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14. ABSTRACT
Plasmodium falciparum malaria kills hundreds of thousands of people every year. A vaccine that blocks red blood cell (RBC) invasion has been an elusive goal. Although anti-merozoite antibodies block invasion in vitro, there is no efficacy in vivo. Since merozoites are able to utilize the complement receptor 1 (CR1) on RBCs to invade we reasoned that complement activation could enhance invasion. To test this hypothesis we studied the role of complement in RBC invasion in vitro and parasite growth in an animal model. Fresh serum enhanced RBC invasion relative to heat-inactivated serum (HIS). Anti-merozoite monoclonal mAb5.2, directed against MSP119, induced invasion enhancement that was inhibited by HIS, the C3 inhibitor compstatin, and soluble CR1 (sCR1). Antibody and complement-mediated invasion led to aggregation and colocalization of CR1, C3, and lipids on the RBC surface at the point of merozoite contact. Total IgG from MSP142 vaccinees enhanced invasion in a complement-dependent manner. Finally, total anti-P. berghei IgG enhanced parasite growth in mice and C3-deficient mice showed decreased parasite growth relative to wild type mice. Our results demonstrate that merozoites are able to use complement to invade RBCs and, thus, hijack the complement system and evade the host immune response.

15. SUBJECT TERMS
Malaria, complement, red blood cells, antibodies, merozoite, invasion

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1. INTRODUCTION:

Plasmodium falciparum is responsible for most of the nearly 1 million deaths from malaria each year. The invasion of red blood cells (RBCs) by the parasite (merozoites) is an appealing target for vaccine development because this process is known to rely on specific ligand-receptor interactions. However, the development of an effective vaccine that blocks RBC invasion has been an elusive goal. When volunteers immunized with experimental vaccines are challenged with malaria, the results have been disappointing despite the presence of antibodies in serum that inhibit parasite growth *in vitro* (1;2). What is the explanation for this discrepancy? The discovery that *P. falciparum* can use the complement receptor 1 (CR1) as a receptor to invade RBCs may be able to answer this mystery (3). Deposition of C3b and/or C4b on merozoites may allow them to bind to the complement receptor 1 (CR1) on RBCs and proceed with invasion. Complement activation could take place via antibody-dependent or independent mechanisms. Complement is activated during malaria infection (4), especially in the presence of antibodies and, thus, these molecules are likely also deposited on merozoites. Although merozoites express a native CR1 ligand called PfRh4 (5), the availability of an alternative host-derived ligand would be useful since the host could make antibodies against parasite antigens but less likely against a self-antigen. Consequently, the parasite could use complement as a second CR1 ligand to enhance RBC invasion. The likely reason why this effect is not seen in *in vitro* growth inhibition assays (GIA) is because these assays are carried out using heat-inactivated serum which lacks complement activity. Hence, the lack of complement activity in GIA likely leads to an overestimation of the inhibitory activity of the antibody. We hypothesized that complement activation during malaria infection enhances the invasion of RBCs by P. falciparum. In this research we tested our hypothesis by investigating the effect of complement activation via antibody-dependent and independent mechanisms on RBC invasion, testing the role of CR1 in this process, and

2. Key Words:

Malaria, complement, merozoites, RBCs, antibodies, CR1

3. Accomplishments:

a. Major Goals of the Project:

The table below summarizes the major goals of the project:

Tasks	% Completion
Specific Aim 1: Determine the effect of monoclonal antibodies and human polyclonal antibodies against merozoite surface antigens on red blood cell invasion in the presence or absence of complement.	100
Major Task 1: Test effect of monoclonal antibodies.	100
Major Task 2: Test effect of human polyclonal antibodies	100
Specific Aim 2: Determine the contribution of CR1 to complement and antibody-mediated enhancement of RBC invasion.	100
Major Task 1: Test effect of sCR1 on invasion.	100
Major Task 2: Test effect of inhibition with anti-CR1	100

monoclonal antibodies.	
Specific Aim 3: Develop a *P. falciparum* PfRh4 deletion mutant.	75%
Major Task #1: Clone PfRh4	100%
Major Task #2: Electroporate, select, and screen mutants.	75%
Specific Aim 4: Determine the effect of passive immunization with anti-malaria antibodies and of complement inactivation on the level of parasitemia of in human CR1 transgenic mice.	100%
Major Task #1: Raise antibodies against merozoite antigens.	100%
Major Task #2: Purify and test antibodies against merozoite antigens.	100%
Major Task #3: Conduct experiment by passively immunizing wild type and CR1 transgenic mice.	100%
Major Task #4: Write proposal for additional funding.	100%

b. Accomplishments:

i. Specific Aim #1: Determine the effect of monoclonal antibodies and human polyclonal antibodies against merozoite surface antigens on RBC invasion in the presence or absence of complement.

We initially heat inactivated serum by heating it at 56 ºC for 30 min which is the standard method for inactivating complement activity. Also, heat inactivation of serum or plasma is routinely done for use in malaria cultures (1). We compared the invasion of *P. falciparum* into RBCs in the presence of heat-inactivated (HI) serum or fresh serum and noticed that the level of invasion in the latter was higher. We then resorted to shorter inactivation times (3 min and 5 min) in an effort to preferentially inactivate complement factors so that the inactivation could be reversed (2). Figure 1 shows that heat inactivation of serum reduces invasion in a dose-dependent manner in two strains of *P. falciparum* (7G8 and 3D7). The greatest effect was seen with 30 min HI. From this point onward we present the data as the percent enhancement of invasion over 30 min HI serum (Figure 2).

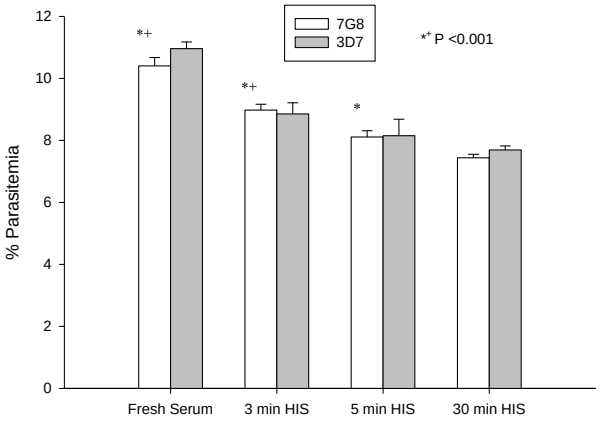

Figure1. Heat inactivation inhibits invasion in a dose-dependent manner. P is for the comparison with 30 min HI serum.

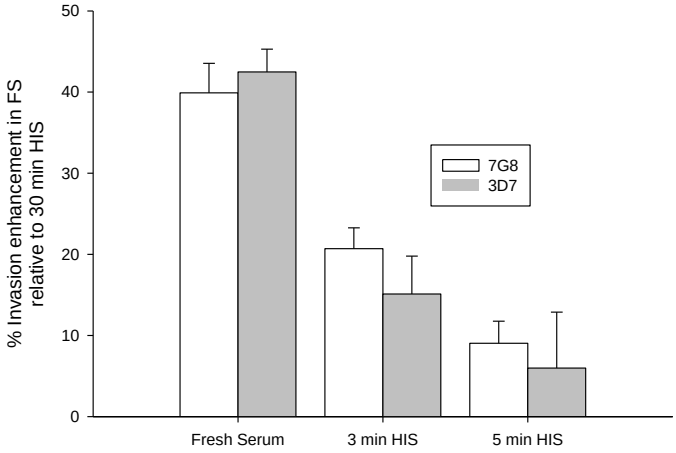

Figure 2. Enhancement effect of fresh serum relative to 30 min HI serum is decreased by shorter periods of heat inactivation.

We used the anti-merozoite antibody MAb5.2 (IgG2b) to test whether it could enhance invasion . We observed that addition of MAb5.2 to fresh serum led to enhanced invasion (Figure 3).

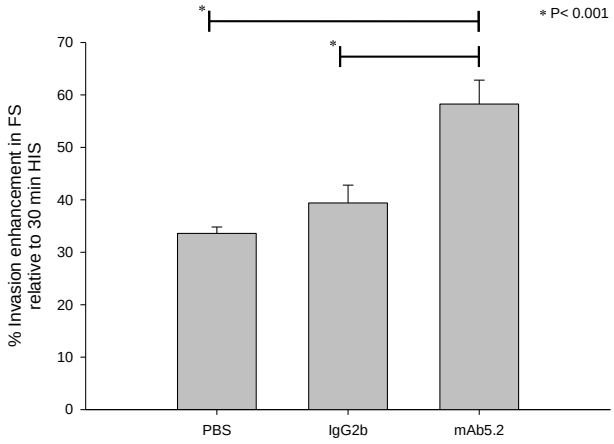

Figure 3. Enhancement of RBC invasion by anti-merozoite monoclonal antibody MAb 5.2

The C3 inhibitor peptide compstatin inhibited the enhancement effect of anti-merozoite antibody MAb5.2 (Figure 4). These data suggest that the enhancement effect of MAb5.2 is complement-mediated.

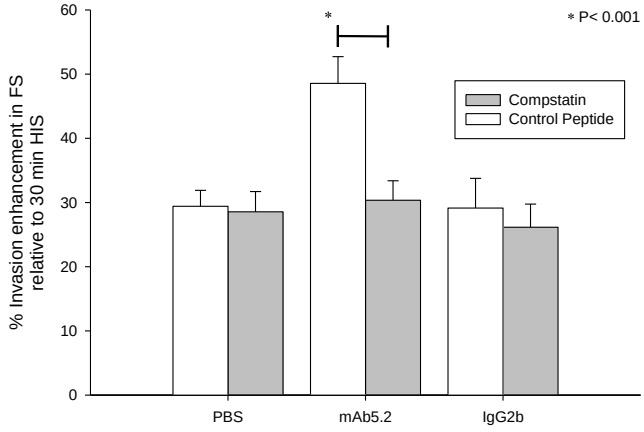

Figure 4. Enhancement of RBC invasion by MAb 5.2 is inhibited by the C3 inhibitor compstatin but not by a control peptide.

Finally, to test our hypothesis with a relevant antibody, we collaborated with Dr. Christian Ockenhouse from the Walter Reed Army Institute of Research to obtain serum samples from merozoite vaccine recipients. We purified total IgG from these samples and from plasma samples of malaria naïve individuals and tested their effect in our invasion assays. Figure 5 shows that IgG from merozoite vaccine recipients enhanced invasion in the presence of complement whereas the IgG from naïve individuals had no effect overall.

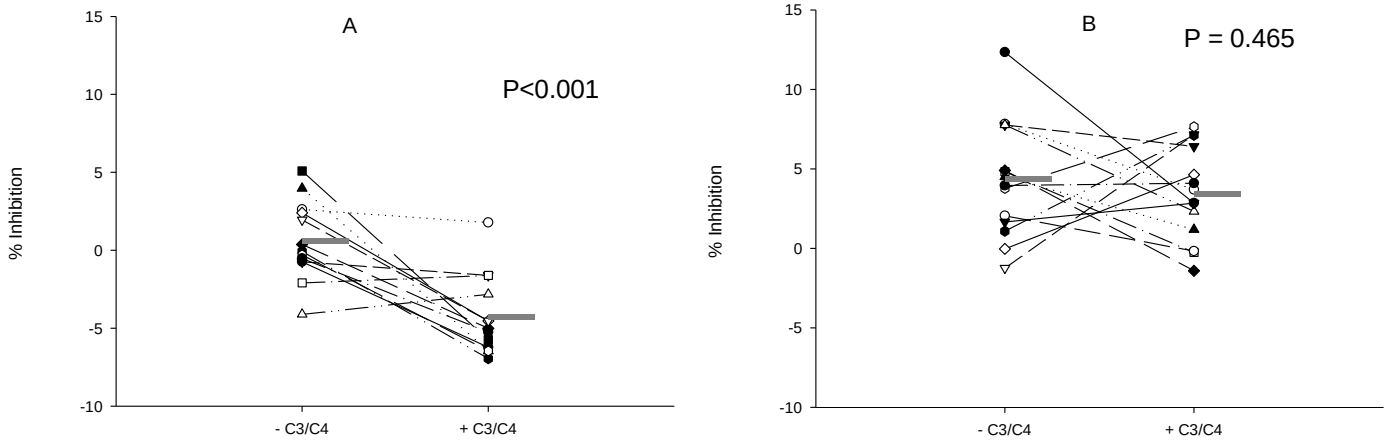

Figure 5. Effect of total IgG from merozoite vaccine recipients and from naïve individuals on RBC invasion. A) Complement decreases inhibitory activity of total IgG from recipients of a merozoite vaccine (MSP142). B) Total IgG from naïve individuals has no effect on inhibitory activity. The horizontal gray lines represent medians.

Conclusions: We have demonstrated that anti-merozoite antibodies can have a paradoxical effect by enhancing RBC invasion in the presence of complement. This effect is mediated by complement activation and could potentially explain the failure of anti-merozoite vaccines.

Future Directions: We would like to determine the antibody subclasses that mediate the enhancing effect of IgG. We anticipate that complement fixing subclasses IgG1 and IgG3 may mediate this effect whereas non-complement fixing subclasses such as IgG2 and IgG4 do not. Thus, the latter may be more inhibitory. In that case, it would be warranted to argue for the development of a vaccine that induces non-complement fixing subclasses. We also would like to do a similar analysis with antibodies from individuals living in endemic areas to determine whether antibodies acquired through natural infection have an enhancing effect on invasion and, if so, what is the mechanism.

ii. Specific Aim #2: Determine the contribution of CR1 to complement and antibody-mediated enhancement of RBC invasion.

Complement receptor 1 (CR1) is a complement receptor on RBCs and we have shown that it mediates sialic acid-independent invasion (3). Therefore, we suspected that it maybe mediating complement-dependent invasion. Figure 6 shows that the enhancement of invasion by anti-merozoite antibody MAb 5.2 is inhibited by sCR1.

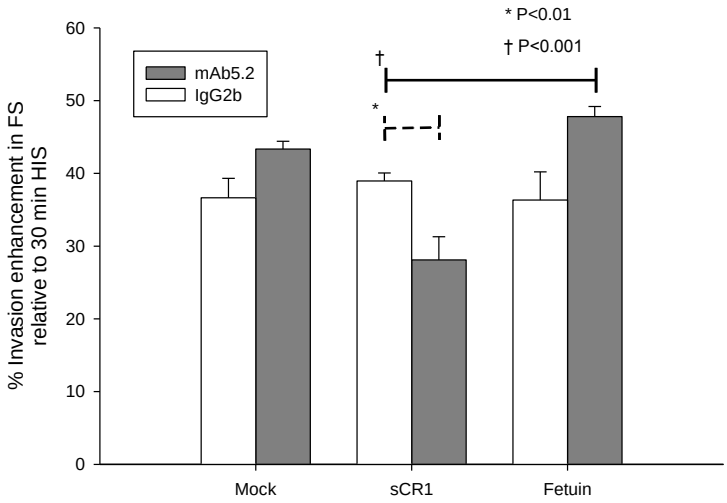

Figure 6. Soluble CR1 (sCR1) inhibits RBC invasion enhancement produced by MAb5.2 but that of serum without antibody.

Figure 7 shows the interaction of merozoites with RBC surface CR1. In the presence of C3 and anti-merozoite monoclonal MAb5.2 there is CR1 aggregation at the point of contact with the RBC as well as lipid accumulation.

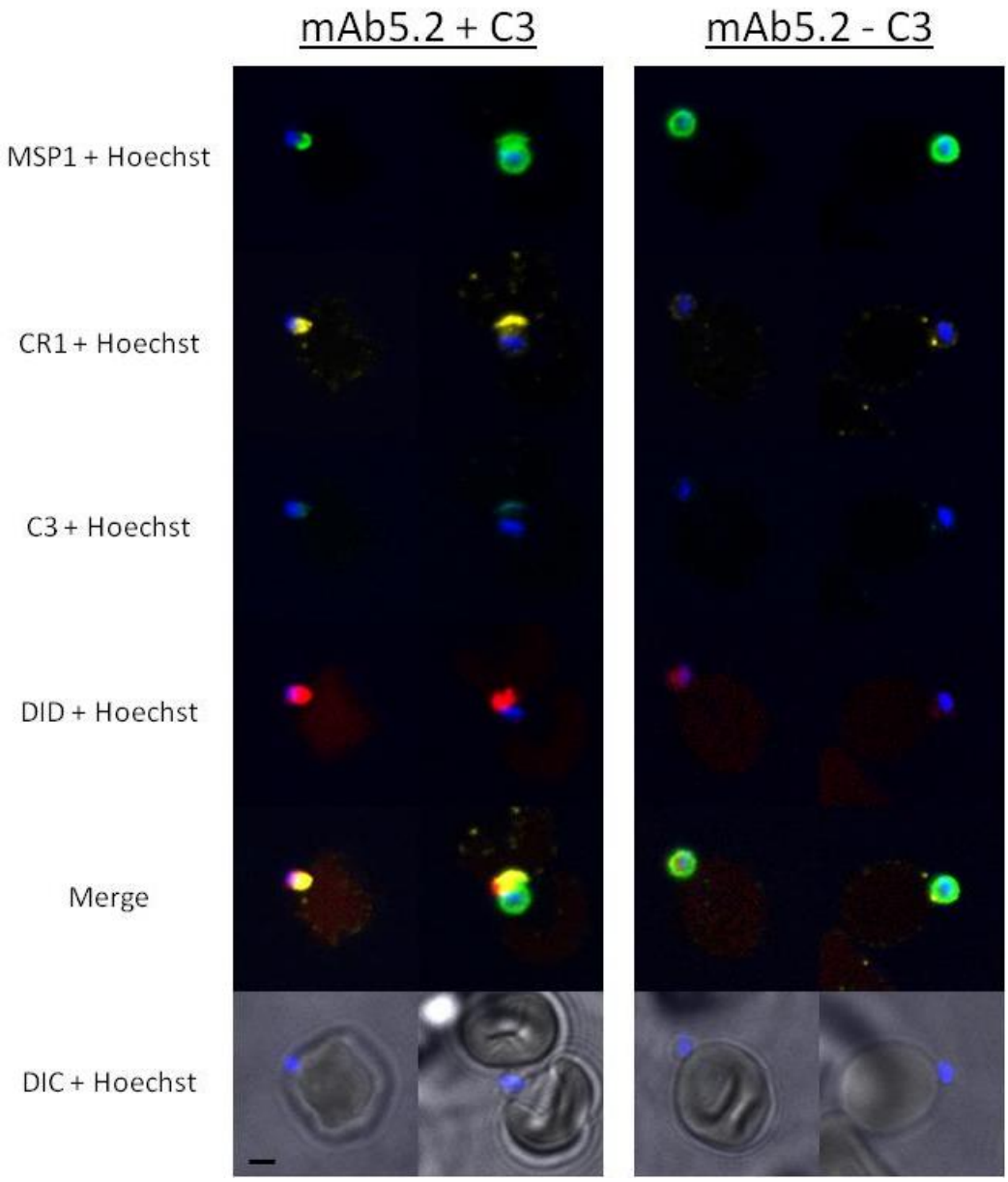

Figure 7: Interaction of merozoites with RBCs in the presence of anti-MSP1 mAb5.2 and in the presence or absence of C3. C3-reconstituted serum and anti-MSP1 mAb5.2 bind to RBCs via CR1 (yellow) which shows intense aggregation at the site of merozoite contact. This area is also positive for C3 (cyan) and shows lipid accumulation by DiD staining (red). Hoechst 33342 was used to stain DNA (blue) and MSP1 staining shows green. On the other hand, using C3-depleted serum there is little or no C3 deposition, and absence of CR1 aggregation or lipid accumulation. Of interest, we observed the characteristic speckled staining of CR1 (yellow) on the surface of all the merozoites. Scale bar is 2 μm.

Conclusion: CR1 is involved in complement and antibody-mediated enhancement by MAb 5.2.

Future Directions: We would like to further explore the role of sCR1 fragments to inhibit antibody-mediated invasion in order to determine which binding site within CR1 is critical. Based

on the inhibitory studies shown above we anticipate that the C3b binding site of CR1 is the one that is involved in binding. We also would like to confirm this by using site specific blocking inhibitory antibodies.

iii. Specific Aim #3: Develop a *P. falciparum* PfRh4 deletion mutant.

We have clone 750 bp fragment of the 5' region of 3D7 PfRh4 including about 100 bp upstream of the start site into PCC1 plasmid. The plasmid was transfected into P. falciparum 3D7 and currently we are in the process of selecting deletion mutants.

iv. Specific Aim #4: Determine the effect of passive immunization with anti-malaria antibodies and of complement inactivation on the level of parasitemia in human CR1 transgenic mice.

To determine the effect of polyclonal IgG anti-malaria IgG in vivo we raised antibodies against P. berghei by carrying out three cycles of infection and treatment with chloroquine of C57BL/6 mice. Total IgG was purified from these mice and from naïve uninfected mice to serve as antibody controls. We infected mice with 10^7 infected RBCs by injection in a retroorbital plexus and simultaneously injected different doses of antibody or PBS in the contralateral plexus. Figure 8 shows the day 6 the enhancement of parasitemia relative to PBS (no antibody). Anti-*P. berghei* antibody resulted in significant enhancement in inverse dose response manner whereas the control antibody was always inhibitory.

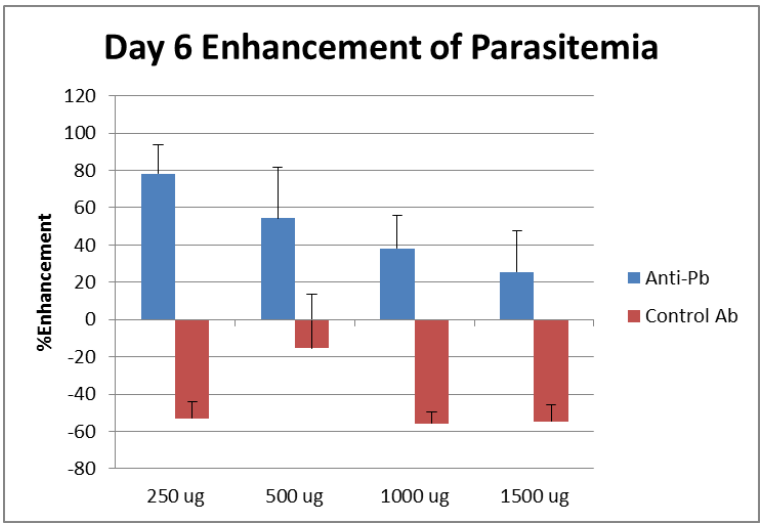

Figure 8. Effect of passive immunization with anti-Pb total IgG on Parasitemia relative to PBS. Control antibody was obtained from naïve uninfected mice.

We then tested whether the same enhancement could occur with passive immunization of human CR1 transgenic mice (4) and whether it could be inhibited by anti-CR1 polyclonal antibody. Figure 9 shows that anti-*P. berghei* antibody results in enhancement of parasitemia. However, unlike wild-type mice, the parasitemia of CR1 mice seems to be enhanced also by control antibody. The enhancement of parasitemia is inhibited by blocking CR1 with a polyclonal antibody. This suggests that the mechanism of increased parasitemia in these mice depends on CR1 availability.

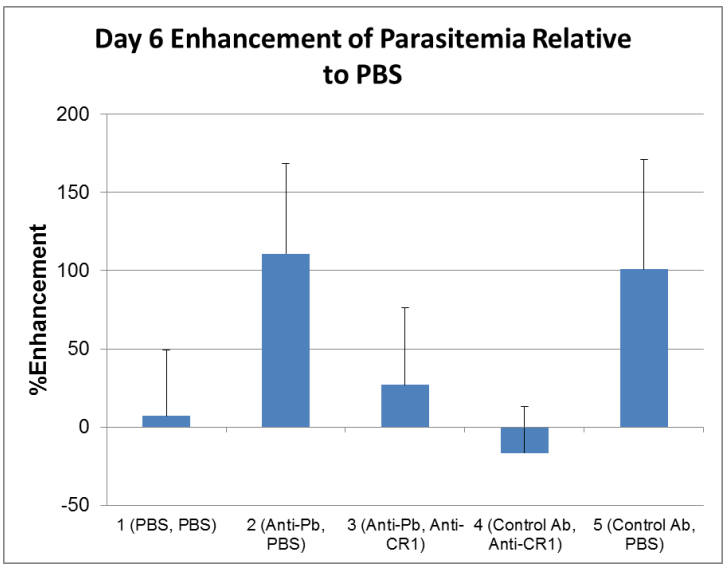

Figure 9. Effect of passive immunization with anti-Pb antibodies on *P. berghei* parasitemia in CR1 mice at 500 ug per mouse. Some mice also received chicken anti-CR1 polyclonal antibody.

Future Directions: We would like to explore this model further in a number of different ways. First, we would like to determine the arm(s) of the complement pathway involved in study the effect of different complement deficiencies by infecting genetically modified mice that are deficient in different complement factors such C3 deficient or mannose binding lectin deficient mice. We would like to purify the different IgG subclasses from the total anti-P. berghei IgG and infused mice with them to determine which subclasses are responsible for the enhancing or inhibitory effect. Further, we would like to determine whether immunizing mice with *P. berghei* MSP-1 can induce similar enhancement of parasitemia and whether purified antibodies from these mice have a parasitemia enhancing effect in naïve mice.

c. Training:

The experiments described provided material for the Ph.D. thesis of Mr. Sergei Biryukov who as graduate assistant in the PI's laboratory.

d. Dissemination of Results:

The results were disseminated via poster and oral presentation at international scientific conferences (see products). A manuscript is also under preparation.

4. Impact:

At the present time our results have not had impact on procedures and approach for vaccine development because they have not been published. A manuscript is being prepared and will be submitted soon (see below):

5. Changes/Problems:

Nothing to report.

6. Products:

These results were presented in the following meetings:

Military Health Systems Research Conference, Ft. Lauderdale Florida, August 2015 (Poster Presentation).

American Society of Tropical Medicine and Hygiene October 28, 2015. Oral Presentation.

Manuscript in preparation.

7. Participants and Other Collaborating Institutions:

Sergei Biryukov, Ph.D., Graduate Assistant, Penn State Hershey College of Medicine. Carried out most of the experiments described in this report.
Mary E. Landmesser, B.Sc., Laboratory Technician. Assisted with mouse experiments.
Liwang Cui, Ph.D. Penn State University, University Park. Assisted with cloning of PfRh4 and development of PfRh4 deletion mutant.

8. Special Reporting Requirements:

None

9. Appendices:

We are submitting the draft of a manuscript that is being prepared for publication.

Classification: BIOLOGICAL SCIENCES: Immunology and Inflammation; Microbiology;

Complement and Antibody-mediated Enhancement of Red Cell Invasion and Growth of *Plasmodium falciparum*

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Abstract

Plasmodium falciparum malaria kills hundreds of thousands of people every year. A vaccine that blocks red blood cell (RBC) invasion has been an elusive goal. Although anti-merozoite antibodies block invasion *in vitro*, there is no efficacy *in vivo*. Since merozoites are able to utilize the complement receptor 1 (CR1) on RBCs to invade we reasoned that complement activation could enhance invasion. To test this hypothesis we studied the role of complement in RBC invasion *in vitro* and in parasite growth in an animal model. Fresh serum enhanced RBC invasion relative to heat-inactivated serum (HIS). Anti-merozoite monoclonal mAb5.2, directed against $MSP1_{19}$, induced invasion enhancement that was inhibited by HIS, the C3 inhibitor compstatin, and soluble CR1 (sCR1). Antibody and complement-mediated invasion led to aggregation and colocalization of CR1, C3, and lipids on the RBC surface at the point of merozoite contact. Sialic acid-dependent strains that are unable to utilize native ligands to bind to CR1 were more dependent on complement for invasion of neuraminidase-treated RBCs than SA-independent strains that are able to use native ligands. Total IgG from $MSP1_{42}$ vaccinees enhanced invasion in a complement-dependent manner. Finally, total anti-*P. berghei* IgG enhanced parasite growth in mice and C3-deficient mice showed decreased parasite growth relative to wild type mice. Our results demonstrate that merozoites are able to evade the host immune response and hijack the complement system for their own benefit.

Significance Statement

Plasmodium falciparum malaria kills hundreds of thousands of people. The parasite invades and destroys red blood cells (RBCs). The development of a vaccine that blocks RBC invasion has been an elusive goal. Here we demonstrate that one possible explanation for this is that activation of the complement cascade by antibodies against merozoites enhances their attachment to and invasion of RBCs via the complement receptor 1 (CR1). Antibodies that target merozoites and activate complement can paradoxically aid the parasite to bind to and invade RBCs. Thus, this is the first demonstration that malaria parasites can utilize the host immune response for their own advantage.

Introduction:

Malaria, a mosquito-borne infectious disease caused by eukaryotic intracellular protists of the genus *Plasmodium*, kills over a million people worldwide each year, predominantly in children under 5 years of age (1, 2). *P. falciparum* accounts for the vast majority of deaths worldwide. *Plasmodium's* complex life cycle involves invasion of hepatocytes and red blood cells (RBCs). Antibodies are thought to play an important role in natural immunity. This has been demonstrated by the reduction in parasitemia and clinical symptoms in *P. falciparum*-infected individuals as a result of passive transfer of immunoglobulins from immune donors but the effector mechanisms are poorly understood (3-5).

Development of a vaccine to block RBC invasion has proven to be an elusive goal. Much of the effort has been focused on the merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA1). Numerous preclinical vaccine studies eliciting antibodies targeting MSP1 and AMA1 of *P. falciparum* have demonstrated *in vitro* inhibitory RBC invasion and growth inhibitory activity in growth inhibition assays (GIA), as well as protective immunity in different *in vivo* animal models (6-11). Unfortunately, to date, these studies have not translated into *in vivo* efficacy in human vaccine trials (12-14). Thus, GIA is a poor predictor of blood stage protective immune responses despite the fact that antibodies do inhibit RBC invasion. The reasons for this discrepancy are unknown.

One possible explanation for this mystery came to light as the result of the discovery that the complement receptor 1 (CR1) is a sialic acid (SA)-independent receptor for *P. falciparum* (15). The complement system is part of the innate immune response and is an important effector arm of humoral immunity. It can be activated via three main pathways: The classical pathway (CP); the lectin pathway (LP); and the alternative pathway (AP) (16). Once activated, the complement cascade induces the formation of opsonins (C3b, C4b) that that promote phagocytosis, induce direct killing by means of membrane lysis of microbial organisms, and promote an inflammatory response (16). Once bound to the pathogen, surface C3b and C4b serve as ligands for CR1, which is present on RBCs as well as most leukocytes (17, 18). Pathogens bound to RBCs can then be taken to reticuloendothelial organs where they are removed by macrophages (19). Complement activation takes place during malaria infection at least via the CP and AP (20-22) and has been linked to the pathogenesis of severe malaria (23, 24). Thus, we hypothesize that *P. falciparum* is capable of exploiting the opsonizing qualities of complement deposition on the merozoite surface which will allow it to bind to CR1 and invade via this invasion pathway. If we are correct, complement activation could negate the inhibitory activity of anti-merozoite neutralizing antibodies generated post vaccination or during natural infection.

Results:

Invasion of RBCs is Enhanced by Fresh Serum Relative to Heat-inactivated Serum and by Anti-merozoite Antibody mAb5.2: To test the hypothesis that complement can enhance erythrocyte invasion, we tested *P. falciparum* SA-independent laboratory strains 7G8 and 3D7 in the presence of fresh or heat-inactivated serum (HIS). Traditional methods of complement inactivation utilize 30 min heat treatment at 56 ^{o}C, but this degree of heat treatment likely results in the inactivation of multiple enzymes in the complement pathway. In order to achieve a more selective inactivation, we incubated serum for 3 min and 5 min at 56 ^{o}C and confirmed inactivation by measuring 50% complement hemolytic activity of the classical and alternative pathways (CH50 and AH50) (Supplementary Figure 1) (25). Figure 1A shows the ring parasitemia of overnight invasion in the presence of 30 min, 5 min, or 3 min HIS or fresh serum. Figure 1B shows the enhancement of parasitemia relative to 30 min HIS in the same experiment. The use of fresh serum resulted in a 40% invasion enhancement relative to 30 min HIS. A significant reduction in invasion enhancement was observed with just 3 or 5 min HIS (Figure 1B). These results suggest that complement may be involved in mediating the enhancement of invasion observed in fresh serum relative to HIS. Subsequent experiments are presented as the relative enhancement over 30 min HIS.

In order to determine whether activation of complement via the classical pathway by anti-merozoite antibodies could enhance invasion, we used mAb5.2 (MR4, Manassas, VA), raised against the C-terminal 19kD fragment of *P. falciparum* MSP1 Uganda-Palo

Alto (26). This antibody was selected due to its availability and relative ease of culture of the hybridoma. Figure 2 shows that, invasion was enhanced by 58% upon addition of mAb5.2 but not by the addition of IgG2b isotype control, suggesting that some antibodies that target the parasite may enhance erythrocyte invasion (Figure 2).

Enhancement of RBC Invasion by Anti-merozoite Antibody mAb5.2 is Complement-dependent: Using CH50 we confirmed that we could reverse the 3 min heat inactivation of complement by addition of complement factor 2 (C2) (Supplementary Figure 1A) (25). Therefore, we tested the effect of 3 min HIS on the ability of mAb5.2 to enhance parasite invasion of RBCs. Use of 3 min HIS abolished the antibody-mediated enhancement of invasion (Figure 3) (25). However, unlike CH50, rescue of antibody-mediated enhancement of invasion required addition of C2 and factor B (fB) to 3 min HIS. C2 and fB added separately only partially rescued the enhancement activity (Supplementary Figure 2).

In order to confirm further that complement is responsible for antibody-mediated enhancement of RBC invasion, we used the C3-selective inhibitor Compstatin, a cyclic 13-amino acid peptide that inhibits C3 cleavage and activation in human serum (27). Enhancement of invasion was observed in fresh serum in the presence of mAb5.2. The addition of compstatin eliminated this enhancement (Figure 4). A scrambled 13-amino acid linear control peptide showed no significant inhibitory activity in the presence of fresh serum and/or mAb5.2. Interestingly, compstatin had no effect on the enhancement of invasion by fresh serum alone. These results support the hypothesis

that the classical pathway of complement activation, mediated by anti-merozite antibodies, can result in enhancement of RBC invasion.

Soluble CR1 Blocks Enhancement of Invasion by Fresh Serum and Anti-merozoite Antibody mAb5.2: CR1 is known to bind C3b and C4b-opsonized immune complexes and was recently shown to be the SA-independent receptor of *P. falciparum* (15, 17, 18). Therefore, it is likely that CR1 is involved in complement and antibody-mediated enhancement of RBC invasion by *P. falciparum*. In order to ascertain the role of CR1 in the enhancement of invasion by fresh serum and anti-merozoite antibody, we used soluble CR1 (sCR1) as an inhibitor. Anti-merozoite antibody-mediated enhancement induced by mAb5.2 in fresh serum was not only negated in the presence of sCR1 but produced inhibition of invasion when compared to the isotype control, IgG2b (Figure 5). sCR1 had no effect in the absence of mAb5.2 (Figure 5).

SA-dependent Strains Demonstrate Greater Enhancement of Invasion in the Presence of Complement than SA-independent Strains: *P. falciparum* can be subdivided into SA-dependent strains that invade neuraminidase (NA)-treated RBCs at a low level and SA-independent strains that are able to invade NA-treated RBCs at a higher level via CR1 and the merozoite ligand PfRh4 (28). Since SA-dependent strains are unable to utilize PfRh4 for CR1-mediated invasion, we hypothesized that these strains may be more reliant on complement to accomplish this function. Hence, we compared invasion levels of SA-dependent and SA-independent stains in fresh and 30 min HIS in untreated and NA-treated RBCs. Figure 6A shows that SA-dependent

strains demonstrated a greater enhancement of invasion with fresh serum compared to HIS in NA-treated RBCs than SA-independent strains, median enhancement 54.8% vs. 31.5%, $P < 0.01$. Similar results, although of lower magnitude, were observed in the presence of C3/C4-inactivated vs C3/C4-reconstituted serum (Figure 6B).

Anti-merozoite mAb5.2 in the Presence of Complement Enhances Merozoite Attachment to RBCs: To address the question of whether the presence of complement enhances the binding of merozoites to the RBC surface we performed attachment assays. In order to inhibit merozoite invagination we allowed merozites to egress in the presence of cytochalasin D (29) and complement deficient or reconstituted serum plus either mAb5.2 or IgG2b isotype control. We then detected RBCs with surface merozoites using flow cytometry with mAb5.2. As expected, in the presence of mAb5.2 and deficient serum reconstituted with either C3/C4 or C3, we observed a significantly greater percent of RBCs with attached merozoites and surface C3 than in the presence of IgG2b isotype control and complement deficient serum (Figure 7A & 7B). In addition to the attachment assay we were also able to identify increased C3b deposition on single merozoites after incubation in fresh serum but not in 30 min HIS using flow cytometry (Supplementary Figure 3).

Anti-merozoite Antibody mAb5.2 Induces CR1 Aggregation and Complement Deposition at the RBC-merozoite Interface: In the interest of depicting the direct interaction of merozoites with RBCs in the presence of complement and mAb5.2 we performed immunofluorescence microscopy. Attachment assays were setup in the presence of C3 depleted and reconstituted serum, with and without mAb5.2. Figure 8

shows that in the presence of C3 and mAb5.2 there was dense aggregation of CR1 and lipids, and C3 deposition at the site of merozoite contact with the RBC. Of further interest is that, as pareviously noted (15), CR1 showed its classical punctate staining not only on RBCs but also on merozoites regardless of the presence of complement. This suggests the possibility that merozoites could have surface CR1.

Complement Decreases Invasion Inhibitory Activity of Human Anti-MSP1 Antibodies: To assess the significance of human antibody-mediated complement activation on RBC invasion we purified total IgG from $MSP1_{42}$ vaccinees who had never been exposed to malaria (30). Using C3/C4-inactivated or reconstituted serum we setup invasion assays in the presence of 2.5 mg/ml total IgG from MSP1 vaccinees or unvaccinated controls. Percent inhibition was calculated relative to PBS-only since we did not have pre-immunization serum. The median inhibitory activity in C3/C4-inactivated serum was around 6% but upon reconstitution of serum with the missing complement factors the inhibitory activity was not only eliminated but became negative signifying enhancement (Figure 9). There was no difference in inhibitory activity with IgG from unvaccinated controls between C3/C4-inactivated and reconstituted serum.

Passive Transfer of Anti-*P. berghei* Antibodies Enhances Parasitemia in a Dose-dependent Manner: To validate the observed *in vitro* antibody-mediated complement enhancement of RBC invasion in an *in vivo* setting we infected C57BL/6 mice with *P. berghei* ANKA and administered varying concentrations of polyclonal total anti-*P. berghei* IgG (anti-Pb), control total IgG from naïve mice, or PBS. The anti-*P. berghei*

antibody resulted in enhancement of endpoint parasitemia in a reverse dose dependent manner when compared to the PBS control group (Figure 10). Surprisingly, control antibodies, independent of the dose administered, produced an inhibitory effect when compared to the PBS control.

C3 Deficient Mice Show Blunted Parasite Growth: To explore further the role of C3 in *in vivo* parasite growth, we subjected $C3^{-/-}$ mice to *P. berghei* infection in the presence of anti-*P. berghei*, control antibodies, or PBS. Figure 11 shows that $C3^{-/-}$ had blunted parasite growth relative to their wild type counterparts across all groups although it was only statistically significant in the case of PBS. $C3^{-/-}$ mice that received anti-Pb antibody still showed enhancement of parasitemia although it did not reach statistical significance. $C3^{-/-}$ mice that received control IgG had an intermediate response.

Discussion:

Following complement activation, C4b or C3b-opsonized pathogens can bind to CR1 on RBCs and are removed by phagocytosis in the liver and spleen (31). This process of pathogen opsonization, binding to CR1, and removal protects the host. However, unlike other pathogens, opsonization of malaria merozoites may facilitate binding to their ultimate target cell, the RBC. There is evidence that complement activation can enhance invasion of target cells by other pathogens such as *Leishmania* and *Babesia* (32, 33). Therefore, we reasoned that opsonization of malaria merozoites may enhance RBC invasion.

We provide compelling evidence that complement activation can enhance invasion of RBCs especially in the presence of anti-merozoite antibodies. We showed that fresh untreated serum enhances RBC invasion over 3 min, 5 min, and 30 min HIS. Although we were able to restore the CP CH50 activity of 3 min HIS by addition of C2 and fB, addition of these factors to 3 min HIS did not rescue the RBC invasion enhancing activity. Thus, we suspect that elements of the LP upstream of C2 are also inactivated in 3 min HIS. On the other hand, we showed that the RBC invasion enhancing activity of mAb5.2 required C2 and fB. Because C2 is an integral part of the CP and fB of the AP amplification loop, these data are strong evidence that both of these pathways of complement activation play a role in antibody-mediated enhancement of RBC invasion. The important role of C3 was demonstrated by the effect of the C3 specific inhibitor compstatin. Use of this inhibitor eliminated antibody-mediated enhancement of invasion but had no effect on enhancement by serum alone (Figure 3). Similarly, we showed

that an intact complement system in the presence of purified total IgG from $MSP1_{42}$ vaccine recipients (30) resulted in decreased invasion inhibitory activity and even enhancement of invasion compared to complement deficient serum. Thus, these results demonstrate that enhancement of RBC invasion can occur with anti-merozoite antibodies in the presence of an intact complement system.

Because SA-dependent strains are unable to use PfRh4 as a CR1 ligand, we reasoned that these strains may rely more on complement for CR1-mediated invasion than SA-independent strains that are able to utilize PfRh4. To test this, we used NA-treated RBCs in which invasion is almost totally CR1-mediated (15). We observed that in both SA-dependent and independent strains invasion of NA-treated RBCs was enhanced by the use of fresh serum compared to HIS, and in C3/C4-reconstituted serum relative to C3/C4-inactivated serum (Figure 6). However, SA-dependent strains exhibited greater level of invasion enhancement into NA-treated RBCs than SA-independent strains. Two properties of NA-treated RBCs contributed to these observations: 1) Unregulated AP activity (34, 35) and 2) lack of sialic acid-dependent invasion. Thus, these experiments, unlike the experiments with intact RBCs, showed that the invasion enhancing properties of serum can be rescued by the addition of complement factors and that inability to use PfRh4 or other SA-independent ligands increases the reliance on complement for RBC invasion.

We showed that complement activation via the CP leads to increased attachment of merozoites to RBCs. Confocal microscopy and the sCR1 inhibitory activity showed that

CR1 plays a critical role in this interaction. Confocal microscopy also allowed us to make a number of novel observations. In the presence of mAb5.2 and complement, we observed alterations in the RBC membrane consisting of aggregation of CR1 and lipids as suggested by the intense fluorescence of the lipid soluble dye DiD at the point of merozoite attachment. The co-localization of C3, CR1, and increased DiD fluorescence suggests that the aggregation of CR1 is an active process brought on by engagement with C3. Ligation of CR1 with opsonized ICs has been shown to cause increased membrane deformability and thus could explain some of these changes (36). By contrast, we did not observe these changes in the absence of C3. Another important observation is the suggestion that merozoites are coated with CR1. Although our results are not definitive, the possibility that merozoites are coated with CR1 could explain their relative resistance to complement-mediated lysis. In addition, if this is correct, there is the possibility of CR1-CR1 interaction between merozoites and the RBC and of merozoites binding opsonized ICs which could lead to engagement of CR1 on the RBC.

Our passive transfer experiments in mice confirmed that anti-malaria antibody and complement can benefit parasite growth. Administration of Anti-Pb IgG enhanced parasite growth in a reverse dose-dependent manner. At first look, these results stand in contrast to previous studies that have shown that antibody from immune rodents inhibits growth *in vivo* when given to naïve animals (37-41). However, the doses of antibody in those studies were much higher, usually given in milligram amounts and sometimes given in several doses prior to and after infection. In addition, several studies have also shown that passive transfer of total IgG from individuals living in

endemic areas to acutely infected children can reduce parasitemia (42-44) but this was achieved at 5-10 fold higher doses per Kg than the ones we have used in our studies. It is unlikely that these levels of antibodies can be achieved by natural infection. Thus, low doses of anti-merozoite antibodies can enhance parasite growth *in vivo*. Our studies suggest that one possible mechanism may be complement activation. By contrast, the inhibitory activity of control polyclonal antibody may be due to its complement scavenging properties (45, 46). Consistent with this hypothesis, $C3^{-/-}$ mice consistently showed significant blunting of parasitemia relative to wild type mice. Yet, they still showed enhancement of parasite growth in response to anti-Pb IgG probably due to residual C4b opsonizing activity.

During the writing of this manuscript, Boyle *et al.* (47) reported that anti-merozoite antibodies from humans inhibit invasion by activating complement and thus, partially contradict our results. The contrasting results are most likely due to a key methodological difference. While we allowed merozoites to egress naturally from IRBCs, Boyle *et al.* (47) used protease-synchronized trophozoites and filter-purified merozoites. Because of the extensive manipulation, these merozoites may be less fit than freshly egressed merozoites and, hence, more susceptible to complement deposition. Nonetheless, we believe that while some anti-merozoite antibodies are capable of enhancing invasion, others are inhibitory. The final outcome will depend on the relative prevalence of the two. We do agree with *Boyle et al.* (47) that complement activation should be included in GIA.

Our studies demonstrate that merozoites are capable of using complement to aid the invasion of RBCs allowing the parasite to not only evade the immune response of the host but also hijack it for its own advantage. These results have important implications for merozoite vaccine development. Based on our data, merozoite vaccines may be more effective if they induce non-complement fixing antibodies.

Methods (See Also Supplementary Information)**:**

Materials and Data Availability: All data and protocols pertaining to this manuscript will be deposited in https://scholarsphere.psu.edu. Access will be granted upon written request.

Invasion Assays: Invasion assays were carried out as described (48). The cyclical C3 inhibitor peptide compstatin (NH3-IIe-Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys-Thr-COOH) (Tocris Bioscience, Bristol, UK), was used to determine the effect of blocking C3 (23). A peptide derived from the linearized and scrambled sequence of compstatin without cysteins (NH3-Arg-Thr-Ala-Trp-Gln-His-Asp-Ala-lle-His-Val-Gly-Val-COOH) was synthesized and used as a control. sCR1 was used as an inhibitor (Celldex Therapeutics, Inc., Hampton, NJ) and fetuin (Sigma-Aldrich) was used as negative control protein where appropriate. mAb5.2 was purified from hybridoma (ATCC) culture supernatant. Mouse IgG2b Clone eBMG2b (eBioscience, San Diego, CA) was used as isotype control. Total human IgG was purified from serum or plasma by protein A/G chromatography (Thermo Scientific, Rockford, IL). After 20 to 24 hours, 5 µL aliquots of individual wells were added to 100 µL 5 µg/ml Hoechst 33342 (Life Technologies, Grand Island, NY) in PBS containing 2% paraformaldehyde. At least 100,000 RBCs were acquired for each sample. Acquisition was done using a LSRII flow cytometer (Becton-Dickinson) equipped with a violet laser and analysis was performed using FCS Express (De novo Software). RBCs with Hoechst-positive ring stage parasitemia (early trophozoite) were used as endpoint for 24-hour invasion assays. The background

staining of an uninfected RBC sample was subtracted. All experiments were repeated 2-3 times.

Merozoite Attachment Assay: Leupeptin (Sigma-Aldrich) 10 µg/ml was added to highly synchronous late stage parasite cultures and incubated for 6 to 8 hours followed by three washes with incomplete media and harvesting in-between by centrifugation at 770 xg for 5 min. Fresh RBCs were added to 2% Hct and 100 µL reactions were set up in 96-well well plates in the presence of 2 µM cytochalasin D (Sigma-Aldrich) along with 10% C3/C4-inactivated serum or C3-depleted serum, and 40 µg/ml mAb5.2 or IgG2b isotype control. For repletion of deficient serum we added C4 to a final concentration of 64 µg/ml and/or C3 to a final concentration of 100 µg/ml (CompTech, Tyler, TX). The plates were gassed and incubated at 37 ^{O}C for 3-4 hours as before. The reaction plate was centrifuged at 400 xg for 1 min 4 ^{O}C and washed twice with 100 µL of 1% BSA/PBS blocking buffer. Primary mouse anti-MSP1 mAb5.2 (2 mg/ml) and goat polyclonal anti-C3 (MP Biomedicals) antibodies diluted 1:33 were added in blocking buffer for 30 min at 4 ºC. Following three washes with blocking buffer, secondary donkey anti-goat IgG-PerCP (R&D Systems) and goat anti-mouse -DyLight 488 (KPL, Kirkegaard & Perry Laboratories) antibodies diluted 1:100 were added in blocking buffer for 30 min at 4 ºC. Following three washes with blocking buffer the pellets were resuspended in 2% formaldehyde/PBS with 5 ug/ml Hoechst 33342 solution. Acquisition was carried out as above.

Immunofluorescence Microscopy of Merozoite Attachment: For detection of RBCs with attached merozoites we followed the same protocol as described above for merozoite attachment assays. However, after the initial assay we resuspended the pellets in 1% BSA/PBS blocking buffer containing 130 µg/ml mouse anti-MSP1 mAb5.2 (if not present during the assay), 190 µg/ml of chicken polyclonal anti-CR1 (Gallus Immunotech, Fergus, ON, CA), and 30 µg/ml of mouse monoclonal 1H8 Anti-C3 IgG1 (Kerafast) and incubated for 30 min at 4 ^{o}C. After three washes with blocking buffer, the pellets were resuspend in 100 µL of blocking buffer containing 1:500 dilutions of goat anti-mouse IgG2b-DyLight 488 (Thermo Fisher Scientific Inc.), goat anti-chicken Alexa Fluor 546 (Thermo Fisher Scientific Inc.), goat anti-mouse IgG1-Alexa Fluor 594 (Thermo Fisher Scientific Inc.), and 30 µM Vybrant DID cell labeling solution (Molecular Probes, Inc, Eugene, OR) and incubated for 30 min at 4 ^{o}C. After an additional three washes, the pellets were resuspended in 4% paraformaldehyde with 10 µg/ml Hoechst 33342. Prior to imaging, 10 µL of the cell suspension was transferred into 0.6 ml tubes and centrifuged at 200xg for 1 min. The supernatant was discarded and the pellet was resuspended in 10 µL of VectorShield Hard Set (Vector Labs, Inc.). 4 µL of this suspension was added to wells of a multi-well slide. Fluorescence z-series were collected on a LEICA SP8 confocal microscope at 40x oil immersion. The images were processed using **Imaris (Bitplane)** image processing software.

Passive Transfer Experiments: Polyclonal anti-*P. berghei* antibody was generated by intraperitoneal infection of C57BL/6 mice with *P*. *berghei* ANKA (1x10^{6} IRBC). Parasitemia was monitored with Giemsa smears and once parasitemia reached (5%-

10%) the mice were treated with sub-curative dose of chloroquine (500 µg/mouse) for three consecutive days. The parasitemia was allowed to rebound and the cycle was repeated for a total of 3 times. Plasma was collected from infected mice two weeks after the third cycle in *citrate phosphate dextrose* solution (CPD) and total antibody was purified using protein A/G columns (GenScript, Piscataway, NJ). Antibody purified from uninfected mice served as control. Wild type and $C3^{-/-}$ mice of C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME) were injected with $1.5x10^7$ IRBCs in 100 µL or RPMI 1640 in one retro-orbital plexus and with 100 µL of anti-*P. berghei*, control antibodies, or 100 µL of PBS in the contralateral plexus. Parasitemia was monitored by staining tail vein blood with Hoechst 33342 (2 µg/ml in PF) (Life Technologies) at pre-determined intervals until day 6 or 7 post-infection. Acquisition and analysis were performed as described above.

Ethics Statement: All human samples were obtained under protocols approved by the pertinent review boards. Consent was obtained from all participants except when the samples were obtained under non-human subject determination.

Statistical Analysis: Statistical analysis was done with SigmaPlot v11.2 (Systat Software, San José, CA). Differences between groups in variables that showed a normal distribution were evaluated by analysis of variance or t-test, whichever was more appropriate. Post-hoc Holm-Sidak tests were used to correct for multiple comparisons. All tests were two-tailed. A $P < 0.05$ was considered statistically significant.

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Figure Legends:

Figure 1: Enhancement of *P. falciparum* invasion of RBCs with fresh serum relative to heat-inactivated serum (HIS). A) Two SA-independent strains of *P. falciparum*, 7G8 and 3D7, were tested in the presence of 10% fresh serum, 3 min HIS, 5 min HIS, or 30 min HIS. Invasion of RBCs decreased with progressively longer heat inactivation times. P is for the comparison with 30 min HIS, One-way ANOVA with post-hoc comparisons. B) Invasion of RBCs in the same experiment expressed as percent enhancement relative to 30 min HIS. P is for the comparison with 5 min HIS. Error bars represent standard deviations.

Figure 2: Anti-merozoite monoclonal antibody mAb5.2 enhances *P. falciparum* invasion of RBCs in the presence of fresh serum. Invasion of *P. falciparum* strain 7G8 was tested in the presence of monoclonal anti-merozoite surface protein 1(MSP1) mAb5.2 (40 µg/ml), an isotype control antibody (IgG2b), or PBS in 10% fresh serum or 30 min HIS. Invasion of erythrocytes is expressed as percent enhancement in fresh serum relative to 30 min HIS. Error bars represent standard deviations. *P <0.001 is for one-way ANOVA with post-hoc comparisons.

Figure 3: Addition of C2 and fB rescues RBC invasion enhancement properties of mAb5.2 in 3 min HIS. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2, 40 µg/ml IgG2b isotype control, or PBS in the presence of 10% fresh serum , 3 min HIS, or 30 min HIS. C2 and fB were added to a final concentration of 25 µg/ml and 200 µg/ml respectively, or the addition of 225 µg/ml fetuin as a control. Error bars represent standard deviations. P is for the comparison between mAb5.2 and IgG2b or PBS.

Figure 4: The C3 inhibitor compstatin inhibits enhancement of RBC invasion by mAb5.2. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2, 40 µg/ml IgG2b isotype control, or PBS in 10% fresh serum or 30 min HIS with the addition of

C3 inhibitor peptide compstatin or control peptide. Invasion of erythrocytes is expressed as percent enhancement in fresh serum relative to 30 min HIS. Error bars represent standard deviations. P is based on the t-test.

Figure 5: Enhancement of RBC invasion by mAb5.2 is abolished in the presence of sCR1. Invasion of *P. falciparum* strain 7G8 was tested in the presence of 40 µg/ml anti-MSP1 mAb5.2 or 40 µg/ml IgG2b isotype control in the presence of 10% fresh serum or 30 min HIS. The final concentration of sCR1 or fetuin was 80 µg/ml. Error bars represent standard deviations. P is based on t-test.

Figure 6: Differences in enhancement of RBC invasion in NA-treated and untreated RBCs between SA-dependent and independent strains. A) SA-dependent strains show greater enhancement of invasion into NA-treated RBC (median 54.8%) in the presence of fresh serum relative to 30 min HIS than SA-independent strains (median 31.5%), $P < 0.01$ by t-test. B) Enhancement of invasion into NA-treated RBCs in the presence of serum C3/C4 reconstituted serum relative to C3/C4-inactivated serum. P values are based on t-test.

Figure 7: Antibody and complement-mediated enhancement of merozoite attachment to RBCs. Attachment of *P. falciparum* strain 3D7 was tested in the presence of anti-MSP1 mAb5.2 or IgG2b isotype control, in the presence of A) C3/C4-inactivated or C3/C4-reconstituted serum and B) C3-depleted serum or C3-reconstituted serum.

Figure 8: Interaction of merozoites with RBCs in the presence of anti-MSP1 mAb5.2 and in the presence or absence of C3. C3-reconstituted serum and anti-MSP1 mAb5.2 bind to RBCs via CR1 (yellow) which shows intense aggregation at the site of merozoite contact. This area is also positive for C3 (cyan) and shows lipid accumulation by DiD staining (red). Hoechst 33342 was

used to stain DNA (blue) and MSP1 staining shows green. On the other hand, using C3-depleted serum there is little or no C3 deposition, and absence of CR1 aggregation or lipid accumulation. Of interest, we observed the characteristic speckled staining of CR1 (yellow) on the surface of all the merozoites. Scale bar is 2 μm.

Figure 9: Complement decreases inhibitory activity of total IgG from $MSP1_{42}$ vaccine recipients. A) Invasion of *P. falciparum* strain FVO in the presence of purified total IgG (2.5 mg/ml) from $MSP1_{42}$ vaccine recipients and either C3/C4-inactivated or C3/C4-reconstituted serum, N = 13. B) Invasion in the presence of total IgG from malaria naïve non-vaccinee controls, N = 14. Horizontal markers represent medians. P is by paired t-test.

Figure 10: Anti-*P. berghei* antibodies enhance endpoint parasitemia in an inverse dose-dependent manner. Wild type (C57BL/6) mice infected with *P.berghei* ANKA ($1.5x10^7$ pRBC/100 μl) and injected with varying doses of anti-*P. berghei* or control antibody in100 μl volume. Control mice were injected with 100ul PBS. Parasitemia was measured from tail vein blood by flow cytometry with Hoechst 33342. Error bars represent standard deviations. *P <0.001 is for one-way ANOVA with post-hoc comparisons.

Figure 11: $C3^{-/-}$ mice have decreased parasite growth relative to wild type mice. $C3^{-/-}$ or wild type mice were injected with 250 μg of anti-*P. berghei* IgG, control IgG, or an equivalent volume of PBS. The y axis shows the enhancement of day 6 post-infection parasitemia relative to wild type mice that received PBS. This is a representative experiment of three. The P value was obtained using t-test.

Figure 1

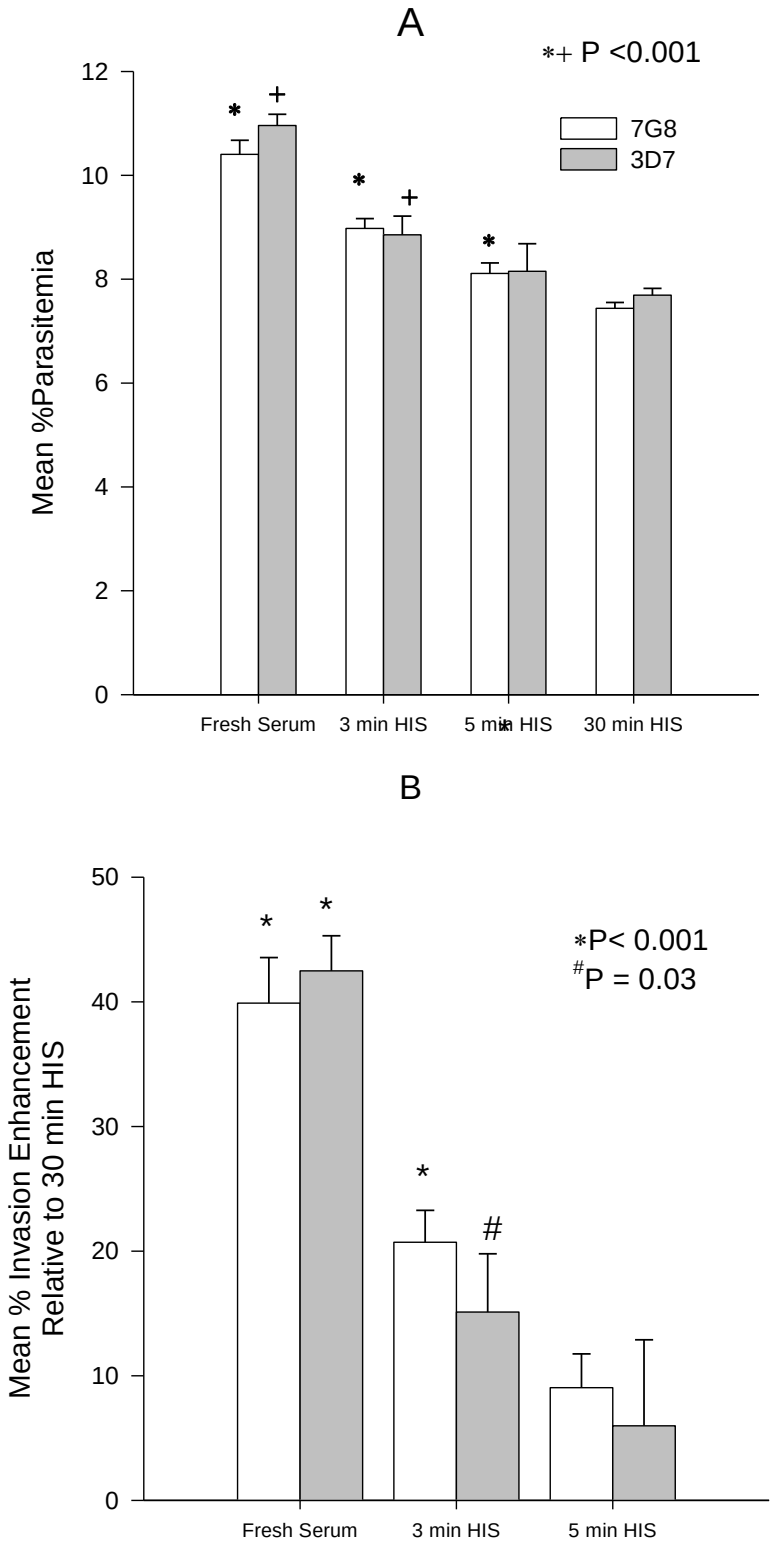

A
*+ P <0.001
7G8
3D7
Mean %Parasitemia
12
10
8
6
4
2
0
Fresh Serum
3 min HIS
5 min HIS
30 min HIS
B
*P< 0.001
#P = 0.03
Mean % Invasion Enhancement
Relative to 30 min HIS
50
40
30
20
10
0
Fresh Serum
3 min HIS
5 min HIS

Figure 2

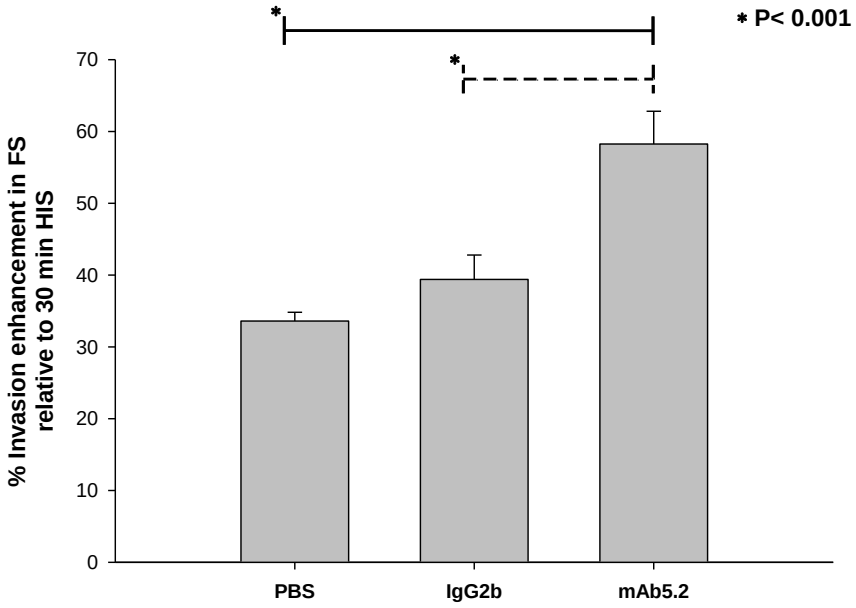

* P< 0.001
*
*
% Invasion enhancement in FS
relative to 30 min HIS
70
60
50
40
30
20
10
0
PBS
IgG2b
mAb5.2

Figure 3

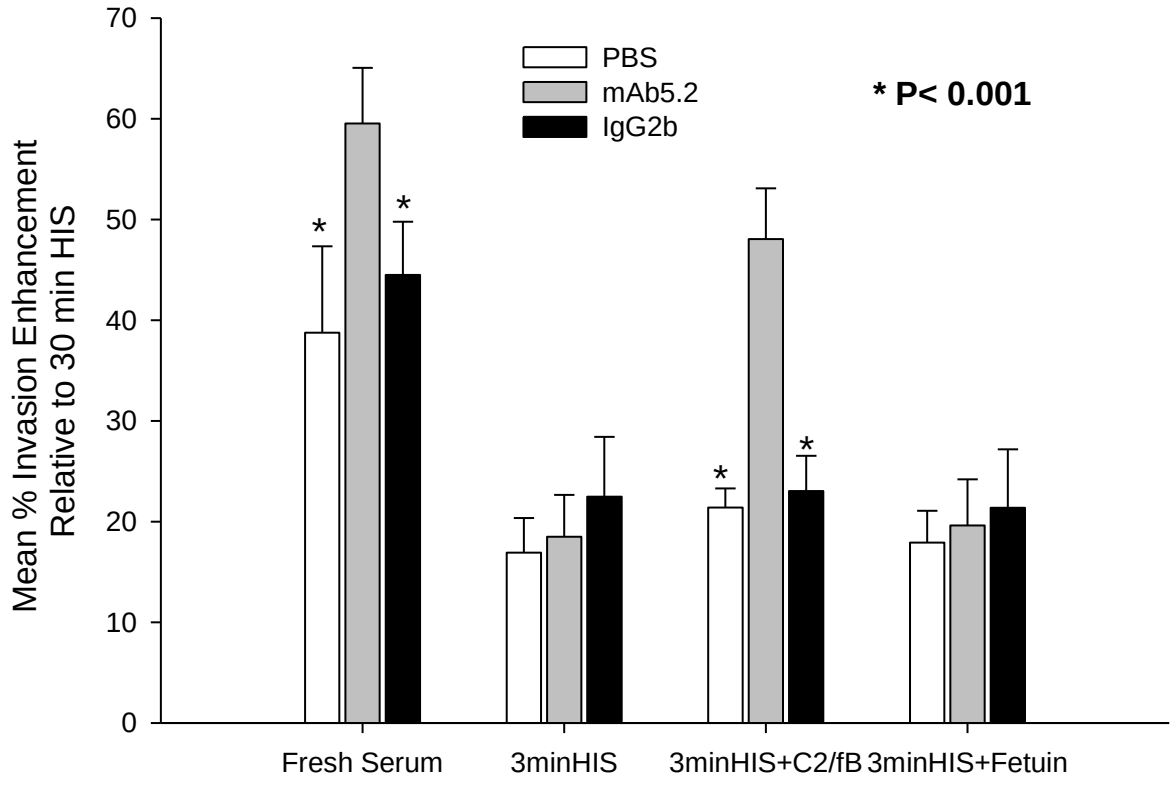

Mean % Invasion Enhancement
Relative to 30 min HIS
70
60
50
40
30
20
10
0
PBS
mAb5.2
IgG2b
* P< 0.001
*
*
*
*
Fresh Serum
3minHIS
3minHIS+C2/fB
3minHIS+Fetuin

Figure 4

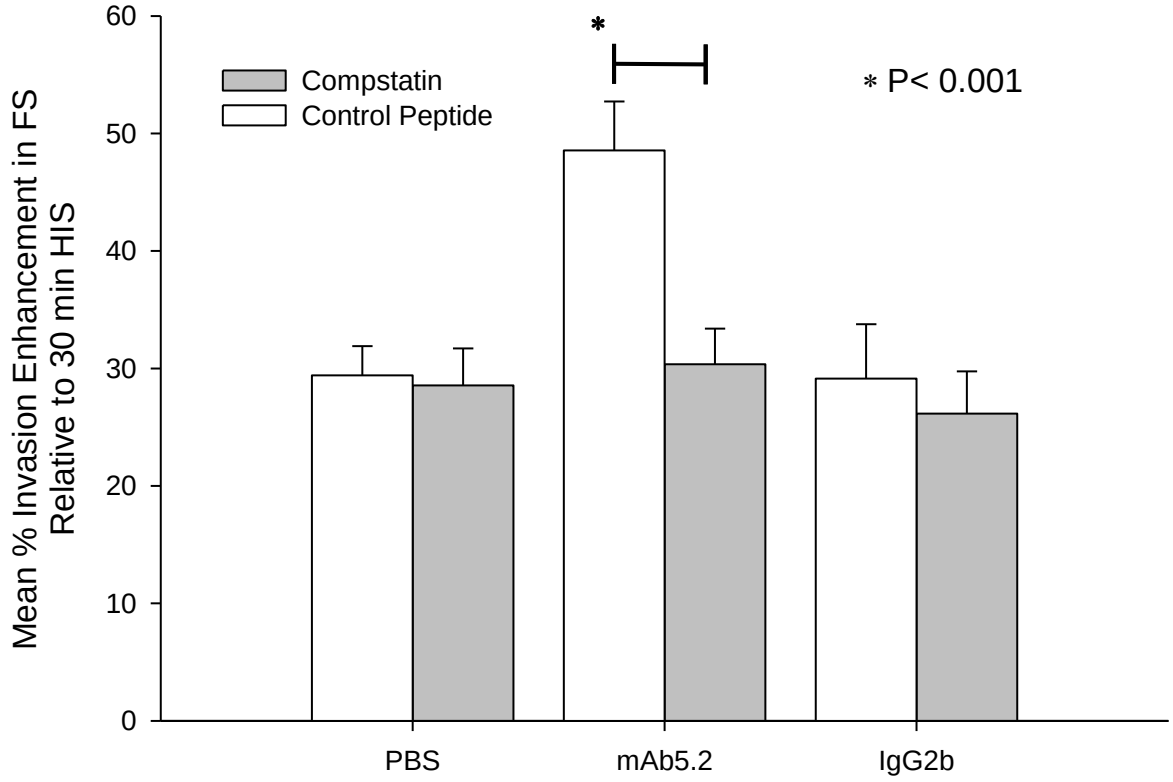

Mean % Invasion Enhancement in FS
Relative to 30 min HIS
60
50
40
30
20
10
0
Compstatin
Control Peptide
*
* P< 0.001
PBS
mAb5.2
IgG2b

Figure 5

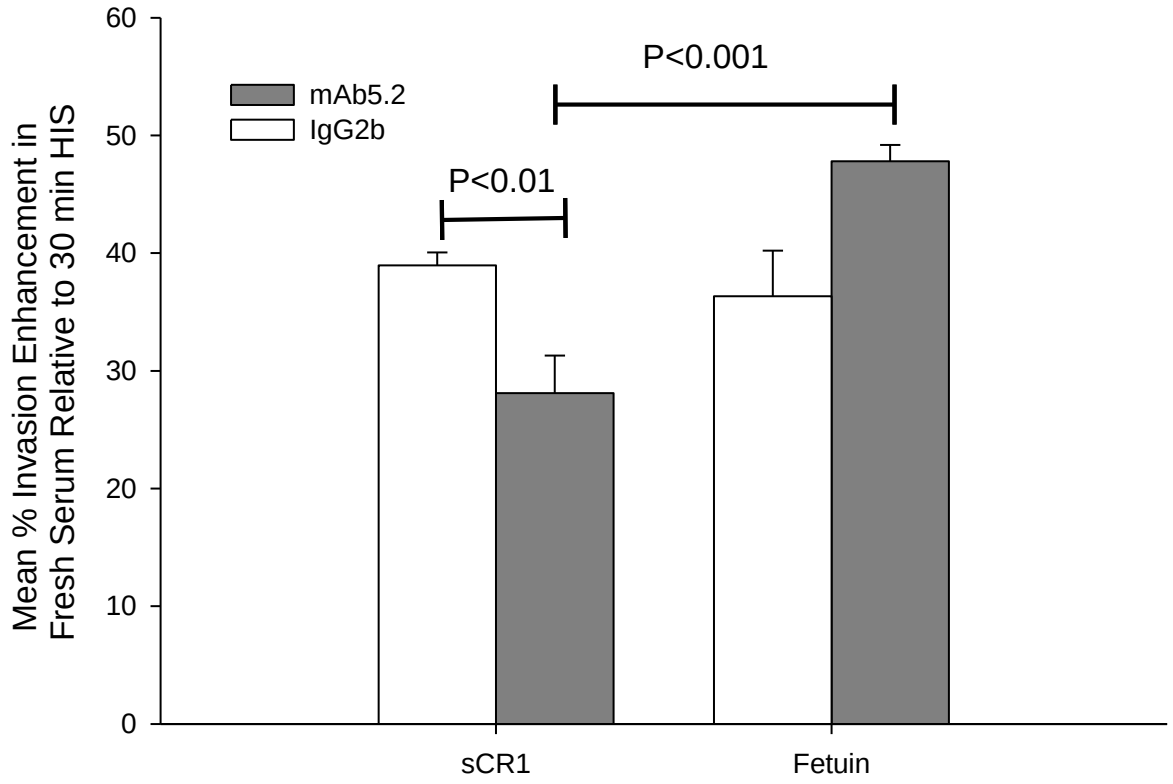

Mean % Invasion Enhancement in Fresh Serum Relative to 30 min HIS
60
50
40
30
20
10
0
mAb5.2
IgG2b
P<0.001
P<0.01
sCR1
Fetuin

Figure 6

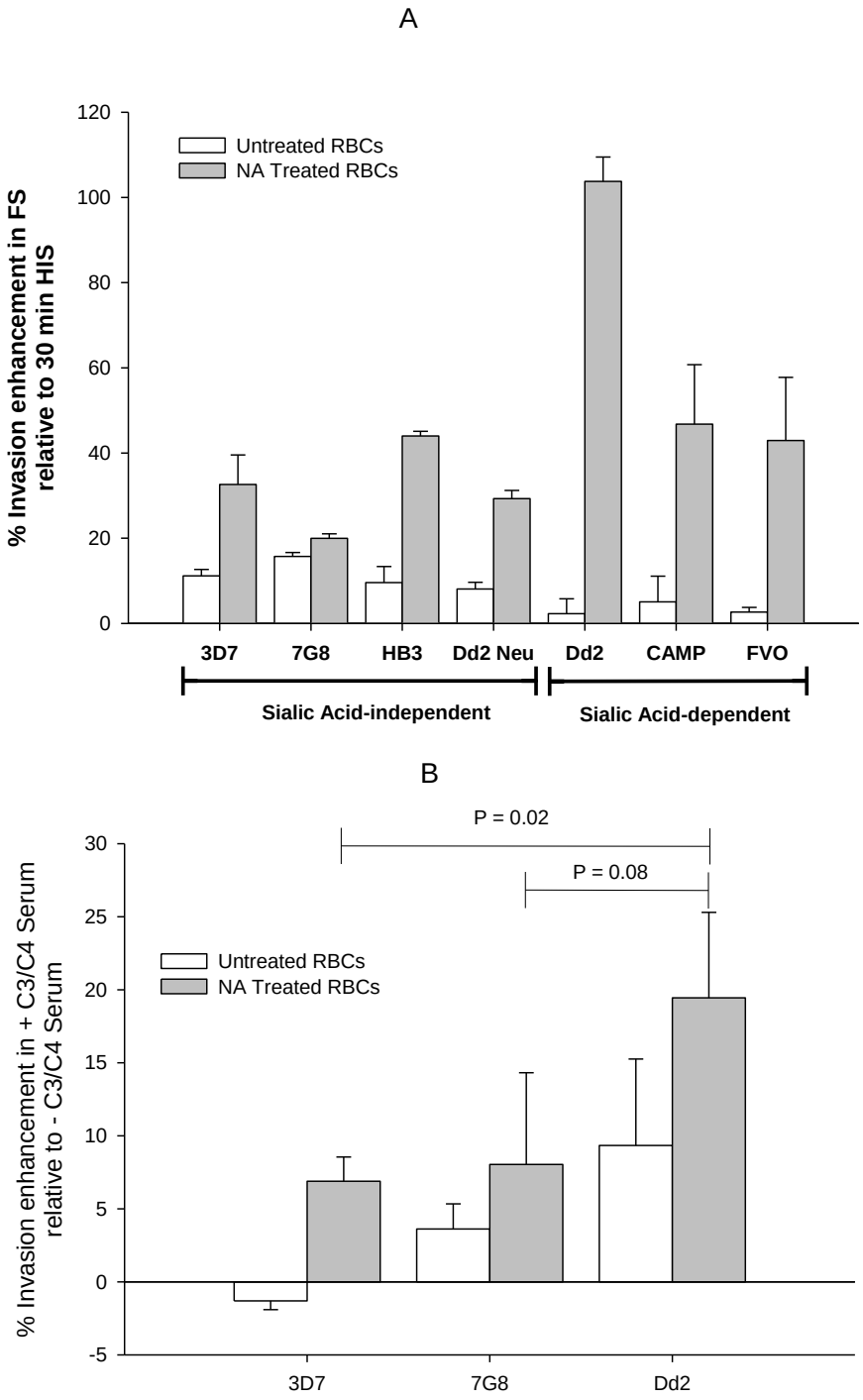

A
120
100
80
60
40
20
0
Untreated RBCs
NA Treated RBCs
% Invasion enhancement in FS
relative to 30 min HIS
3D7
7G8
HB3
Dd2 Neu
Dd2
CAMP
FVO
Sialic Acid-independent
Sialic Acid-dependent
B
P = 0.02
P = 0.08
30
25
20
15
10
5
0
-5
Untreated RBCs
NA Treated RBCs
% Invasion enhancement in + C3/C4 Serum
relative to - C3/C4 Serum
3D7
7G8
Dd2

Figure 7

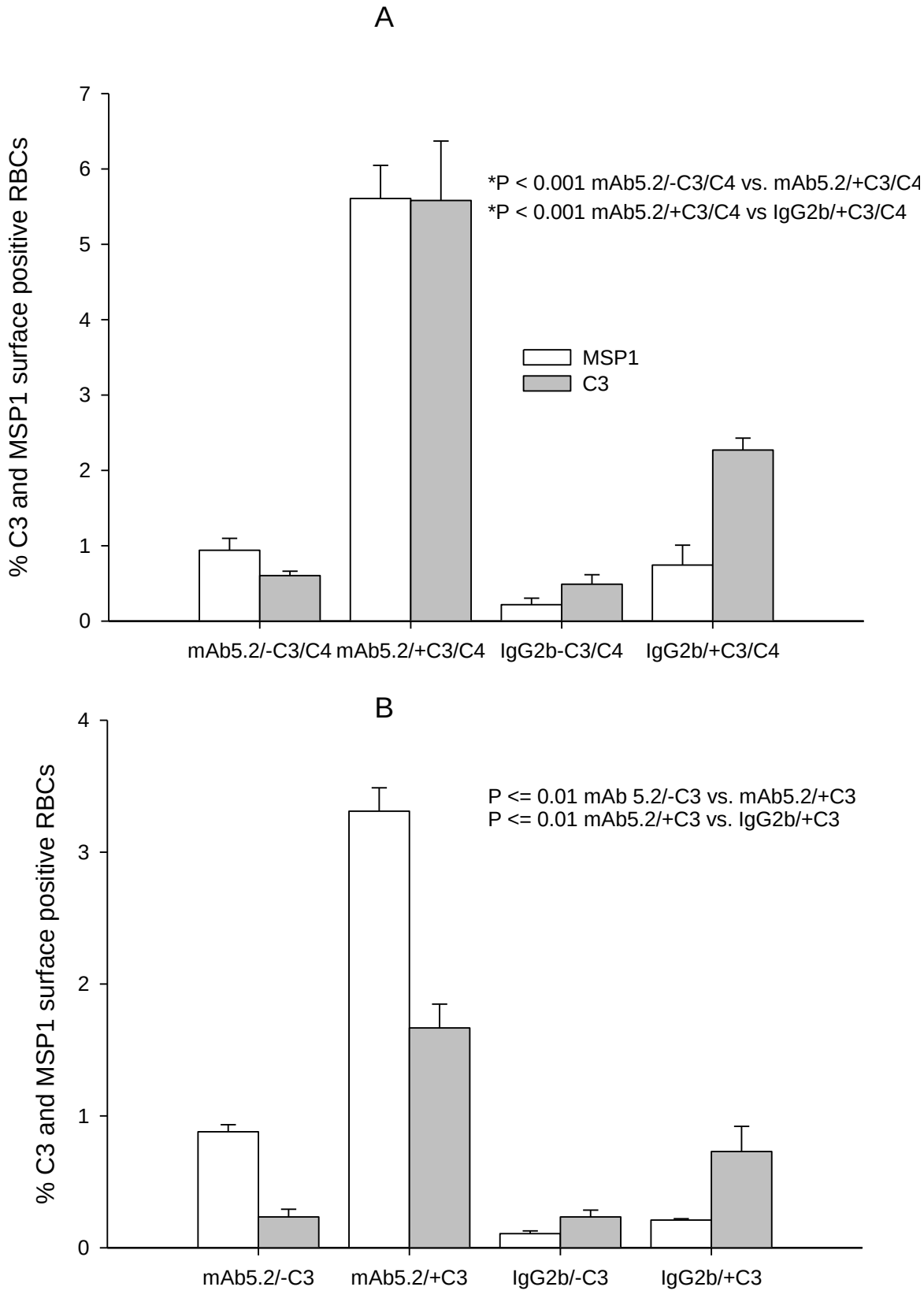

A
% C3 and MSP1 surface positive RBCs
7
6
5
4
3
2
1
0
*P < 0.001 mAb5.2/-C3/C4 vs. mAb5.2/+C3/C4
*P < 0.001 mAb5.2/+C3/C4 vs IgG2b/+C3/C4
MSP1
C3
mAb5.2/-C3/C4
mAb5.2/+C3/C4
IgG2b-C3/C4
IgG2b/+C3/C4
B
% C3 and MSP1 surface positive RBCs
4
3
2
1
0
P <= 0.01 mAb 5.2/-C3 vs. mAb5.2/+C3
P <= 0.01 mAb5.2/+C3 vs. IgG2b/+C3
mAb5.2/-C3
mAb5.2/+C3
IgG2b/-C3
IgG2b/+C3

Figure 8

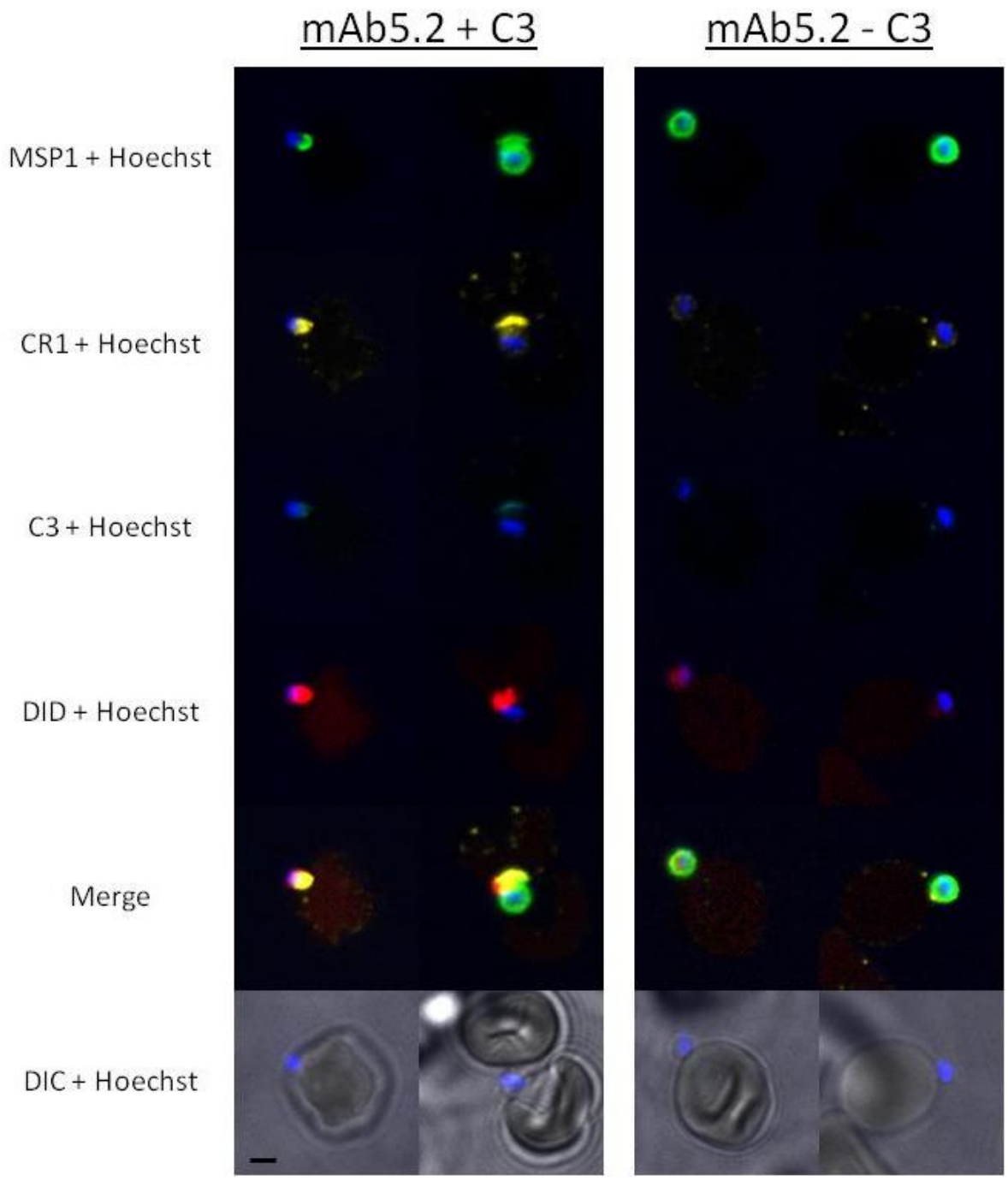

mAb5.2 + C3
mAb5.2 - C3
MSP1 + Hoechst
CR1 + Hoechst
C3 + Hoechst
DID + Hoechst
Merge
DIC + Hoechst

Figure 9

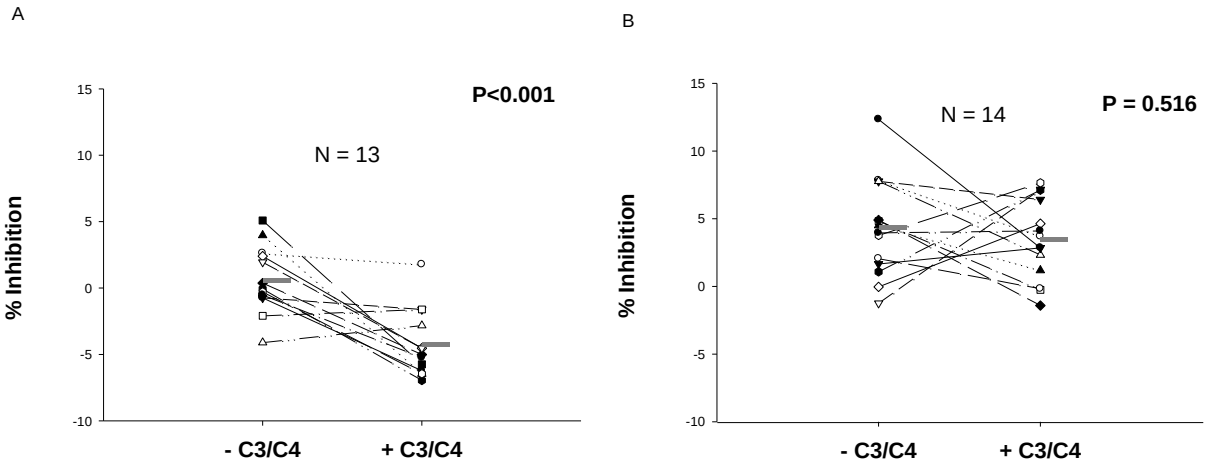

A
P<0.001
N = 13
% Inhibition
15
10
5
0
-5
-10
- C3/C4
+ C3/C4
B
N = 14
P = 0.516
% Inhibition
15
10
5
0
-5
-10
- C3/C4
+ C3/C4

Figure 10

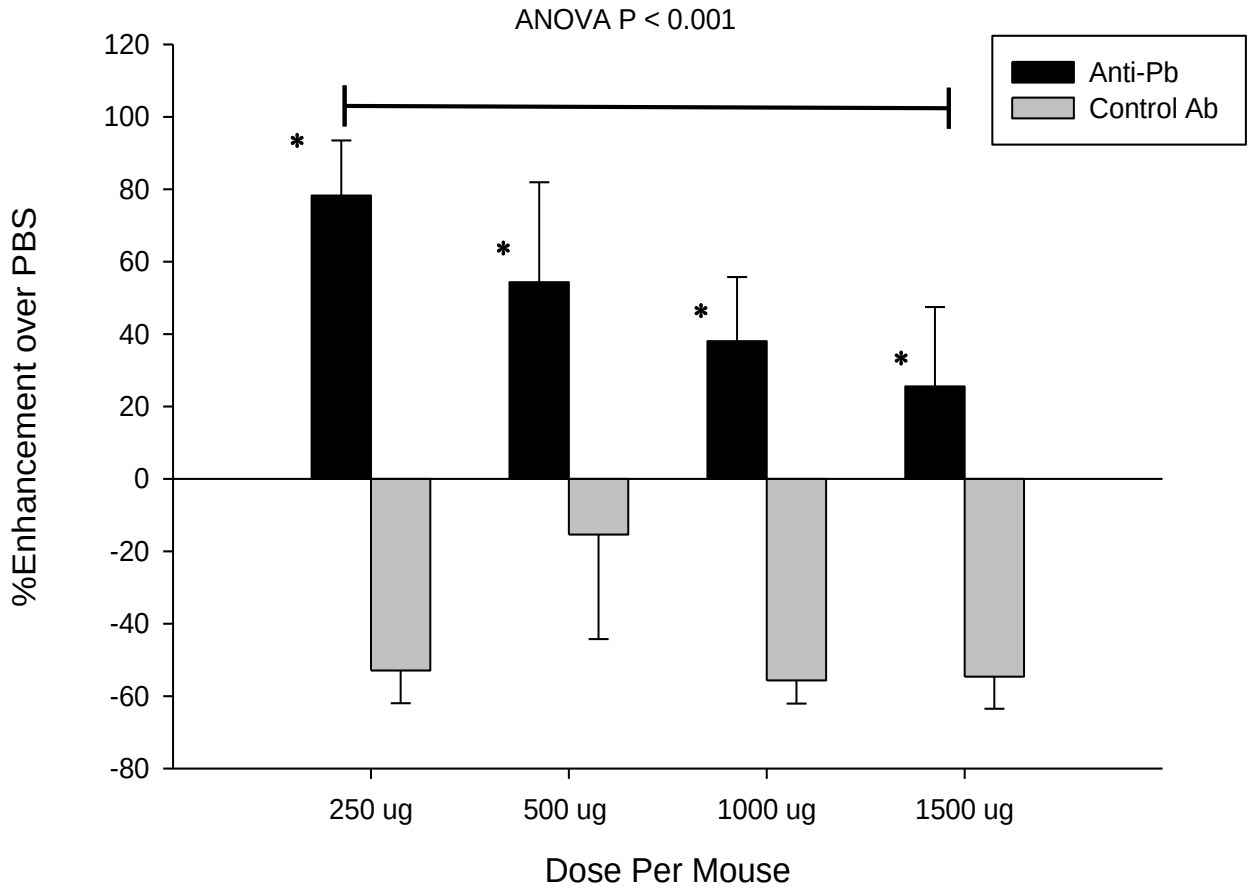
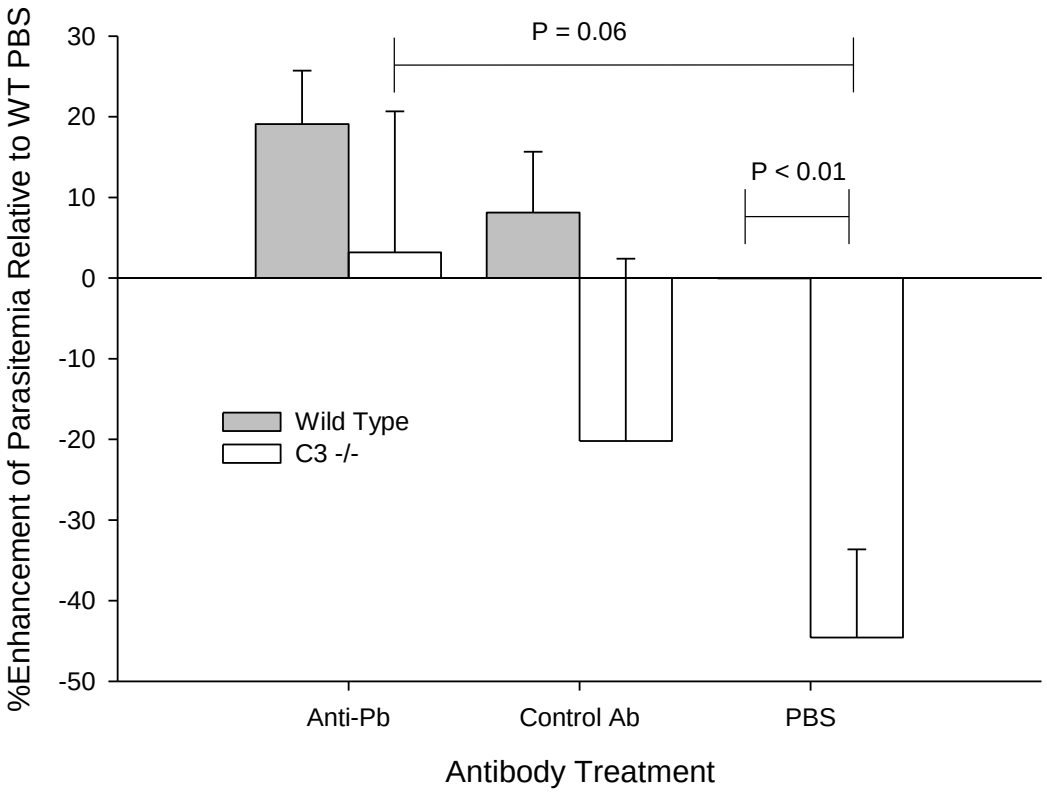

ANOVA P < 0.001
Anti-Pb
Control Ab
%Enhancement over PBS
120
100
80
60
40
20
0
-20
-40
-60
-80
*
*
*
*
250 ug
500 ug
1000 ug
1500 ug
Dose Per Mouse

Figure 11


%Enhancement of Parasitemia Relative to WT PBS
30
20
10
0
-10
-20
-30
-40
-50
P = 0.06
P < 0.01
Wild Type
C3 -/-
Anti-Pb
Control Ab
PBS
Antibody Treatment