody GF28R (MA5-15256, Invitrogen), and anti-c-myc-HRP antibody (A00173, Genscript) were commercially procured. The nanopatterned antigens were characterized by ELISA to confirm the ability to block recognition to desired regions. Briefly, 50 µL of 0.002 mg/mL of the antigen solution was coated per well on ELISA plates (Maxisorp NUNC, Thermo Scientific) overnight at 4°C in PBS. Plates were blocked with 100 µL of 5% Bovine serum albumin (BSA) (12659, EMD Millipore) in PBST (PBS with 0.05% Tween-20) for 1 hr at RT. After 3x washes with PBST, plates were incubated with 50 µL of primary antibody solutions in 1% BSA in PBST for 1 hr at RT. After 3x washes with PBST, plates were incubated with an appropriate HRP-conjugated secondary antibody for 1 hr at RT. After 3x washes with PBST, plates were developed with TMB substrate solution (00-2023, Thermo Fisher) for 5 min and stopped using 160 mM sulphuric acid solution. Plates were read on a Spectramax i3x plate reader (Molecular Devices) at 450 nm. Experiments were performed in triplicates.

Immunizations in mice

BALB/c mice were immunized with the nanopatterned GFP mutants or controls adjuvanted with AddaVax (InVivoGen, CA) by ProSci, Inc. (Poway, CA). Samples included tetrameric scaffolds presenting: (i) wtGFP; (ii) Mut1-NB*; (iii) Mut1-NB*-PEG; (iv) Mut2*; (v) Mut2*-PEG as well as (vi) tetrameric scaffold alone as a control. Five mice were immunized per group. The protein

antigens were diluted to 40 µg/mL in PBS (on a GFP basis). Each mouse (groups i-v) was injected subcutaneously with 2 µg of GFP. Mice in the control group (vi) received an equivalent amount of scaffold as mice in the other groups. Mice were injected with antigen on days 0, 21, and 42. On day 70, the mice were terminally bled and serum was stored at -20°C until further analysis. This mouse study was performed at ProSci Inc.'s USDA licensed, registered and NIH/OLAW assured animal facility. All the protocols that included experimental animal procedures were carried out in accordance with the US Animal Welfare Act and approved by ProSci Inc.'s Institutional Animal Care and Use Committee.

For $MSP1_{19}$ immunizations, groups of five female CB6F1/J (H-2d/b) mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory. The animals were immunized subcutaneously on days 0, 20, 40 and 88, in the base of the tail and the interscapular area. 7.5 µg of antigens (*Py*MutI*-PEG, *Py*MutB*-PEG, PBS control) were emulsified in the adjuvant Montanide ISA 51 VG (Seppic, Fairfield, NJ) prior to injection. All the protocols that included experimental animal procedures were carried out in accordance with the US Animal Welfare Act and approved by Emory University's Institutional Animal Care and Use Committee.

Serum analysis and Immunodepletions

The terminal bleed serum was assessed for endpoint titer against wtGFP or *Py*$MSP1_{19}$ (from isolates 17XNL and N67). A version of antigens with a Strep-tag® II were used for ELISAs to avoid signal from anti-His serum antibodies. Briefly, 50 µL of 0.002 mg/mL antigen was coated per well on ELISA plates overnight at 4°C in PBS. Plates were blocked with 100 µL of 5% BSA in PBST for 1 hr at RT. After 3x washes with PBST, plates were incubated with 50 µL of sera diluted four-fold starting at 1:100 in 1% BSA in PBST for 1 hr at RT. After 3x washes with PBST,

plates were incubated with a secondary HRP-conjugated anti-mouse antibody (115-035-003, Jackson ImmunoResearch) for 1 hr at RT. After 3x washes with PBST, plates were developed with TMB substrate solution (00-2023, Thermo Fisher) for 5 min and stopped using 160 mM sulphuric acid solution. Plates were read on a Spectramax i3x plate reader (Molecular devices) at 450 nm. Titers were expressed as area under the curve (AUC) determined using GraphPad Prism software. Appropriate controls were included.

Immunodepletion assays for the GFP set were carried out with the pooled terminal bleed serum for Mut1-NB*-PEG, Mut2*-PEG, and scaffold alone. Briefly, GFP Nanobody was first immobilized on Ni-NTA beads via the hexa-histidine tag on the Nanobody. The beads were thoroughly washed to remove any unbound NB. Next, GFP with the orthogonal Strep-tag® II purification tag was incubated with the NB-coated NTA beads, resulting in a complex of GFP and NB immobilized on NTA beads. After thorough washing to remove any unbound GFP, the GFP-NB-NTA depletant was incubated with serum (at a 1:200 dilution) for 3-4 hrs at 4 °C. A total of 4 rounds of depletion were performed for all three serum samples. All incubations, washes, and serum dilutions were carried out in 1% BSA solution in 20 mM Tris, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol, pH 8.0. As a control, NB-negative NTA beads were used as a depletant, and serum samples were similarly depleted 4 times. The depleted and undepleted sera from all 3 groups were characterized by ELISA as described above.

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