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A New Rodent Model to Assess Blood Stage Immunity to the *Plasmodium falciparum* Antigen Merozoite Surface Protein 1₁₉ Reveals a Protective Role for Invasion Inhibitory Antibodies

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Abstract

Antibodies capable of inhibiting the invasion of *Plasmodium* merozoites into erythrocytes are present in individuals that are clinically immune to the malaria parasite. Those targeting the 19-kD COOH-terminal domain of the major merozoite surface protein (MSP)-1₁₉ are a major component of this inhibitory activity. However, it has been difficult to assess the overall relevance of such antibodies to antiparasite immunity. Here we use an allelic replacement approach to generate a rodent malaria parasite (*Plasmodium berghei*) that expresses a human malaria (*Plasmodium falciparum*) form of MSP-1₁₉. We show that mice made semi-immune to this parasite line generate high levels of merozoite inhibitory antibodies that are specific for *P. falciparum* MSP-1₁₉. Importantly, protection from homologous blood stage challenge in these mice correlated with levels of *P. falciparum* MSP-1₁₉-specific inhibitory antibodies, but not with titres of total MSP-1₁₉-specific immunoglobulins. We conclude that merozoite inhibitory antibodies generated in response to infection can play a significant role in suppressing parasitemia in vivo. This study provides a strong impetus for the development of blood stage vaccines designed to generate invasion inhibitory antibodies and offers a new animal model to trial *P. falciparum* MSP-1₁₉ vaccines.

Key words: malaria • transfection • *P. berghei* • merozoite • invasion inhibition

Introduction

It is well established that antibodies targeting merozoite antigens can mediate a degree of immunity to *Plasmodium falciparum* malaria and this knowledge underpins the development of blood stage vaccines against this devastating pathogen (for review see references 1 and 2). Such antibodies are thought to operate by a number of different mechanisms including the prevention of merozoite release (3), the direct neutralization of merozoites (4, 5), and the induction of monocyte-mediated parasite killing (6). However, the relative contribution of these different mechanisms to controlling blood stage parasitaemia remains unclear. A better understanding of this is particularly important for the development of functional "correlate-of-protection" assays for use in clinical trials of malaria vaccine candidates.

A protective role for merozoite invasion inhibitory antibodies, which are those that act in a manner that is

independent of complement or other cellular mediators, has been difficult to formally demonstrate and quantify. There are several reasons for this. First, there has been a lack of robust in vitro inhibition assays that account for confounding factors present in serum that can cause non-specific inhibitory, or indeed growth-promoting, effects. Although in vitro inhibition assays have been used for some time to assess antibodies to *P. falciparum* merozoite antigens and have provided a useful guide as to the inhibitory activity of a particular serum or monoclonal antibody, the problems associated with accurate quantification of this activity, especially in whole serum, are well recognized in the field (4, 5, 7, 8). With respect to one important antigen, the 19-kD COOH-terminal domain of *P. falciparum* merozoite surface protein (MSP)-1₁₉, this problem has now been overcome with the development of an assay that allows accurate quantification of MSP-1₁₉-specific inhibitory antibodies in

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Abbreviations used in this paper: EGF, epidermal growth factor; GST, glutathione S-transferase; IFA, immunofluorescence assay; MSP, merozoite surface protein; UTR, untranslated region.

whole serum (9, 10). This assay involves a comparison of the inhibitory effect of a given serum on two isogenic parasite lines that differ only in MSP-1₁₉. One expresses the *P. falciparum* domain and the other expresses an antigenically distinct domain from a rodent malaria parasite *Plasmodium chabaudi*. Using this assay in a previous study, we determined that the invasion inhibitory activity of antibodies present in serum obtained from adults that are clinically immune to malaria, as a result of frequent exposure to *P. falciparum*, is dominated by those targeting MSP-1₁₉ (9). This finding was consistent with many studies that had implicated a protective role for MSP-1₁₉-specific antibodies generated in response to infection or vaccination with recombinant proteins (for review see references 11 and 12). Despite the surprising knowledge that antibodies of this single specificity make such an important contribution to the process of invasion inhibition, it remained uncertain whether these antibodies actually contribute significantly to immunity.

The major remaining obstacle to addressing the protective role of inhibitory antibodies is the lack of a simple and robust in vivo challenge system that can be used in parallel with an in vitro inhibition test. In this paper, we have developed such an in vivo model by generating a rodent malaria parasite line that expresses *P. falciparum* MSP-1₁₉ in place of its own domain. Using this model, we show that the level of MSP-1₁₉-specific invasion inhibitory antibodies generated in mice that had been repeatedly exposed to this chimeric parasite line correlates with the ability of these animals to control a subsequent blood stage infection. The availability of this novel rodent malaria model also provides an alternative to non-human primates for assessing and monitoring *P. falciparum* MSP-1₁₉-based vaccines.

Materials and Methods

Plasmids and Plasmodium berghei Transfection. To create pPb-PfM19, 1.3 Kb *P. berghei* MSP-1 targeting sequence was fused in frame to the MSP-1₁₉ region of *P. falciparum* (see Fig. 1). PCR was performed on *P. berghei* ANKA and *P. falciparum* D10 genomic DNA (gDNA) using oligonucleotides PbF (5'-CGGGG-TACCATCGATAAATACTTTACCTCTGAAGCTGTTCC) and PbR1 (5'-TACATGCTTAGGGTCTATACCTAATAAATC), and PbPfF (5'-GGTATAGACCCTAAGCATGTATGCGTAAAAAACAATGTCCAGAA) and PbR (5'-TGCTCTAGATTAAATGAAACTGTATAATATTAAC), respectively, and sewing products together via PCR using the primers PbF and PbR. The resulting fragment was cloned into the KpnI/XbaI sites of pGem4Z (Promega) that harbored the *hsp86* 3' untranslated region (UTR; reference 13). The *MSP-1/hsp86* 3' sequence was excised with KpnI/HindIII, the HindIII site filled in with Klenow reagent and the fragment cloned into the KpnI/HincII site of pD_BD_{TmΔH}D_B (14). A 0.55 Kb 3' targeting sequence was then cloned into the EcoRV/BamHI site of this vector to create pPb-PfM19. The 3' targeting region comprising the *P. berghei* MSP-1 3' UTR was identified by library screen (15) and PCR amplified from *P. berghei* ANKA gDNA using oligonucleotides PbM3'F (5'-GGCGATATCATAAATTATTGAAATATTTGTTGGA) and PbM3'R (5'-CGCGGATCCTATACAAAACATATACAAAC). The plasmid pPb-PfM19 is analogous to that of pPb-PfM19 with the exception that the entire *MSP-1* 5' targeting sequence is that of *P. berghei*. This fragment was amplified from *P. berghei* ANKA gDNA using the oligonucleotides PbF and PbR2 (5'-TGCTCTAGATTAAAATATATTAATACAATTAATGTG). Linearized plasmids were transfected into *P. berghei* ANKA parasites essentially as previously described (16, 17, 18).

MSP-1₁₉ Glutathione S-Transferase (GST) Fusion Proteins and Generation of Antisera. The DNA sequence corresponding to amino acids Gly 1672-Ser 1766 of *P. berghei* MSP-1₁₉ was amplified using the oligonucleotides PbM19eF (5'-CGCGGATC-CGGTATAGACCCTAAGCATGTATG) and PbM19eR (5'-

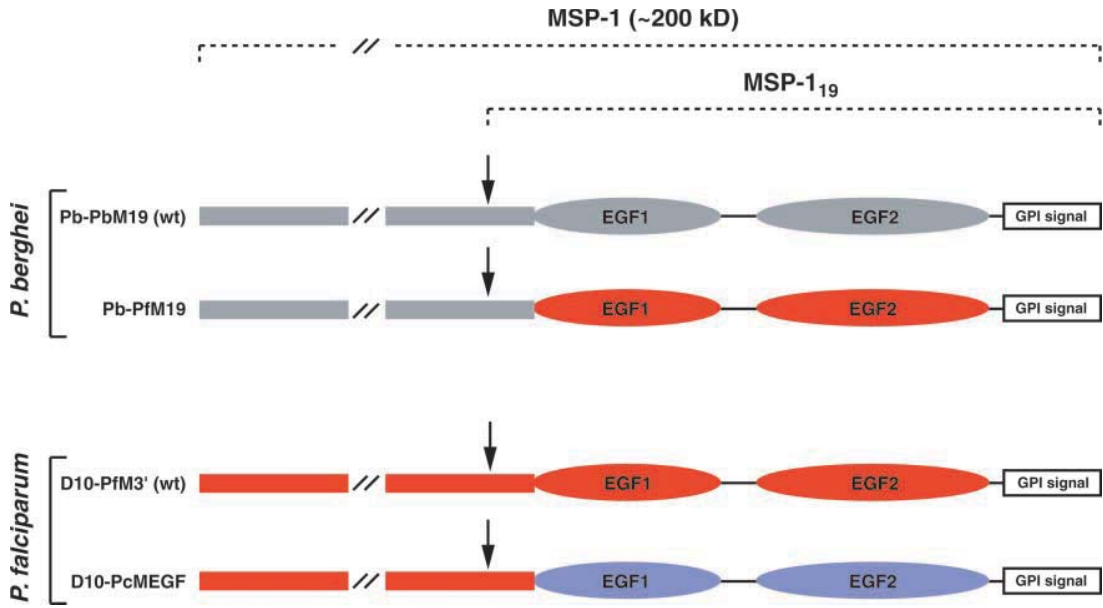


Figure 1. Schematic representation of *P. berghei* and *P. falciparum* MSP-1 chimeras. The MSP-1 sequences of *P. berghei* (gray), *P. falciparum* (red), and *P. chabaudi* (blue) are represented. The arrows indicate the MSP-1 secondary cleavage site.

GGAAGATCTTAGCTACAGAATACACCATCATAAT). The resulting product was ligated into the BamHI site of pGEX-4T-1 and expressed as a GST fusion protein (termed GST-PbM19). Rabbit antisera to GST-PbM19 was derived as previously described (9). Western blot and indirect immunofluorescence assay (IFA) were performed as previously described (9, 19, 20).

Generation of Semi-immune Mice. Semi-immune BALB/c mice were generated by the administration of 10^4 erythrocytes infected with either Pb-PbM19 or Pb-PfM19. At 5–10% parasitemia, mice were treated consecutively for 5 d with chloroquine (10 mg/kg body weight, injected i.p.). Recrudescence was typically observed 1 wk after this primary infection, after which mice were administered another five doses of chloroquine. 1 mo later mice were experimentally reinfected and then drug cured as described above. Sera were obtained from individual mice 10 d after the final drug treatment to monitor MSP-1₁₉ antibodies. For challenge infections, mice were injected i.p. with 5×10^6 Pb-PbM19- or Pb-PfM19-infected erythrocytes and the course of parasitemia was monitored by microscopy.

Serology. Antibodies reacting with recombinant *P. berghei* or *P. falciparum* MSP-1₁₉ were detected by ELISA (9). Blood taken from mice before primary infection were used as negative controls. The ELISA endpoint titres were taken as the highest serum dilution that gave an OD reading five times above that of the control sera. Inhibition of invasion assays were performed as previously described (9).

Results

Allelic Replacement of *P. berghei* MSP-1₁₉ with *P. falciparum* MSP-1₁₉: Functional Complementation of Divergent MSP-1₁₉ Sequences. To establish whether *P. falciparum* MSP-1₁₉ can complement the in vivo function of the divergent *P. berghei* MSP-1₁₉ domain, we created a *P. berghei* MSP-1 chimera that expresses *P. falciparum* MSP-1₁₉ in place of the endogenous molecule (Fig. 1). The transfection vector pPb-PfM19 was designed to integrate into *P. berghei* MSP-1 and replace the endogenous sequences encoding epidermal growth factor (EGF) domains 1 and 2, and the GPI recognition sequence, with the corresponding *P. falciparum* (D10 line) sequence (Figs. 1 and 2 A). A second plasmid, pPb-PbM19, designed to integrate in an identical manner but resulting in a homologous MSP-1₁₉ replacement, was also constructed to generate a control transfectant. Both plasmids were electroporated into the *P. berghei* (ANKA) line and transgenic parasites were cloned by limiting dilution. Southern blot analysis of gDNA showed that integration had occurred in these parasites by the expected double crossover event into MSP-1 (Fig. 2). The resulting *P. berghei*/*P. falciparum* chimeric line, which we have termed Pb-PfM19, could be distinguished from a control *P. berghei* transfection line, termed Pb-PbM19, by digestion with PstI

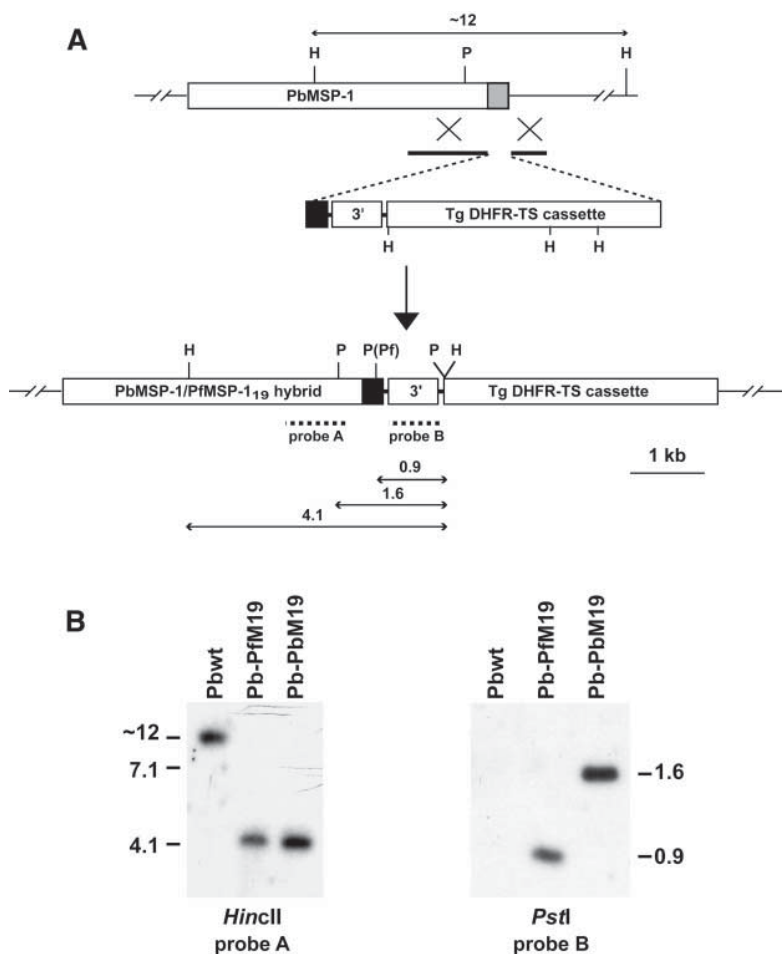


Figure 2. Generation of *P. berghei* chimera lines containing either *P. berghei* or *P. falciparum* MSP-1₁₉. (A) Schematic diagram of the *P. berghei* MSP-1 locus, the transfection vector (pPb-PfM19) used to replace the endogenous MSP-1₁₉ molecule, and the predicted MSP-1 locus of the Pb-PfM19 chimera line after integration. The gray box represents endogenous *P. berghei* MSP-1₁₉ sequence and the black box represents *P. falciparum* MSP-1₁₉ sequence (or *P. berghei* MSP-1₁₉ sequence for creation of the Pb-PbM19 chimera line). The solid lines in pPb-PfM19 depict targeting sequence, TgDHFR-TS, selectable marker cassette; 3', *HSP86* 3' UTR. The expected sizes of fragments resulting from digestion with either HincII (H) or PstI (P) are shown. (B) Southern blot analysis of digested genomic DNA from *P. berghei* wild-type and chimera lines.

(Fig. 2 B). In addition, PCR amplification of gDNA using oligonucleotides specific for the integration events confirmed the expected integration event (unpublished data).

To determine whether the Pb-PfM19 and Pb-PbM19 lines expressed the expected MSP-1₁₉ domains, Western blot analysis was performed on late stage parasite extracts using specific anti-MSP-1₁₉ antibodies (Fig. 3 A). *P. falciparum* MSP-1₁₉ antibodies recognized both MSP-1₁₉ and an ~200-kD band corresponding to full-length MSP-1 in Pb-PfM19 parasites but not in Pb-PbM19 parasites. In contrast, antibodies specific for *P. berghei* MSP-1₁₉ only recognized MSP-1₁₉ and full-length MSP-1 in wild-type *P. berghei* and the transfection control line, Pb-PbM19. This demonstrates that *P. falciparum* MSP-1₁₉ can be correctly expressed and processed in *P. berghei* and that the endogenous MSP-1₁₉ gene is no longer expressed in Pb-PfM19 parasites. The localization of MSP-1₁₉ in *P. berghei* lines was also assessed by double-labeling IFA. Characteristic merozoite surface labeling was observed in both chimera lines, with Pb-PfM19 parasites reacting only with

the *P. falciparum*-specific monoclonal antibody 4H9/19, whereas *P. berghei* wild-type and Pb-PbM19 chimeric parasites reacted only with rabbit anti-*P. berghei* MSP-1₁₉ antibodies (Fig. 3 B). This confirms that the appropriate MSP-1₁₉ domain is correctly localized in both Pb-PbM19 and Pb-PfM19 parasite lines. The growth rates of the transfected lines were also examined and compared with the wild-type parasite line (Fig. 3 C). All mice injected with 10⁴ parasites succumbed to infection over a similar time frame, regardless of which parasite line they were given. These results extend our previous finding that the function of MSP-1₁₉ during in vitro culture is conserved across divergent *Plasmodium* species (9, 20) to show that MSP-1₁₉ function is also conserved during the erythrocytic cycle in vivo.

A Key Role for MSP-1₁₉-specific Invasion Inhibitory Antibodies in Protection Elicited by Repeated Infection/Drug Cure. Sera from BALB/c mice that were rendered semi-immune to either Pb-PfM19 or Pb-PbM19 as a result of a low dose infection/drug cure regimen were tested for total MSP-1₁₉

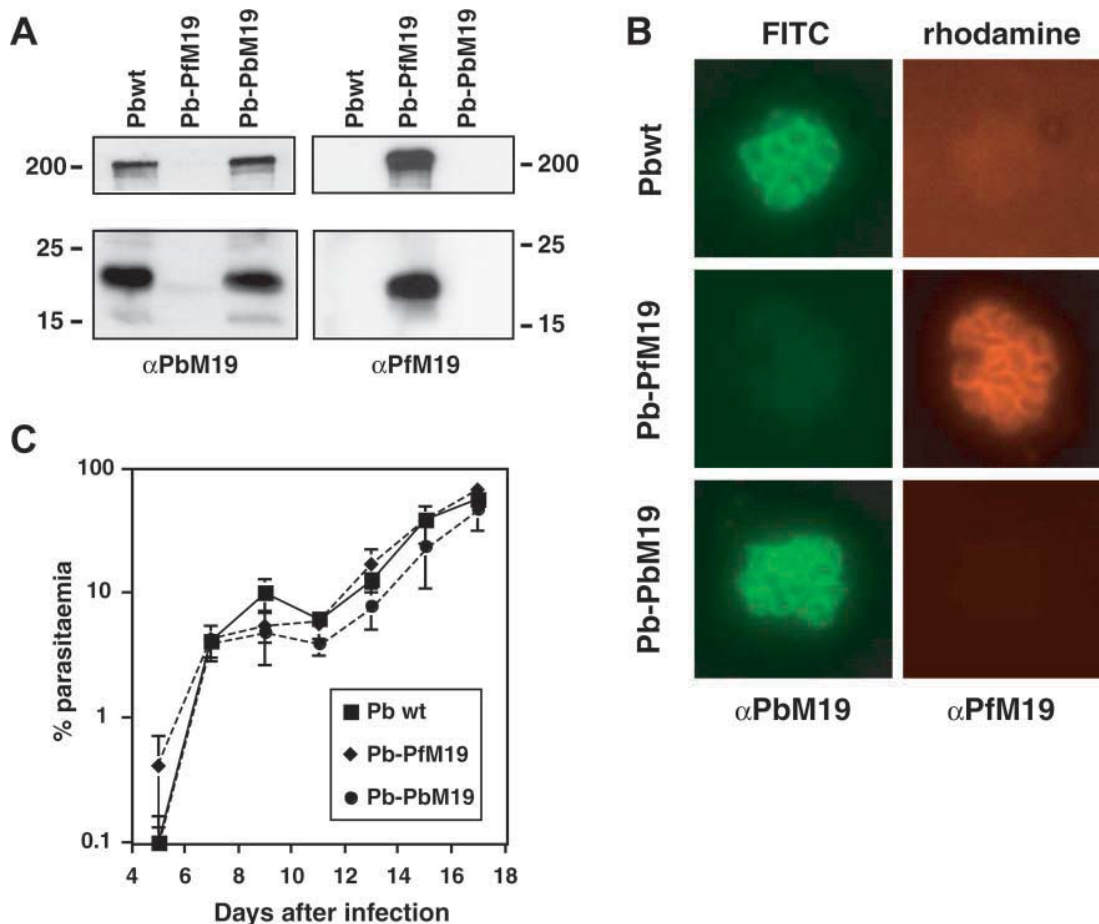


Figure 3. Phenotypic analysis of *P. berghei* chimeric lines. (A) Western blot analysis of late stage parasite extracts using rabbit αPbM19 or αPfM19 antibodies (both diluted 1/4,000) detecting both full-length MSP-1 (~200 kD) and MSP-1₁₉ (~19 kD). (B) Localization of MSP-1₁₉ by IFA. Schizont stage parasites were incubated with a mixture of rabbit αPbM19 (1/1,000) and 4H9/19 (αPfM19; 1/100) antibodies, followed by a mixture of FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse immunoglobulins (both diluted 1/200). (C) Course of blood parasitemia in mice after infection at day 0 with *P. berghei* wild-type, Pb-PbM19, or Pb-PfM19. Shown is the mean ± SD of the parasitemia observed in five mice.

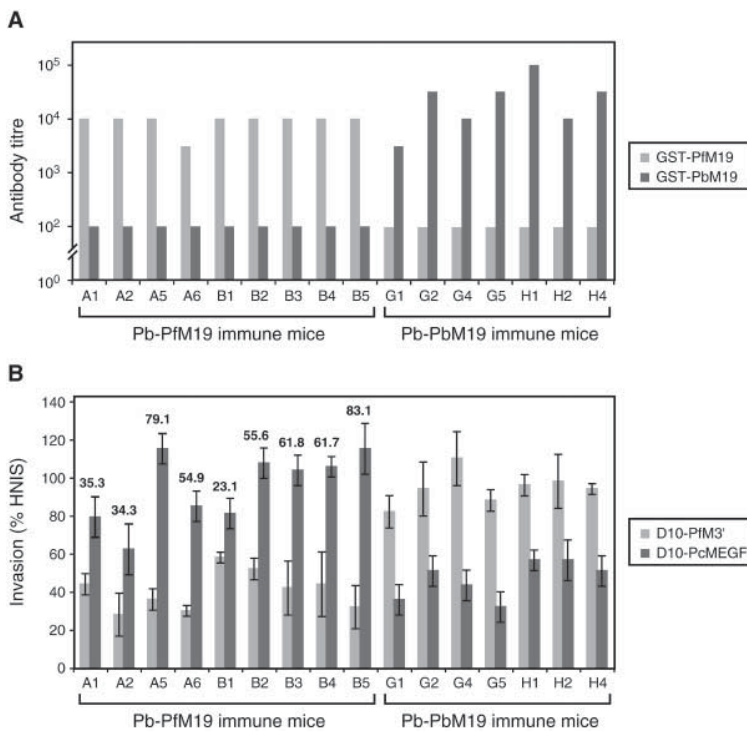


Figure 4. Mice repeatedly infected with *P. berghei* transfectants elicit MSP-1₁₉-specific inhibitory antibodies. (A) Reciprocal anti-MSP-1₁₉ antibody endpoint titres of serum from Pb-PfM19 and Pb-PbM19 immune mice against recombinant *P. falciparum* and *P. berghei* MSP-1₁₉-GST fusion proteins. (B) Invasion inhibition assay of D10-PfM3' and D10-PcMEGF parasite lines in the presence of individual serum from Pb-PfM19 and Pb-PbM19 immune mice. Invasion is expressed as a percentage of the invasion observed in parasites cultured in human nonimmune sera (HNIS). The numbers shown represent the *P. falciparum* MSP-1₁₉-specific invasion inhibitory activity of a given serum, calculated by subtracting the invasion rate of D10-PfM3' from that of D10-PcMEGF.

antibodies in ELISA and for MSP-1₁₉-specific invasion inhibitory antibodies. Using MSP-1₁₉ GST fusion proteins as antigen in an ELISA, all mice generated a strong MSP-1₁₉ antibody response that was specific for the relevant MSP-1₁₉ domain (Fig. 4 A). This data highlights the immunogenicity of this domain in the context of a low dose blood stage infection procedure and validates the expression of the appropriate MSP-1₁₉ domains in the transfected *P. berghei* lines.

For the in vitro inhibition assay, the ability of a given serum to inhibit the invasion of RBC by two isogenic *P. falciparum* lines, D10-PfM3' and D10-PcMEGF (Fig. 1), was compared. D10-PcMEGF expresses the antigenically diverse *P. chabaudi* MSP-1₁₉ polypeptide and so is not recognized by *P. falciparum* MSP-1₁₉-specific antibodies. This line was used as an alternative to a *P. falciparum* chimera expressing *P. berghei* MSP-1₁₉ because the latter is currently not available. Hence, *P. falciparum* MSP-1₁₉-specific invasion inhibitory activity of a given serum can be calculated by determining the difference in invasion rates of D10-PfM3', which uses the wild-type *P. falciparum* MSP-1₁₉ domain for invasion, and D10-PcMEGF in the presence of the test serum. All sera from Pb-PfM19 mice inhibited D10-PfM3' parasites far more effectively than D10-PcMEGF parasites (Fig. 4 B). Conversely, all sera from Pb-PbM19 immune mice inhibited D10-PcMEGF more effectively than wild-type *P. falciparum*. Because *P. chabaudi* and *P. berghei* are closely related rodent parasites with somewhat conserved MSP-1₁₉ domains (73% identity), a degree of antigenic cross-reactivity was expected here. However, because many epitopes differ between the MSP-1₁₉ domains of rodent malaria parasites (21), the invasion inhibitory activity of these sera cannot be accurately determined.

To determine if there is an association between the levels of MSP-1₁₉-specific invasion inhibitory antibodies present in mouse serum and degree of protection from a subsequent parasite challenge, Pb-PfM19 semi-immune mice were administered a high dose (5×10^6) of Pb-PfM19-infected erythrocytes 3 d after they had been bled for the serological analyses. After challenge, the course of parasitemia was determined and plotted against levels of *P. falciparum* MSP-1₁₉-specific invasion inhibitory antibodies (Fig. 5). Strong evidence of regression was observed ($R^2 = 0.63$; $P = 0.01$ by ANOVA), implicating a substantial role for MSP-1₁₉-specific inhibitory antibodies in controlling a blood stage infection. A similarly significant regression curve was evident when invasion inhibition was plotted against cumulative parasitemia in these mice ($R^2 = 0.56$; $P = 0.02$). The apparent linear relationship shown in Fig. 5 might be explained by the fact that *P. berghei* growth in BALB/c mice in our hands has two periods of almost log phase expansion (see Fig. 3 C). We also performed a two-sided rank correlation test, a more stringent analysis that does not assume a linear relationship between two parameters. This analysis also demonstrated significance for peak parasitemia versus MSP-1₁₉-specific invasion inhibition ($P = 0.05$). Importantly, all mice had very similar anti-PfMSP-1₁₉ ELISA antibody endpoint titres when measured against MSP-1₁₉ GST fusion proteins (Fig. 4 A) or a baculovirus-expressed *P. falciparum* MSP-1₄₂ antigen (MAD20 allele). The latter antigen is likely to represent MSP-1₁₉ in a more appropriate conformation and the reciprocal endpoint titres obtained in this case ranged from $10^{4.5}$ – 10^5 for Pb-PfM19 mice and $\geq 10^2$ for

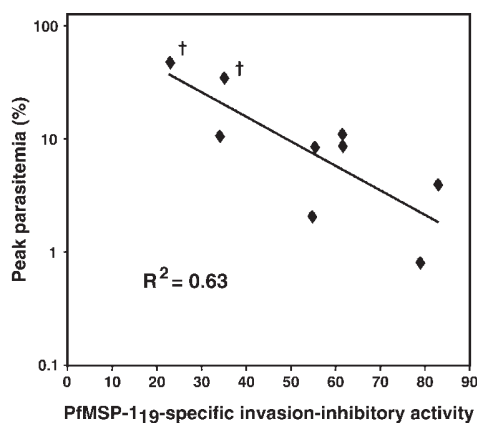


Figure 5. Evidence that MSP-1₁₉-specific inhibitory antibodies control a blood stage infection. MSP-1₁₉-specific invasion inhibitory activity of serum from individual Pb-PfM19 immune mice plotted against peak parasitemia attained after challenging corresponding mice with Pb-PfM19.

Pb-PbM19 mice. Hence, no association between IgG levels and protection were observed.

To assess the overall contribution of MSP-1₁₉ to blood stage immunity, in parallel with the homologous challenge experiment described above, groups of Pb-PbM19 and Pb-PfM19 immune mice were cross-challenged with the heterologous line. In both cases the data suggested that the groups challenged with the homologous parasite line were better protected. When homologous and heterologous challenge groups were combined for analysis, a difference was observed for mean peak parasitemia (14.8% in the homologous groups compared with 18.8% in the heterologous groups), cumulative parasitemia (586% compared with 1,033%), and time until parasite clearance (<0.01%; 12.6 compared with 15.1 d). However, although this trend was consistent across all parameters measured, only the latter approached statistical significance ($P = 0.056$ using log-rank test). Thus, to further explore the overall importance of MSP-1₁₉ antibodies to immunity, sera from *P. berghei* immune mice was pooled and transferred into two groups of five BALB/c mice (150 μ l serum/mouse on days -1 and 0), which were subsequently challenged (on day 0) with 10^4 Pb-PbM19 or Pb-PfM19 parasites. Mice challenged with the homologous line were significantly better protected than those challenged with Pb-PfM19 when comparing the parasitemia of the mice in the two groups over the first 10 d after challenge ($P = 0.05$ using a one-sided permutation test) or when comparing the time taken to reach 2% parasitemia ($P < 0.05$ by log-rank test). Together, these data are consistent with a significant role for MSP-1₁₉ antibodies in the control of blood stage parasites.

Discussion

Although antibodies directed against merozoite antigens are known targets of protective immunity, the relative importance of such antibodies to clinical immunity and their mode of action remain uncertain. In this paper we provide

strong evidence implicating a key role for merozoite invasion inhibitory antibodies in reducing the parasite burden in animals previously exposed to blood stage parasites. In addition, consistent with our previous findings (9), we demonstrate that antibodies that are specific for the EGF domains of MSP-1₁₉ comprise a large component of the inhibitory activity of the sera from these animals.

This study involved the generation of a *P. berghei* parasite line (Pb-PfM19) that expresses the *P. falciparum* MSP-1₁₉ domain in place of its own domain and an experiment that demonstrates that the in vivo function of MSP-1₁₉ is fully conserved across distantly related *Plasmodium* species. It is now clear that the extensive amino acid differences between rodent and human (*P. falciparum*) malaria MSP-1₁₉ domains, including an absent disulfide bond in the first EGF domain of the rodent malaria form, are not involved in species-specific erythrocyte invasion events either in vivo (this study) or in vitro (9, 20).

Our observation that the level of inhibitory antibodies specific for MSP-1₁₉ correlates with the control of parasitaemia upon subsequent blood stage challenge has a number of important implications. First, the dominant protective role for antibodies of this single specificity provides strong support for the development of MSP-1₁₉-based vaccines. Second, our findings validate the quantification of MSP-1₁₉-specific inhibitory antibodies as a useful correlate-of-protection assay. It should be stressed that the measurement of total MSP-1₁₉-specific immunoglobulins is not particularly useful in this regard as some B cell epitopes formed by this protein are not protective (7, 22) and indeed we found no correlation between IgG levels and either invasion inhibition or protection.

The model developed here may prove to be a useful tool to test and monitor the potency of *P. falciparum* MSP-1₁₉-based vaccines. At present there are numerous candidate vaccines based on this domain that are undergoing preclinical testing including different recombinant forms, conformers, and vaccine combinations that incorporate MSP-1₁₉ as one component. MSP-1₄₂, a protein that incorporates MSP-1₁₉ and MSP-1₃₃, is the most advanced of these candidates. It appears that the protective antibodies elicited by MSP-1₄₂ are directed against the MSP-1₁₉ fragment (the MSP-1₃₃ fragment may contain T cell epitopes) and hence the animal model developed here should be useful to analyze this vaccine. Currently, the only *P. falciparum* challenge model available to test MSP-1₁₉ antigens involves the use of nonhuman primates. Although vaccine testing in these animals is probably an important precursor to human trials, it is clearly substantially less practical for routine efficacy testing than the simple rodent challenge model described here.

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