

Strain-Transcending Fc-Dependent Killing of *Plasmodium falciparum* by Merozoite Surface Protein 2 Allele-Specific Human Antibodies^{∇†}

Janine Stubbs,^{1*} Sope Olugbile,² Balam Saidou,³ Jacques Simpoire,⁴
Giampietro Corradin,³ and Antonio Lanzavecchia¹

Institute for Research in Biomedicine, Bellinzona, Switzerland¹; Centre Hospitalier Universitaire Vaudoise CHUV, Lausanne, Switzerland²; Department of Biochemistry, University of Lausanne, Epalinges, Switzerland³; and Saint Camille Hôpital, Ouagadougou, Burkina Faso⁴

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It is widely accepted that antibody responses against the human parasitic pathogen *Plasmodium falciparum* protect the host from the rigors of severe malaria and death. However, there is a continuing need for the development of *in vitro* correlate assays of immune protection. To this end, the capacity of human monoclonal and polyclonal antibodies in eliciting phagocytosis and parasite growth inhibition via Fc γ receptor-dependent mechanisms was explored. In examining the extent to which sequence diversity in merozoite surface protein 2 (MSP2) results in the evasion of antibody responses, an unexpectedly high level of heterologous function was measured for allele-specific human antibodies. The dependence on Fc γ receptors for opsonic phagocytosis and monocyte-mediated antibody-dependent parasite inhibition was demonstrated by the mutation of the Fc domain of monoclonal antibodies against both MSP2 and a novel vaccine candidate, peptide 27 from the gene *PFF0165c*. The described flow cytometry-based functional assays are expected to be useful for assessing immunity in naturally infected and vaccinated individuals and for prioritizing among blood-stage antigens for inclusion in blood-stage vaccines.

Merozoite surface protein 2 (MSP2) is a leading *Plasmodium falciparum* vaccine candidate. Antibody responses to MSP2 have been associated with protection from malaria in humans (1, 11, 37, 43, 50, 53) and afford protection in mouse models (35, 46). However, extreme sequence diversity in MSP2 is considered an obstacle for vaccine design. Hundreds of alleles encoding MSP2 are classified into two main allelic families represented by the 3D7 and FC27/D10 MSP2 sequences (15, 28, 49, 57). The coexistence of parasites bearing the dimorphic alleles at similar frequencies in globally disparate locations indicates their maintenance by selective pressures (9). Given that MSP2 is a blood-stage antigen, it is considered likely that sequence diversification may be driven by the host immune response by allele-specific antibodies. Consistent with this hypothesis, vaccine recipients in the Combination B phase IIb vaccine trial, whose humoral responses against the MSP2-3D7 allele were boosted, were rendered more susceptible than the placebo controls to parasitization with heterologous FC27-type parasites (16, 19). Early studies showed that mouse monoclonal antibodies (MAbs) that discriminated between heterologous parasites affected homologous *in vitro* growth inhibition (7, 13). Curiously, there is scant evidence of the inability of allele-specific MAbs to inhibit heterologous parasites *in vitro* (45).

Like other *P. falciparum* antigens, MSP2 antibody responses are skewed to cytophilic (IgG1 and IgG3) isotypes (37, 43, 50,

53, 54), which have been positively associated with protection from malaria (4, 11, 21, 37, 44, 50, 54). These isotypes bind via their Fc domain to Fc γ receptors, thereby eliciting cellular immune responses of the innate immune system. Antibody opsonization of *P. falciparum*-infected erythrocytes and merozoites enhances their phagocytosis (6, 12, 22, 30, 33, 55). The respiratory burst that is elicited by Fc receptor cross-linking furnishes the phagosome with cytotoxic compounds and has been positively associated with protection (20). Cytotoxicity occurring outside the confines of the phagolytic vacuole can result in the destruction of the surrounding environment. Plasmodia appear to be susceptible to this nonspecific cytotoxicity (5, 29, 38), possibly accounting for the accumulation of dead intraerythrocytic parasites *in vivo* (51). Like opsonic phagocytosis, monocyte-mediated antibody-dependent inhibition of the *in vitro* growth of parasites (3, 24, 32, 36, 48, 56) positively correlates with *in vivo* protection (3). The central importance of Fc receptor biology in malaria is indicated by the observation that polymorphisms in Fc receptors modify the outcome of infection (8, 10, 40, 47, 65).

We tested the hypothesis that sequence diversity in MSP2 renders parasites bearing heterologous MSP2 alleles differentially susceptible to Fc-dependent functions of allele-specific MSP2 antibodies. Antibodies from clinically immune adults living in regions where malaria is endemic were isolated using long synthetic peptides of the family-specific MSP2 sequences devoid of the adjoining conserved domain. Functional analysis was conducted using parasites bearing heterologous MSP2 alleles, namely, D10, 3D7, and allele-swap parasites isogenic with 3D7-AEX which, due to an allelic exchange (AEX), express MSP2-FC27 in place of MSP2-3D7 (64). Functional assays included opsonic phagocytosis and monocyte-mediated antibody-dependent parasite inhibition. These were compared

* Corresponding author. Present address: Centre for Immunology, Burnet Institute, GPO Box 2284, Melbourne, 3001 Victoria, Australia. Phone: 61-3-9282-2111. Fax: 61-3-9282-2100. E-mail: janine@burnet.edu.au.

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for wild-type (WT) and Fc domain mutant monoclonal antibodies to both MSP2 and an alpha-helical coiled-coil (peptide 27) from the gene *PFF0165c*. Peptide 27 has recently been identified to be a novel, highly conserved, *P. falciparum* vaccine candidate (34, 39, 61). Contrary to prediction, a remarkably high level of heterologous function by allele-specific MSP2 human antibodies was observed. These results underscore the importance and feasibility of complementing seroepidemiological surveys with high-throughput assays of antibody function.

MATERIALS AND METHODS

Peptide synthesis. Synthetic peptides of the MSP2-FC27 and MSP2-3D7 family-specific and conserved domains are listed in Table S1 in the supplemental material. Peptide synthesis was performed as described before (61) using solid-phase 9-fluorenylmethoxy carbonyl chemistry with an Advanced ChemTech AC T348 Omega multichannel synthesizer and Applied Biosystems synthesizers 431A and 433A, followed by purification by reverse-phase high-pressure liquid chromatography (C_{18} preparative column) and matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis (Applied Biosystems).

Immune donors. Ethical clearance was obtained from the individual state health authorities before blood donation. Blood samples were collected from seven healthy adult donors from Nigeria and Burkina Faso. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation and cryopreserved. Previous *P. falciparum* exposure was confirmed by serology against malaria peptides.

Memory B cell immortalization and B cell cloning. IgG-positive (IgG⁺) memory B cells were isolated using CD22 microbeads (Miltenyi), followed by staining with antibodies against IgD (BD Biosciences), IgM (Jackson), and IgA (Jackson), before they were sorted using a FACSAria cell sorter (Beckton Dickinson). Cells were immortalized using Epstein-Barr virus (EBV) as described previously (59) and directly cloned by limiting dilution in 384-well plates in the presence of irradiated PBMCs in complete RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum FCS (HyClone), 50 units/ml penicillin, 50 μ g/ml streptomycin (Gibco), 100 μ g/ml kanamycin (Gibco), 2 mM L-alanyl-L-glutamine (Gibco), 1% minimal essential medium (MEM) nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 50 μ M 2-mercaptoethanol (Gibco), 30 μ g/ml transferrin (Holo; Lubioscience), and 2.5 μ g/ml CpG oligodeoxynucleotide 2006 (Microsynth). Following 2 weeks of culture, B cell supernatants were screened by enzyme-linked immunosorbent assay (ELISA).

Antibody purification. Polyclonal IgG from the serum of six malaria-experienced donors were purified with peptide-bound CNBr-Sepharose 4B (Amersham) as described previously (61), and the absence of IgM, IgE, or IgA was confirmed by ELISA. Control IgG from healthy human serum was isolated using HiTrap protein G HP columns (GE Healthcare). Monoclonal antibodies from the supernatants of EBV-immortalized human B cells or transiently transfected HEK293T cells were purified by fast protein liquid chromatography on an ÄKTApure chromatograph (GE Healthcare) with 1-ml HiTrap protein A HP columns (GE Healthcare), followed by desalting with 5 ml HiTrap desalting columns (GE Healthcare). Purified antibodies were monitored for potential endotoxin contamination with the quantitative chromogenic *Limulus* amoebocyte lysate assay (Cambrex) or for their inability to upregulate costimulatory molecules CD80 and CD86 on day 4 granulocyte-macrophage colony-stimulating factor (50 ng/ml)- and interleukin-4 (1,000 U/ml)-differentiated monocyte-derived dendritic cells.

ELISA. MSP2 long synthetic peptides and recombinant proteins (kindly provided by R. Anders, La Trobe University, Australia) were coated at 1 μ g/ml and MSP2-FC27 15-mer and 20-mer peptides and peptide 27 were coated at 5 μ g/ml in phosphate-buffered saline (PBS) in 96-well (Corning) or 384-well (Spectra) ELISA plates. Positive reactions were revealed with goat anti-human IgG (anti-hIgG) coupled to alkaline phosphatase (AP; Jackson ImmunoResearch) at 1:500 and AP substrate solution *p*-nitrophenylphosphate (pNPP; Sigma). To quantify IgG, ELISA plates were coated with goat anti-hIgG (Southern Biotech) at 10 μ g/ml. Twelve-point 1:2 serial dilutions of the antibodies were made and the concentration was determined relative to that for the standard control ERM-DA470 human serum reference reagent by four-parameter logistic regression. For isotype ELISAs, capture antibodies were coated as follows: mouse anti-hIgG1 (1:250) and mouse anti-hIgG3 (1:500) (both from AbD Serotec) in bicarbonate buffer and mouse anti-hIgG2 (1:500) and mouse anti-hIgG4 (1:500) (both from BD Pharmingen) in PBS. Titrated antibodies were revealed with

mouse anti-hIgG coupled to biotin (BD Pharmingen) at 1:1,000, followed by streptavidin-AP conjugate (Jackson) at 1:250, and developed with pNPP as described above.

Parasites and genotyping. 3D7 and 3D7-AEX parasites expressing MSP2-FC27 in place of MSP2-3D7 (64) were kindly provided by A. Cowman (Walter and Eliza Hall Institute, Australia). D10 parasites were obtained from the Malaria Research and Reference Reagent Resource Center (MR4; Manassas, VA). Parasites were maintained in human type O-positive erythrocytes (Swiss Red Cross) at 4% hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES (Gibco), 4 mM Glutamax cell culture medium (Gibco), 0.2 mM hypoxanthine (Sigma), 3 μ g/ml gentamicin, and 0.5% Albumax II (Gibco) in 3% O₂-5% CO₂-92% N₂. To control for parasite genotype, PCR for MSP2 was performed on genomic DNA isolated by standard methods with forward primer 5'-GAGTATAAGGAGAAGTATG-3' and reverse primer 5'-GGACATATGCATGGTTCTAG-3' with 28 cycles of 95°C for 30 s, 56°C for 45 s, and 68°C for 45 s (14). The absence of *Mycoplasma* contamination in parasite cultures was controlled for by VenorGeM PCR (Sigma).

Immunoblot analysis. Reduced parasite lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Filters were blocked with 10% milk; incubated with primary antibodies (MAb 5J18, 100 ng/ml; polyclonal antibody 2 [pAb2], 10 ng/ml; pAb5, 25 ng/ml), followed by horseradish peroxidase-conjugated mouse anti-hIgG secondary antibody (GE Healthcare) at 1:2,500; and developed with an ECL Plus Western blotting detection system (GE Healthcare).

Immunofluorescence microscopy. Blood smears were prepared on SuperFrost Plus slides (Menzel Gläser), air dried, fixed in ice-cold 90% acetone–10% methanol for 2 min, air dried again, and stored at –80°C until use. Thawed slides were blocked in PBS containing 1% fetal calf serum (Gibco) and reacted with primary antibodies, as indicated, or with rabbit anti-erythrocyte binding antigen 175 (anti-EBA-175; MRA-2, MR4) at 1:500 or rabbit anti-MSP1₁₉ (MRA-35, MR4) at 1:1,000, followed by secondary reagents: A488 anti-hIgG or A594 anti-recombinant IgG (both from Molecular Probes) at 1:500. The DNA stain 4,6-diamidino-2-phenylindole (DAPI) was added at 2 μ g/ml in the second to the last wash, and slides were mounted in polyvinyl alcohol antifading medium with 1,4-diazabicyclo [2.2.2] octane (Fluka). Fluorescence was captured using a Nikon Eclipse E800 microscope with an Hamamatsu EM-CCD C9100 digital camera and OpenLab software.

Flow cytometry. Schizont-stage parasites (as a source of merozoites) at 4% parasitemia in 30 ml of culture were enriched over magnet LS columns (Miltenyi) and incubated with primary antibodies, as indicated, for 30 min at 37°C, followed by three washes and incubation with the secondary antibody A647 anti-hIgG (Molecular Probes) at 1:500 for 15 min at room temperature. Following two further washes, labeled parasites were resuspended in 100 μ l of the DNA stain Retic-COUNT (Becton Dickinson) and analyzed with a FACSCalibur cytometer (Beckton Dickinson).

IgG variable-chain cloning and production of wild-type and LALA mutant antibodies. Recombinant IgG1 was produced by cloning the variable regions of heavy-chain and light-chain genes into expression vectors (kindly provided by M. Nussenzweig) (58). Both wild-type IgG1(λ) and L234A L235A (LALA) mutant heavy-chain expression vectors were utilized; the latter has been shown to greatly diminish Fc γ receptor binding (25, 26). RNA was isolated from EBV-immortalized B cell clones 5J18 and 24G4 with an RNeasy kit (Qiagen), and cDNA was synthesized with Moloney murine leukemia virus (Invitrogen). Primer pools for amplifying the region upstream of the variable region through to the constant region are shown in Table S2 in the supplemental material. Sequencing was performed with *Pfu* Turbo (Stratagene) in both directions using the sequencing primers shown in Table S2 in the supplemental material. Gene-specific primers (see Table S2 in the supplemental material) were designed for cloning into expression vectors, and equal amounts of the resulting heavy-chain and light-chain vectors were transfected using Eugene reagent (Roche) into 80% confluent HEK293T cells (ATCC) in Dulbecco MEM (Gibco) supplemented with 10 units/ml penicillin, 10 μ g/ml streptomycin (Gibco), and 10% FCS (HyClone). Antibodies were recovered from supernatants harvested 3 and 5 days after transfection.

Phagocytosis assays. Parasites at 4% hematocrit and 5% ring-stage parasitemia were synchronized 4 h apart, at 42 h prior to coculture with monocytes that were isolated using CD14 microbeads (Miltenyi). Titrated antibodies diluted in parasite growth medium supplemented with 1% MEM nonessential amino acids (Gibco) and 1 mM sodium pyruvate (Gibco) were added in 50- μ l aliquots to round-bottom 96-well plates. To label parasites with carboxyfluorescein succinimidyl ester (CFSE), late-stage schizont-infected erythrocytes were purified using Miltenyi LS columns, labeled with 0.5 μ M CFSE in PBS, and washed well in complete medium. Equal numbers of parasites were preincubated with anti-

bodies at 37°C for 30 min before addition of 100,000 CD14⁺ monocytes. After 2 h, monocytes were labeled with anti-CD14 allophycocyanin (APC; Beckman Coulter) at 1:200 for 15 min on ice, washed, and analyzed by flow cytometry. Median fluorescence intensities (MFIs) were determined for the FL-1 (CFSE) channel on gated CD14⁺ cells, and results were expressed relative to the result for nonopsonic phagocytosis.

Monocyte-mediated antibody-dependent parasite inhibition assays. Parasites were synchronized 4 h apart, at 32 h before coculture with CD14⁺ monocytes. On the day of assay, antibodies were diluted 20-fold (pAbs) to 100-fold (MAbs) in parasite growth medium supplemented with 1% MEM nonessential amino acids (Gibco) and 1 mM sodium pyruvate (Gibco) and were then prepared in a concentration gradient of 50- μ l aliquots in flat-bottom 96-well plates. Late-stage parasites at 4% hematocrit and 0.5% parasitemia were added to wells in 50- μ l aliquots, followed by the addition of 50 μ l of medium or 50 μ l containing 200,000 CD14⁺ monocytes. Fresh medium was added to the assay in 50- μ l aliquots, at 48 and 72 h after they were set up. At 86 h after they were set up, trophozoite-monocyte cocultures were incubated with anti-CD45 APC (Caltag) at 1:100 for 15 min on ice, washed, resuspended in 100 μ l of the DNA stain Retic-COUNT (Becton Dickinson), and analyzed by flow cytometry with the high-throughput screening option for the FACSCanto apparatus (Beckton Dickinson) to enumerate parasitemia. The results are expressed as the percent inhibition relative to that for the no-antibody control.

RESULTS

Isolation and characterization of MSP2 and peptide 27 antibodies. Long synthetic peptides representing the MSP2 family-specific FC27/D10 and 3D7 domains as well as the conserved domain (Fig. 1A; see Table S1 in the supplemental material) were used to isolate human monoclonal and polyclonal antibodies. In addition, monoclonal antibodies were isolated on peptide 27, an alpha-helical coiled-coil from the vaccine candidate *PFF0165c* (34, 39, 61). Screening for monoclonal antibodies was performed by ELISA using 12-day supernatants of CD22⁺ IgG⁺ memory B cell cultures seeded at 40 cells/well that were polyclonally stimulated and differentiated into antibody-secreting cells using EBV, CpG, and allogeneic PBMCs (59). Out of 10,752 immortalized memory B cell cultures screened from one donor, 1 secreted antibody specific for MSP2-FC27 (Mab 5J18); 5 secreted antibody specific for MSP2-3D7, but these were not stably transformed lines; and 10 secreted antibody specific for peptide 27, of which 2 were stably transformed (and 1, Mab 25G4, was pursued in detail).

Monoclonal antibody 5J18 against MSP2-FC27 and polyclonal antibodies (pAb1 to pAb3 isolated on peptide MSP2-FC27; pAb5 to pAb8 isolated on peptide MSP2-3D7) bound in ELISA in an allele-specific manner to the corresponding peptide (Fig. 1B, upper panels) and to recombinant full-length MSP2-FC27 or MSP2-3D7 (Fig. 1B, lower panels). Polyclonal antibodies purified using the conserved region of MSP2 (pAb9-cons) bound with equal measure to both recombinant MSP2-3D7 and MSP2-FC27 (Fig. 1B, lower panels) but not to the peptides where the conserved sequence is absent (Fig. 1B, upper panels). To examine binding of parasite-derived MSP2 in Western blots, three different *P. falciparum* parasites bearing heterologous MSP2 alleles were studied: D10 parasites (a clone of FC27 parasites) expressing MSP2-FC27; 3D7 parasites expressing MSP2-3D7; and 3D7-AEX parasites, isogenic to parental 3D7, which, due to an allele exchange, express MSP2-FC27 in place of MSP2-3D7 (64) (see Fig. S1 in the supplemental material). As expected, Western blotting with Mab 5J18 and pAb2 against MSP2-FC27 revealed a band at 44 kDa for D10 and 3D7-AEX parasites but not for 3D7 parasites

expressing the heterologous allele (Fig. 1C). Conversely, pAb5 against MSP2-3D7 detected a band of the same molecular mass only in 3D7 parasites (Fig. 1C). In immunofluorescence microscopy, Mab 5J18 against MSP2-FC27 displayed classic merozoite rim fluorescence that overlaid with MSP1₁₉ expression (Fig. 2D). The epitope recognized by Mab 5J18 within the MSP2-FC27 long synthetic peptide was mapped using 20-mer and 15-mer overlapping peptides (Fig. 1E; see Table S1 in the supplemental material), which showed that the majority of binding was accounted for by the sequence KTDGKGESE (Fig. 1E). Together, these data confirm that antibodies isolated on MSP2 allele-specific long synthetic peptides bind to the MSP2 molecule in an allele-specific manner.

The Mab 25G4 against peptide 27 displayed the expected punctate staining in merozoites (61), and this was located within the perimeter defined by MSP1₁₉ (Fig. 2A). Furthermore, the staining was concentrated toward the apical end of merozoites defined by the localization of EBA-175 (Fig. 2B).

Quantification of merozoite binding by allele-specific MSP2 antibodies. To determine if the allele-specific antibodies against MSP2 discriminated merozoites bearing heterologous MSP2 alleles, antibody binding was measured by immunofluorescence and flow cytometry. Fluorescence microscopy confirmed preferential binding of Mab 5J18 to homologous parasites D10 and 3D7-AEX (Fig. 3A). Nevertheless, at an optimized signal intensity at which the control consisting of secondary antibody alone did not account for fluorescence (data not shown), robust binding of Mab 5J18 to heterologous 3D7 parasites was detected, and this colocalized with MSP1₁₉ (Fig. 3B). This cross-reactivity was quantified using flow cytometry with freshly released merozoites that were identified on the basis of their appearance at schizont burst during the synchronous blood-stage cycle (see Fig. S2 in the supplemental material). Both Mab 5J18 against MSP2-FC27 and Mab 25G4 against peptide 27 labeled freshly released merozoites (Fig. 3C). Consistent with the immunofluorescence microscopy results, flow cytometry of merozoites with Mab 5J18 revealed stronger binding to homologous parasites D10 and 3D7-AEX than to the heterologous parasite 3D7, for which the staining was nevertheless conspicuous (Fig. 3D and E). In parallel, parasites were stained with Mab 25G4 against peptide 27. As expected for this conserved sequence (34), there was no difference in the staining between the three parasites (Fig. 3E). In contrast to Mab 5J18, strain-specific binding could essentially not be detected for polyclonal antibodies pAb2 against MSP2-FC27 and pAb5 against MSP2-3D7. Together, the data reveal a surprisingly high level of heterologous merozoite binding by allele-specific MSP2 antibodies.

Cloning, expression, and validation of wild-type and Fc-mutated human monoclonal antibodies. Two leucine-to-alanine point mutations at L234 and L235 have previously been shown to greatly reduce antibody binding to the Fc γ receptor and, consequently, to disrupt effector functions (25, 26). To test the hypothesis that allele-specific antibodies against MSP2 affect differential parasitocidal activity dependent on Fc γ receptor interactions, monoclonal antibodies 5J18 and 25G4 were cloned and expressed from HEK293T cells as WT and Fc-mutated (LALA) IgG1 molecules. To control for preserved antigen specificity, WT and LALA antibodies were tested in parallel with the original EBV-immortalized B cell-secreted

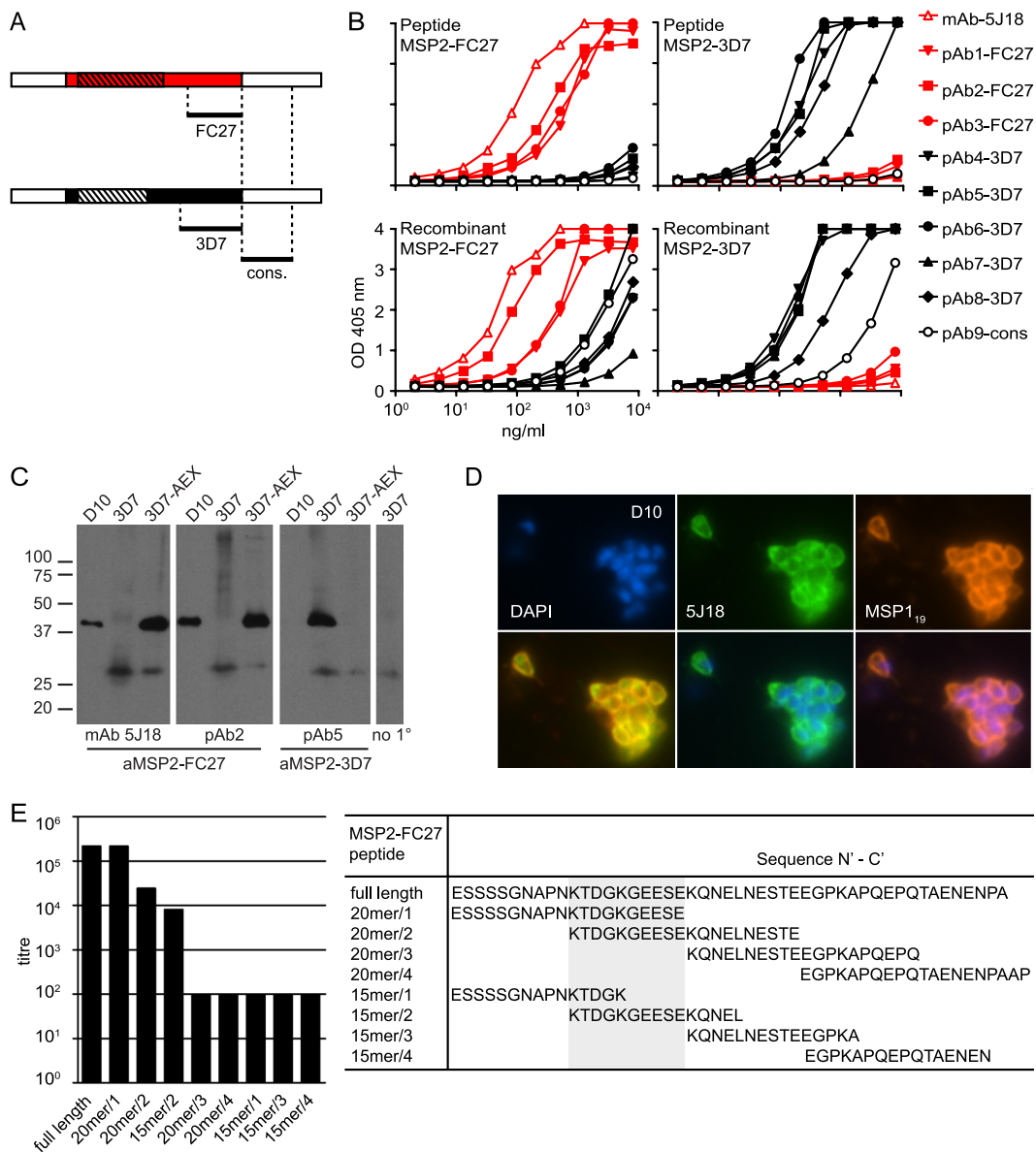


FIG. 1. Allele-specific recognition of *P. falciparum* MSP2 by human monoclonal antibody 5J18 and human polyclonal antibodies. (A) Schematic of the dimorphic allelic families of MSP2. The internal region contains repetitive (hatched) and nonrepetitive sequences that define the allelic families (red, FC27/D10; black, 3D7), while the N- and C-terminal regions (white) are highly conserved (49, 57). The locations of long synthetic peptides used to isolate antibodies are marked (see also Table S1 in the supplemental material). (B) Binding in ELISA of monoclonal antibody 5J18 and polyclonal antibodies pAb1 to pAb9 to MSP2 family-specific peptides (upper panels) or to family-specific full-length recombinant proteins (lower panels). (C) Western blots of *P. falciparum* late trophozoites comparing D10, 3D7, and 3D7-AEX parasites (see Fig. S1 in the supplemental material) for expression of MSP2 alleles, using MAb 5J18 and pAb2 against MSP2-FC27 and pAb5 against MSP2-3D7. Molecular mass markers (in kilodaltons) are shown on the left. (D) Immunofluorescence microscopy showing colocalization of binding by MAb 5J18 (green) with staining for MSP1₁₉ (red) relative to the merozoite nucleus stained by DAPI (blue). (Upper panels) Single colors; (lower panels) two-color overlays. (E) ELISA to map the binding specificity of MAb 5J18 within the MSP2-FC27 peptide using 20-mer and 15-mer overlapping peptides. Titer refers to the final antibody dilution giving a reading 3 standard deviations above the average of the negative control.

IgG1 antibodies (see Fig. S3 in the supplemental material). The levels of antigen binding by the WT and LALA antibodies were the same as those of the original EBV-immortalized B cell-secreted antibodies, as determined by ELISA (see Fig. S3, upper panels, in the supplemental material) and flow cytometry of merozoites (see Fig. S3, lower panels, in the supplemental material).

Opsonic phagocytosis of *P. falciparum* by monoclonal and polyclonal antibodies. Monocytes ubiquitously populate the blood and interface with humoral immunity through Fc receptors to perform effector functions such as opsonic phagocytosis. To determine if heterologous blood-stage parasites are differentially phagocytosed when they are opsonized with allele-specific MSP2 antibodies, magnetically purified schizonts

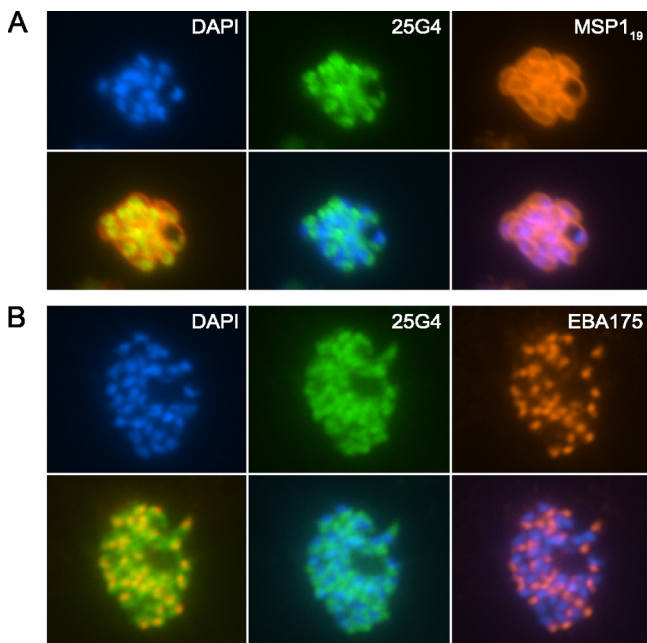


FIG. 2. Merozoite apex staining by human monoclonal antibody 25G4 directed against alpha-helical coil peptide 27 of *P. falciparum* PFF0165c, detected by immunofluorescence microscopy with MAb 25G4 (green) relative to MSP1₁₉ (red) (A) or EBA-175 (red) (B), with the merozoite nucleus stained by DAPI (blue). (upper panels) single colors; (lower panels) two-color overlays.

were labeled with CFSE (Fig. 4A) and returned to culture with titrated opsonizing antibody. Coculture for 2 h with CD14-enriched human monocytes resulted in parasite phagocytosis (Fig. 4A), which was read out by flow cytometry (Fig. 4B). Opsonization by MAb 5J18 against MSP2-FC27 greatly increased the level of phagocytosis above the background non-opsonic phagocytosis, and this activity was abolished by mutation of the antibody Fc domain (Fig. 4C, upper panels). Surprisingly, the extent of opsonic-phagocytosis by MAb 5J18 was equivalent for homologous (3D7-AEX and D10) and heterologous (3D7) parasites. The MAb 25G4 against peptide 27 also potentiated phagocytosis but to a lesser extent than that observed for MAb 5J18, and this was also abolished by mutation of the Fc domain. As for MAb 5J18, allele-specific polyclonal antibodies against MSP2 (pAb2 and pAb5) potentiated phagocytosis equally for all three parasites, while polyclonal antibodies from normal human serum (NIg) showed no functional activity. Thus, strain-transcending opsonic phagocytosis was observed for the allele-specific MSP2 and peptide 27 antibodies, for which the antibody Fc domain is essential.

***In vitro* parasite killing by monoclonal and polyclonal antibodies in the presence of human monocytes.** To determine if blood monocytes in the presence of allele-specific MSP2 antibodies differentially inhibit the *in vitro* growth of parasites bearing heterologous alleles, a flow cytometry-based assay was used to determine parasitemia (Fig. 5A). In the absence of antibodies, monocytes from different individuals were capable, with a wide degree of variability, to reduce the parasitemia of *in vitro* *P. falciparum* cultures (Fig. 5B). Irrespective of the monocyte capacity for nonopsonic parasite killing, addition of

antibodies against MSP2 to the parasite-monocyte coculture consistently increased the level of parasite inhibition (Fig. 5B to D). Unlike polyclonal antibodies from normal serum (NIg), the polyclonal antibodies against MSP2 on their own somewhat inhibited parasite growth (Fig. 5C, top panels), and their function was further revealed by the presence of monocytes (Fig. 5C, bottom panels). There was a modest trend to allele-specific function, with the clearest result being the preferential inhibition of homologous parasites 3D7-AEX and D10 by pAb2 against MSP2-FC27. The presence of monocytes also revealed the function of MAb 5J18 against MSP2-FC27 (Fig. 5D, bottom panels) relative to the complete lack of inhibition in the absence of monocytes (Fig. 5D, top panels). Mutation of the antibody Fc domain essentially abrogated monocyte-mediated antibody-dependent parasite inhibition (Fig. 5D, bottom panels). The LALA mutation greatly reduces but does not abolish binding to the Fc γ receptor (25); the residual inhibition of homologous parasites likely reflects the superior binding of MAb 5J18 to homologous parasites compared to that to heterologous parasites. Consistent with the opsonic phagocytosis results, the parasite-inhibitory effect of the wild-type version of MAb 5J18 was potent for the heterologous parasite 3D7 and did not clearly differ from homologous interactions (Fig. 5D, bottom panels). In addition, monocyte-mediated antibody-dependent parasite growth inhibition was measurable for MAb 25G4 against peptide 27 (Fig. 5D, far right panels), with the antibody on its own not inhibiting parasite growth. Together, these data indicate an unexpectedly high level of heterologous function of allele-specific MSP2 antibodies in *in vitro* Fc-dependent correlate assays of *in vivo* immunity.

DISCUSSION

Notably limited evidence demonstrates that extensive genetic diversification of MSP2 impacts the functional consequences of antibody binding (19, 45, 50). To address this issue, monoclonal and polyclonal antibodies were isolated on the basis of binding exclusively to the family-specific domains of MSP2 without the conserved flanking sequences. Although the heterologous MSP2 alleles have been described to exhibit “striking conservation of the overall amino acid composition, net charge, and hydrophobicity” (49), both the novel monoclonal antibody against MSP2-FC27 (MAb 5J18) and polyclonal antibodies against the family-specific domains reacted to MSP2 in an allele-specific manner.

While monoclonal antibody 5J18 revealed antigenic dissimilarity between free merozoites expressing heterologous MSP2 alleles, there was conspicuous cross-reactivity with the heterologous parasite, 3D7. In contrast, polyclonal antibodies against MSP2 barely discriminated between parasites bearing heterologous alleles. The *P. falciparum* genome encodes approximately 5,000 predicted genes (18). As the isogenic parasites 3D7 and 3D7-AEX differ only in the expression of the MSP2 allele (64) and the KTDGKGEESE binding site is not present in the 3D7-MSP2 sequence, it may be concluded that the cross-reactive binding is accounted for by merozoite antigens other than MSP2 with sufficient similarity to the mapped site. Indeed, pentamers of the KTDGKGEESE sequence occur numerous times in the genome of *P. falciparum* strain 3D7 (<http://plasmodb.org>), and identifying the surface proteins that interact most strongly with MAb 5J18 is

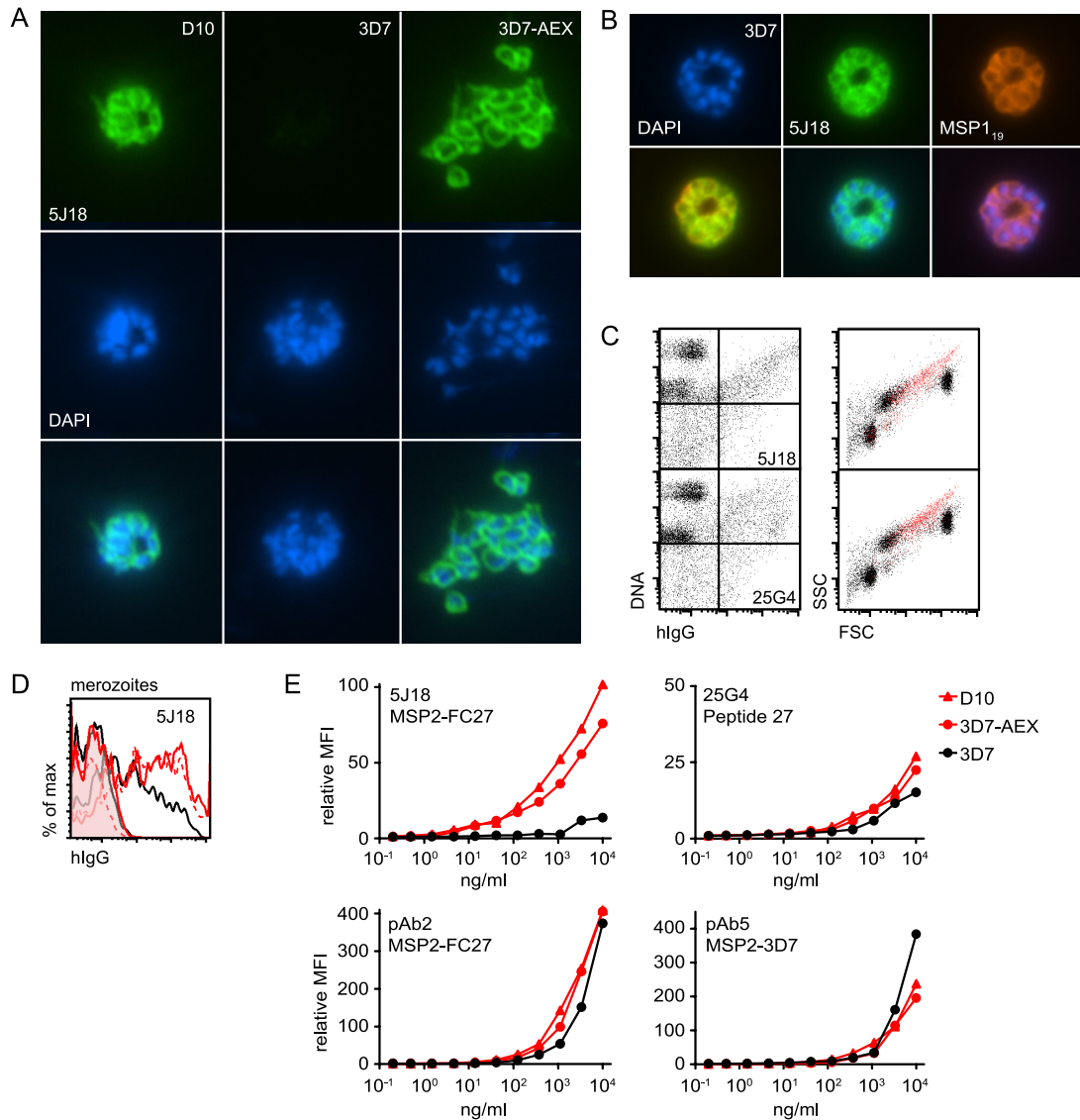


FIG. 3. Cross-reactive binding of allele-specific MSP2 antibodies to heterologous *P. falciparum* parasites. (A) Immunofluorescence microscopy of parasites D10, 3D7, and 3D7-AEX with MAb 5J18 at 80 ng/ml and exposure length of 100 ms counterstained with nuclear dye DAPI (blue). (B) Staining of heterologous 3D7 parasites stained with MAb 5J18 at 800 ng/ml and exposed for 900 ms, suggesting cross-reactive staining that colocalizes with MSP1₁₉. (C) Flow cytometric analysis of MAb 5J18 and MAb 25G4 binding to free merozoites (red) from magnet-purified cultures backgated from the antibody-positive, DNA-positive population (see also Fig. S2 in the supplemental material). FSC, forward scatter; SSC, side scatter. (D) Relative fluorescence intensity in flow cytometry of gated merozoites stained with MAb 5J18 at 5 μ g/ml; stippled red line, D10; solid red line, 3D7-AEX; black line, 3D7. Control stainings without the primary antibody are shown as filled traces. (E) Titration of MAb 5J18, MAb 25G4, pAb2, and pAb5 in flow cytometry of D10, 3D7-AEX, and 3D7 merozoites. Results are expressed as MFI relative to the intensity for the control, consisting of secondary antibody alone. Shown are the results of one representative of three independent experiments.

the subject of ongoing work. Precedence exists for the cross-reactive binding of allele-specific MSP2 antibodies between parasites bearing heterologous alleles. The mouse MAb 8G10/48 raised against FC27 parasites recognized a dominant band at 51 kDa and the sequence STNS, which occurs twice in the MSP2-FC27 allele (13, 45). It was proposed that cross-reactivity with heterologous parasites (15) was due to the binding of proteins, other than MSP2, containing epitopes sufficiently similar to STNS (45). Thus, allele-specific MSP2 antibodies do not necessarily resolve large antigenic differences at the whole-parasite level.

Nevertheless, genetic diversification and the consequent dis-

crimination by antibodies of heterologous parasites predict disparate outcomes in *in vitro* assays of presumed *in vivo* correlates of immunity (17, 23, 27, 31). Since the skewing of the MSP2 antibody response to cytophilic antibodies is associated with protection (11, 37, 43, 50, 53, 54), the influence of opsonizing antibody on Fc γ receptor-dependent functions of monocytes was investigated. As has been noted previously (48), monocytes from different donors displayed heterogeneous anti-parasite activity; nevertheless, they consistently collaborated with antibody to affect additional parasite inhibition. While polyclonal antibodies against MSP2 at 2 μ g/ml collaborated

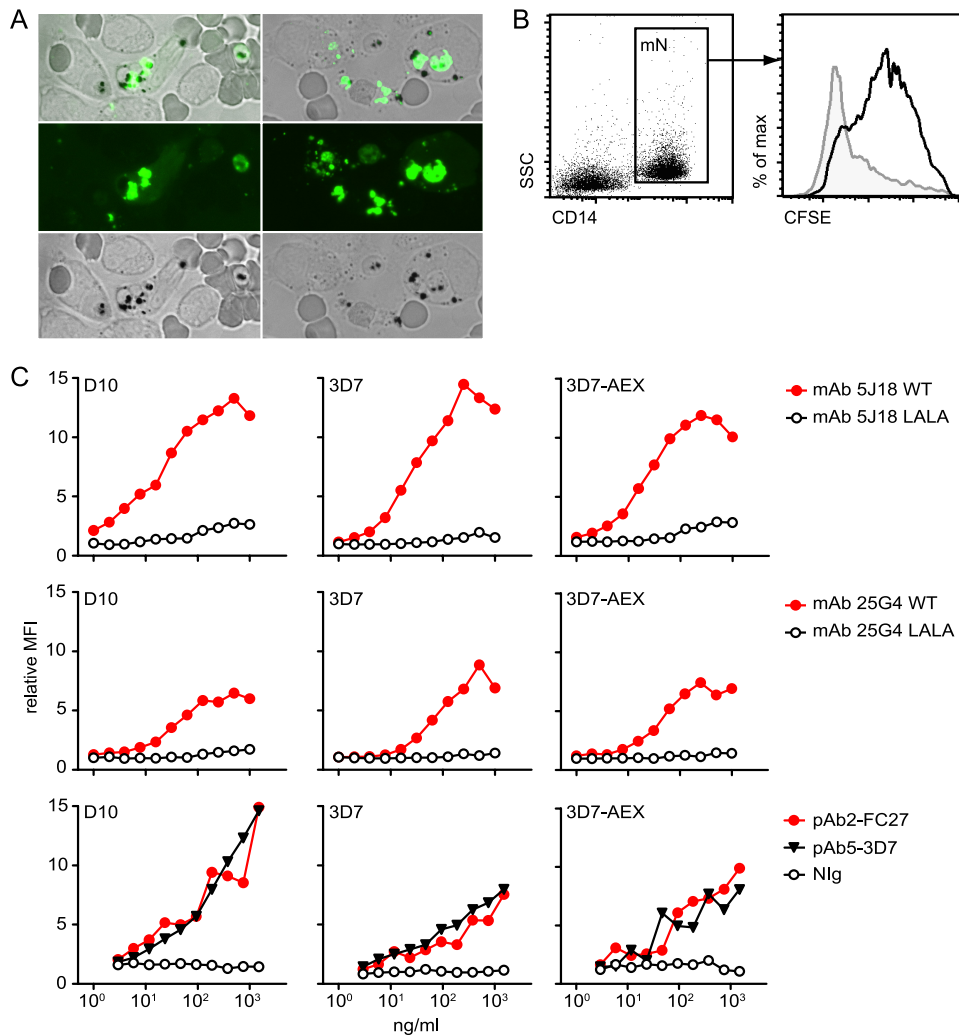


FIG. 4. Strain-transcending Fc-dependent phagocytosis of *P. falciparum* by MSP2 allele-specific human antibodies. (A) Fluorescence microscope images showing phagocytosis of CFSE-labeled parasites (green) by human monocytes after a 2-h coculture at 37°C. (B) Flow cytometric analysis of CD14⁺ monocytes (mN) cocultured with CFSE-labeled 3D7 merozoites in the absence (filled trace) and presence (unfilled trace) of MAb 5J18 against MSP2-FC27 at 1 μ g/ml. SSC, side scatter. (C) Opsonic phagocytosis of D10, 3D7, and 3D7-AEX parasites by WT and LALA versions (see Fig. S3 in the supplemental material) of MAb 5J18 against MSP2-FC27 (upper panels), MAb 25G4 against peptide 27 (middle panels), and polyclonal antibodies pAb2 (MSP2-FC27), pAb5 (MSP2-3D7), and NIg (bottom panels). Results are expressed as MFI relative to the intensity for nonopsonic phagocytosis. Shown are representative data from one of four independent experiments.

with monocytes to inhibit parasite *in vitro* growth, 10 to 20% inhibition was accounted for by the activity of antibodies alone, as has been observed for other antiparasite antibodies at this concentration (13, 27, 41, 62). Low concentrations of monoclonal antibodies against MSP2 and peptide 27, on the order of nanograms and within the range required to trigger Fc-dependent functions (29, 52), sufficed to measure opsonic phagocytosis and monocyte-mediated parasite inhibition. As expected, antibody function in both assays was abrogated by mutation of the antibody Fc domain. Surprisingly, results from all functional assays of allele-specific antibodies against MSP2 were essentially indistinguishable for homologous and heterologous interactions. The lack of clear strain-specific function by allele-specific MSP2 antibodies suggests that the level of cross-reaction between parasites bearing heterologous MSP2 alleles occurs above a minimum threshold that abolishes their biological

functions. Particularly in light of the relatively limited evidence of strain-specific functional immunity against MSP2 that exists for other strain- and variant-specific responses (2, 17, 23, 27, 31, 42, 60), the hypothesis arises that allele-specific MSP2 responses generally function in a strain-transcending manner. However, strain-transcending antibody responses are difficult to reconcile with immune pressure that confers a survival advantage to parasites bearing novel MSP2 alleles (9). Despite this, strain-transcending antibody responses would be consistent with the rapid acquisition of heterologous antibody responses thought to protect from severe disease. Functional analysis of larger panels of allele-specific MSP2 antibodies and field parasites to which the antibody responses were initially raised could resolve these questions.

Although the flow cytometry-based method to measure phagocytosis probably measures a combination of both para-

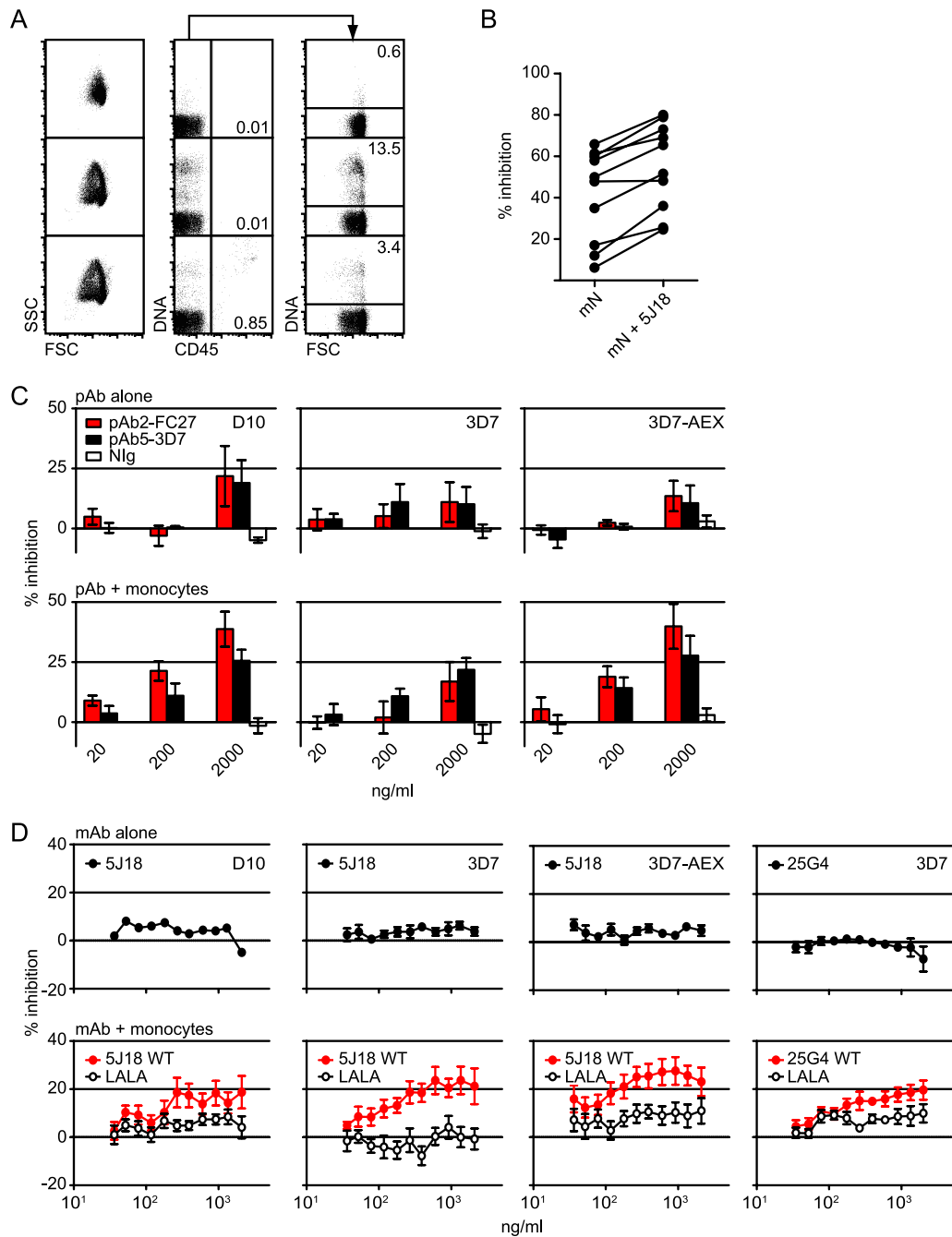


FIG. 5. Strain-transcending Fc-dependent killing of *P. falciparum* by MSP2 allele-specific human antibodies. (A) Flow cytometric gating strategy for the determination of parasitemia after two cycles of invasion. Uninfected erythrocytes (top panels) are discriminated from infected erythrocytes with the DNA stain thiazole orange (middle panels), while monocytes in the coculture with infected erythrocytes (bottom panels) are labeled with the surface marker CD45. Parasitemia is determined on the basis of the CD45-negative population. Shown is a 75% reduction in parasitemia when 3D7 parasites are cultured with 2 μ g/ml 5J18 in the presence of 200,000 monocytes. FSC, forward scatter; SSC, side scatter. (B) Nonopsonic *in vitro* growth inhibition by human monocytes (mN) compared to killing in the presence of 2 μ g/ml MAb 5J18. Each data set represents a different donor assayed at least in triplicate. The percent inhibition relative to the final parasitemia of the culture containing infected erythrocytes alone is shown. (C and D) Dose-dependent parasite growth inhibition by titrated antibody for D10, 3D7, and 3D7-AEX parasites in the absence of antibodies (top panels) and presence (bottom panels) of monocytes. The percent inhibition relative to parasitemia in the absence of antibodies is shown. (C) pAb2 against MSP2-FC27, pAb5 against MSP2-3D7, and IgG from normal human serum (NIg); (D) WT and LALA mutant versions of MAb 5J18 against MSP2-FC27 and MAb 25G4 against peptide 27 (see Fig. S3 in the supplemental material). Parasites and antibodies were tested in parallel. Data points are the means and standard errors of at least three independent experiments, with the exception of D10, which shows inhibition with MAb 5J18 in the absence of monocytes from two independent experiments. Experiments in the presence of monocytes were performed in at least three independent experiments with monocytes from at least five healthy donors.

site adherence to the monocyte surface and internalization, the results presented here suggest that phagocytosis contributes to monocyte-mediated antibody-dependent parasite growth inhibition (5, 29). Teasing out the molecular mechanisms of phagocytosis and cytotoxicity will shed light on the relative contribution of these two processes to parasite killing.

Factors governing antibody function include the avidity of antigen interaction and antigen accessibility (52). The apical location of peptide 27 may account for the lower potency of MAb 25G4 relative to that of MAb 5J18, which targets MSP2 and thus coats the entire merozoite surface. Overall, the data suggest that not all antibody-antigen interactions are equal regarding effector function. Identifying the most potent interactions will be important to assess levels of immunity in naturally infected individuals and for prioritizing among antigens for vaccine development. Flow cytometry-based functional assays coupled with defined monoclonal reagents could help to fast-track the realization of these aims.

In conclusion, this study highlights that molecular assays of allele-specific antibodies are silent on the possibility for cross-reactivity at the whole-organism level and are thus limited with regard to predicting antibody function. Functional assays based on flow cytometric readouts lend themselves to high-throughput application. Thus, the interpretation of ELISA measurements in epidemiological studies of naturally acquired and vaccine-induced immunity (1, 37, 43, 53, 63) may be strengthened by the functional assays described here.

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