Variant-specific immunity to *Plasmodium berghei* in pregnant mice

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Abbreviations: IE: infected erythrocyte; IFn: immunized, non-pregnant female mice of parity n; IM: immunized male mice; IPn: immunized mice at week 16 of pregnancy n; IPPn: immunized mice shortly after pregnancy n; MFI: mean fluorescence index; NI: non-immunized, non-infected mice; NP1: non-immunized, non-infected mice at week 16 of first pregnancy; PAM: pregnancy-associated *P. falciparum* malaria; PfEMP1: *P. falciparum* erythrocyte membrane protein 1; VSA: variant surface antigens; VSAPAM: PAM-specific VSA
ABSTRACT

We have investigated the immunological basis of pregnancy-related *Plasmodium berghei* recrudescence in immune mice with substantial pre-existing immunity. Specifically, we examined the relevance of this experimental model to the study of pregnancy-associated *P. falciparum* malaria (PAM) in women with substantial pre-existing protective immunity.

We used mice with immunity induced prior to pregnancy, and used flow cytometry to assess their plasma levels of IgG recognizing antigens on the surface of infected erythrocytes (IEs). After immunization, the mice did not possess IgG specific for antigens on IEs obtained during pregnancy-related recrudescence, but they acquired recrudescence-specific IgG over the course of several pregnancies and recrudescences. In contrast, levels of antibodies recognizing IEs from non-pregnant mice did not increase with increasing parity. Furthermore, maternal hemoglobin levels increased and pregnancy-related parasitemia decreased with increasing parity. Finally, litter sizes and pup weights were lower in parasitemic than in non-parasitemic mice. Taken together, these observations suggest that levels of antibodies specific for recrudescence-type IEs are related to protection of pregnant mice from maternal anemia, low birth weight, and decreased litter size. We conclude that the model replicates many of the key parasitological and immunological features of PAM, although the *P. berghei* genome does not encode proteins homologous to the *P. falciparum* erythrocyte membrane protein (PfEMP) 1 adhesins of key importance in *P. falciparum* malaria. The study of *P. berghei* malaria in pregnant, immune mice can be used to gain significant new insights regarding malaria pathogenesis and immunity in general, and regarding PAM in particular.
INTRODUCTION

PAM is a major cause of mother-offspring morbidity and mortality in areas with stable transmission of *P. falciparum* parasites, despite acquired protective immunity to *P. falciparum* malaria acquired by the mother prior to first pregnancy (7,29). Susceptibility to PAM declines with increasing parity due to acquisition of protective IgG with specificity for parasite-encoded, clonally variant surface antigens (VSA) that are selectively expressed on placenta-sequestering IEs (14,34). The PAM-specific VSA (VSA\textsubscript{PAM}) are functionally and antigenically distinct from the VSA expressed by *P. falciparum* parasites infecting non-pregnant hosts, and lack of VSA\textsubscript{PAM}-specific IgG appears to be the main reason for the high susceptibility to PAM in primigravidae possessing substantial pre-existing protective immunity (4,13,28). The best-studied VSA are the high-molecular weight PfEMP1 molecules encoded by the *var* gene family with about 60 members per haploid parasite genome (17,35). VAR2CSA appears to be the only PfEMP1 involved in the pathogenesis of, and protective immunity to, PAM (30,31). Studies of VSA-specific immunity to *P. falciparum* malaria have been frustrated by lack of convenient and relevant animal models. Although rodent malaria parasites lack *var* gene orthologs, antigenic variation and IE sequestration occur in several *Plasmodium* species (1,19,23,41) and they possess multi-gene families that appear to encode IE surface-expressed VSA (6,16). This notwithstanding, only limited information is available regarding the role of the products of these gene families in pathogenesis and immunity (21,22).

In a series of papers in the eighties, Eling and co-workers developed a mouse model to study the impact of pregnancy on immunity to *P. berghei* infection. Importantly, they used the model to demonstrate pregnancy-related recrudescences accompanied by severe clinical symptoms in mice with pre-existing acquired protective immunity (38). Furthermore, they found that susceptibility to recrudescence appeared to decrease with increasing
parity (39,40). In these aspects, their model resembles PAM caused by *P. falciparum* in malaria-endemic areas, where women generally develop substantial clinical immunity to malaria before reproductive age. In the present study we re-evaluated the model developed by Eling *et al.* in view of the recent evidence pointing to the clinical importance of VSA-specific antibody responses in PAM. We show that the apparent breakdown of pre-existing protective immunity to *P. berghei* K173 infection during pregnancy is in fact the consequence of emergence of parasites expressing pregnancy-specific VSA to which the animals do not possess antibodies if they have never been pregnant. Furthermore, antibodies to these pregnancy-specific VSA are acquired in a parity-dependent manner, and appear to be related to protection from pregnancy-related recrudescence, maternal anemia, low birth weight, and reduced litter size.
MATERIALS AND METHODS

Mice
We used BALB/c mice purchased from Taconic, Lille Skensved, Denmark (http://www.taconic.com). The animals were maintained on a 12h/12h dark/light cycle with feed and water ad libitum at the Department of Experimental Medicine, University of Copenhagen, Denmark, in accordance with institutional, Danish and European guidelines for animal experimentation and welfare. All mice used were pathogen-free. The Danish Animal Experiments Inspectorate (Dyreforsøgstilsynet) approved all experiments reported in this report as required under Danish law (permission: 2006/561-1093).

Parasites and infections
We used P. berghei strain K173 parasites (12) for all the experiments reported here. The parasites were originally obtained as a kind gift from Wijnand Eling. The parasites were maintained by weekly blood passage in non-immunized mice. Infections were initiated by intraperitoneal injection of 1x10^6 IEs in 200 µl normal saline, and parasitemias were monitored from the third day of infection by microscopic examination of Giemsa-stained thin smears of blood obtained from tail nicks. This blood was also used to assess hemoglobin levels using a HemoCue instrument (http://www.hemocue.com). Mice with fulminant parasitemia or severe clinical symptoms were killed.

Immunization
We used a modification of the immunization protocol described by Eling and Jerusalem (10). In brief, 6-8 week old mice were infected as described above. The infection was suppressed by adding 15 mg/L sulfadiazine (http://www.sigmaaldrich.com) to the drinking water on days 4-11 and days 18-25. On day 32, the mice were challenged using the same inoculum and route.
used for immunization. Mice showing very low or microscopically undetectable parasitemia after one week were considered immune.

**Mating and pregnancy monitoring**

The weights and peripheral blood parasitemias of females to be mated were recorded. On the following day (D0), they were put together with males (2-3 females and one male per cage) for 4 days. The animals were not disturbed during this period to minimize stress-induced early pregnancy failure. The females were weighed when the males were removed on D4, and then left undisturbed until D10. We took an increase in body weight (D4-D10) as evidence of pregnancy. Subsequent abrupt weight loss was taken as indicator of pregnancy interruption. Parasitemias and body weights of the animals were monitored daily from D10. Although parasite recrudescence often occurred spontaneously in pregnant mice (see Results), we generally re-infected mice on D11-D12 (0.2-1x10^7 IE from pregnant mice) to increase the frequency of recrudescence in immune mice. To determine litter sizes and pup weights, female mice were kept in separate cages from D19. The cages were examined every morning, and all newborn pups were counted and weighed. The mating protocol was repeated, until mice of parity 1 through 4 had been obtained.

**Plasma for analysis of VSA-specific IgG**

Blood samples were obtained at various time-points from different groups of mice, and are identified in the text as described below. We used samples obtained from immunized females before first pregnancy (IF0), around Day 16 of first pregnancy (IP1), or shortly after first delivery (IPP1). We also used similar samples obtained during or shortly after the second (IP2, IPP2) or third (IP3, IPP3) pregnancy, or during the fourth pregnancy (IP4). It should be noted that mating did not always result in pregnancy, and therefore there was no clear-cut relationship between age and time of exposure to parasites between the different IP and IPP.
samples. Control samples were collected from immunized, non-pregnant females of parity 1-3 (IF>0), from immunized males (IM), and from age-matched, non-immunized animals that had never been mated or infected (NI samples). Finally, we used blood samples collected from primigravid, non-immunized, and never-infected mice around Day 16 of gestation (NP1).

**Measurement of variant surface antigen-specific antibodies**

Levels of plasma IgG antibodies reacting with antigens on the surface of *P. berghei*-IEs were measured by flow cytometry, using a modification of the protocol for *P. falciparum*-IEs previously developed by us (33). In brief, IEs were collected in heparinized Eppendorf from the peripheral blood of either primigravid (Day 14-16 of pregnancy) or non-pregnant mice with parasitemias >5%. After centrifugation and removal of plasma, 50 µL packed IEs were re-suspended in 10 mL Krebs-Henseleit medium (Sigma Aldrich) supplemented with 1% BSA, and matured overnight (3%O₂, 6% CO₂, 91% N₂, 37°C). The next day, the cultures were enriched for hemozoin-containing late-developmental stages by exposure to a strong magnetic field. The DNA of purified late stage IEs (3x10⁶/mL) was labeled with hydroethidin (1 µg/mL). Labeled cells were incubated at 37°C for 20 min in 96-well microtiter plates (100 µL/well) with murine plasma samples (5 µL/well), washed, and stained with secondary, FITC-conjugated horse anti-mouse IgG (H+L) affinity purified antibody (FI-2000, 1:100, http://www.vectorlabs.com) at 4°C for 30 min. The samples were analyzed by flow cytometry, and levels of IgG reacting with antigens on the IE surface were quantified as the mean FITC fluorescence index (MFI) of hydroethidin-positive cells, using WinMDI software (http://facs.scripps.edu/software.html). Although levels of VSA-specific antibodies measured by flow cytometry depend on a number of variables that make inter-species comparisons difficult, the levels we observed in the present study were comparable to levels observed with placental *P. falciparum* isolates.
Histology

Mice were anesthetized by intraperitoneal injection (10 µL/g body weight) of a 1:1 mixture solution of Hypnorm (http://www.janssenpharmaceutica.be) and Dormicium (http://www.roche.com), each reconstituted 1:1 in sterile water. After collecting a blood sample from the retroorbital plexus, the animals were killed by cervical dislocation. Tissue from the placenta, kidney, liver, spleen, lung, and brain were collected and fixed by immersion in Zamboni’s fixative solution for 24 h at room temperature. The organs were then transferred into 70% ethanol, dehydrated, embedded in paraffin, and cut in a microtome. For the illustrations we used 4 µm sections, whereas 2 µm sections were used for determination of parasitemia in solid tissues. All sections were stained by hematoxylin-eosin and examined by light microscopy. Hemozoin pigment crystals were examined under polarized light to increase their visibility.

Statistical analysis

We used the SigmaStat (http://www.systat.com) and CIA (2) software packages for the statistical analyses. Results are reported as means or medians with corresponding 95% confidence intervals. Student’s t-test and Mann-Whitney rank-sum test were used to evaluate inter-group differences. The Spearman rank-order coefficient (r_s) was used to evaluate parameter association, while multiple linear regression analysis was used to identify significant predictors of hemoglobin levels and birth weights. Differences with P<0.05 were considered statistically significant.
RESULTS

Infection and sub-curative treatment result in immunity to virulent *P. berghei* infection

Infection of non-immune Balb/c mice with the K173 strain of *P. berghei* is uniformly lethal (12). However, the mice can be rendered immune to *P. berghei* by repeated infection and sub-curative treatment (10,11). Using a modification of this protocol, we obtained immunized animals that were highly resistant to challenge. Thus, all of 40 animals infected with $1 \times 10^6$ IEs followed by two rounds of suppressive sulfadiazine treatment had parasitemias <0.6% one week following challenge on Day 32 (Fig. 1). In contrast, 3/3 non-immunized mice infected on the same day developed parasitemia >15% over this period (Fig. 1).

Hemoglobin levels were slightly lower in immunized animals about 14 days after challenge compared to non-immunized, non-infected control animals (median difference [95% confidence interval]=1.1 [0.5; 1.9] g/dL, P(T)=0.001), probably caused by the episodes of patent parasitemia during the immunization period. These results show that immunization by infection and sub-curative treatment induces a marked, but non-sterile immunity that provides a high degree of resistance to challenge infection.

Pregnancy causes recrudescence of *P. berghei* parasitemia in immunized mice

It has long been known that a proportion of *P. berghei*-immune mice develop recrudescent parasitemia during pregnancy (38,39). This finding is confirmed in the present study, where we observed that 24/24 first-time pregnant mice immunized prior to mating recrudesced (median max. parasitemia=2.8%, range=0.02% to 35.8%), including seven with fulminant parasitemia (Fig. 2). Although episodes of patent parasitemia were also observed in all of 12 immune non-pregnant control females during the same period of time, the parasitemias were much lower (median max. parasitemia=0.15%, range=0.008% to 6.8%), and the difference in the highest observed parasitemias between pregnant and non-pregnant animals was significant.
(median difference=2.5% [0.73% to 12%], P(T)<0.01). These results show that pregnancy can cause recrudescence of parasitemia previously controlled at very low levels by acquired immunity.

Pregnancy-associated recrudescence of *P. berghei* parasitemia in immunized mice causes maternal anemia

Pregnancy-associated *P. berghei* recrudescence in immune mice caused a significant reduction in hemoglobin levels (IF0 vs. IP1; median difference=3.5 [2.4; 4.8] g/dL, P(T)<0.001) (Fig. 3A). This difference was not simply related to pregnancy *per se*. Thus, although hemoglobin levels in non-immunized and never-infected controls (NI group) and non-immunized and never-infected primigravidae (NP1 group) were also significantly different (median difference=1.7 [0.8; 2.8] g/dL, P(T)=0.001) (Fig. 3A), hemoglobin levels in NP1 mice and IF0 mice were similar (median difference=0.5 [-0.2; 1.4] g/dL, P(T)=0.17) (Fig. 3A). These results show that pregnancy-associated recrudescence can cause maternal anemia.

Susceptibility to pregnancy-associated anemia and recrudescence decreases with increasing parity

It has been shown previously that recrudescence rates are lower during the second than during the first pregnancy (38), and that this appears to be due to some kind of pregnancy-dependent immune response (40). Our preliminary data supported these findings, and also showed that pregnancy-associated recrudescences during second and third pregnancies were more common if mice were re-infected around Day 11 of their second or third pregnancies with IEs obtained from primigravid mice with pregnancy-associated recrudescence (data not shown). Taking this approach, we found that hemoglobin levels around Day 16 of pregnancy correlated with parity (Fig. 3A, groups IP1, IP2, and IP3) (P(r=0.40)<0.001), as did the...
proportion of animals with anemia (Hb<12 g/dL) (Fig. 4A, groups IP1, IP2, and IP3) 
\( \text{P}(\chi^2=14.5)<0.001 \). Corresponding correlations were observed with respect to levels of 
parasitemia (Fig. 3B, groups IP1, IP2, and IP3) (\( \text{P}(r_e=-0.40)<0.001 \)) and the proportion of 
mice with patent parasitemia (Fig. 4B) (\( \text{P}(\chi^2=9.3)=0.009 \)). Both parasitemia and parity were 
significant predictors (\( \text{P}<0.001 \) for each) of hemoglobin levels in a multiple linear regression 
model. Taken together, these results show that \( P. \text{berghei} \) parasitemia adversely affects 
maternal hemoglobin levels, and that acquired immunity reduces recrudescent parasitemias 
and thereby protects the pregnant mice from anemia.

**Immunized mice acquire high levels of IgG with specificity for antigens on the surface of** 
**infected erythrocytes**

The immunization protocol used here has been shown to result in acquisition of antibodies 
with specificity for antigens on the surface of \( P. \text{berghei} \)-IEs (32) (Fig. 5). In agreement with 
this, we found that levels of surface-reactive IgG with specificity for VSA expressed on the 
surface of \( P. \text{berghei} \)-IEs from non-pregnant mice were significantly different (median 
difference=17.6 [15.3; 19.9] MFI units, \( \text{P}(\text{T})<0.001 \)) in non-immunized (NI) and immunized 
(IF+IM) mice of comparable age (Fig. 5A). Also as expected, levels of IgG in the immunized 
animals did not depend on sex (IF vs. IM, median difference=0.7 [-2.5; 3.3] MFI units, 
\( \text{P}(\text{T})=0.64 \)) or parity (IF0 vs. IF>0, median difference=2.1 [-0.7; 5.3] MFI units, \( \text{P}(\text{T})=0.15 \)) 
(Fig. 5A). These results show that immunization results in acquisition of IgG with specificity 
for antigens on the surface of IEs obtained from non-pregnant mice, and that this acquisition 
is independent of sex and parity.
Pregnancy-related recrudescence is caused by parasites expressing distinct variant antigens on the surface of infected erythrocytes

Based on the above findings, we proceeded to address the hypothesis that the susceptibility to pregnancy-related recrudescence in *P. berghei*-immune mice is due to expression of pregnancy-specific VSA by the recrudescing parasites; VSA to which the mice do not have antibodies, despite high levels of antibodies to VSA expressed on the surface of *P. berghei*-IEs from non-pregnant animals. We found that IgG levels to VSA expressed by pregnancy-associated recrudescence-type IEs in non-immunized (NI) and immunized (IF+IM) mice were significantly different (median difference=25.2 [17.6; 31.2] MFI units, \( P(T)<0.001 \)) (Fig. 5B), similar to the difference observed when assessing IgG levels to VSA expressed by IEs from non-pregnant mice (Fig. 5A). However, among the immunized animals (IF and IM) the levels of IgG to VSA expressed on the surface of IEs obtained from pregnant mice varied with both sex and parity. Thus, antibody levels in immunized males (IM) and immunized females (IF) were different (median difference=7.4 [-0.2; 13.2] MFI units, \( P(T)=0.06 \)) (Fig. 5B) due to the difference between males (IM) and previously pregnant females (IF>0) (median difference=9.7 [4.1; 14.8] MFI units, \( P(T)=0.002 \)) (Fig. 5B). Levels in the males (IM) were similar to levels in never-pregnant females (IF0) (median difference=6 [-9.1; 9.1] MFI units, \( P(T)=0.84 \)) (Fig. 5B). Taken together, these results show that the antigens on *P. berghei*-IEs from non-pregnant and pregnant mice are partially different. The simplest explanation for this finding is that there are antigens on the recrudescence-type IEs that are not found on other *P. berghei*-IEs, in addition to antigens that are similar or identical to antigens on IEs from non-pregnant animals.
Levels of antibodies with specificity for the variant surface antigens expressed by parasites causing pregnancy-related recrudescences increase with increasing parity

To further substantiate the hypothesis that the susceptibility to pregnancy-related recrudescence in *P. berghei*-immune mice is due to expression of pregnancy-specific VSA to which the host does not have specific antibodies, we examined the relation between IE-specific IgG levels and parity. While there was no apparent association between parity and levels of IgG with specificity for VSA on the surface of IEs from non-pregnant animals (\(P_{r_s} = 0.21\)) (Fig. 6A), there was a clear correlation between parity and levels of IgG with specificity for recrudescence-type IEs (\(P_{r_s} = 0.50\)) (Fig. 6B). These results show that IgG with specificity for recrudescence-type IEs is acquired in a selective and parity-dependent manner, reinforcing the hypothesis of a causal relationship between resistance to pregnancy-related recrudescence and IgG with specificity for antigens on the surface of erythrocytes infected by recrudescence-type *P. berghei* parasites.

Erythrocytes infected by mature *P. berghei* parasites accumulate in the placenta

Erythrocytes infected by parasites of all maturation stages can be seen in the peripheral blood of *P. berghei*-infected mice. Nevertheless, erythrocytes infected by mature *P. berghei* parasites can sequester in various tissues (1,24). Of particular importance here is the recently observed chondroitin sulfate A (CSA)-dependent binding of *P. berghei*-IEs in the placenta (24), which resembles that observed in PAM (13). We found that the placental intervillous space of mice with pregnancy-associated recrudescence showed numerous erythrocytes infected by mature, pigment-containing parasites (19.0% [15.1 to 27.3]) but was almost devoid (<1%) of early developmental stage parasites (ring forms) (Fig. 7A). In contrast, ring-stage parasitemia (15.0% [8.6% to 18.2%]) dominated over mature-stage parasitemia (7.2% [4.0% to 9.8%]) in the peripheral blood (median difference 6.8% [3.0% to
accumulation of *P. berghei*-IEs is a feature of pregnancy-related recrudescence (24). We also looked for evidence of sequestration of IEs in brain, kidney, liver, lung, and spleen. Brains were essentially free of both IEs and hemozoin (presence of hemozoin is evidence of phagocytosis of IEs) (data not shown). Hemozoin dominated in spleen, liver, and lung, consistent with the expected phagocytosis of IEs in these organs (Fig. 7C and data not shown). Low levels of IEs were seen in the kidneys (Fig. 7D). Taken together, these results provide additional support for the hypothesis of preferential sequestration of recrudescence-type IEs in the placenta.

**Pregnancy-associated recrudescence is associated with small litter size and low pup weight**

Pregnancy-related parasite recrudescence has been reported to be associated with intra-uterine growth retardation and reduced pup weight in non-immune mice (24). We found offspring from immunized mice to be smaller (N=44, mean birth weight 1.33 [1.27; 1.38] g) than offspring from non-immunized, non-infected mice (N=9, mean birth weight 1.40 [1.24; 1.56] g) (Fig. 8). Furthermore, the average litter size among the control mice (median litter size=9.0 [6; 9]) was bigger than that of immunized mice (litter size=5.5 [5.0; 7.0]) (median difference=3 [1; 4], P(T)=0.004) (Fig. 8), and litter size was the strongest predictor of birth weight in a linear regression model (P=0.013). Among immunized mice, those with above-average parasitemia between D10 and D18 of pregnancy, produced smaller pups (mean birth weight=1.26 [1.17; 1.39] g) than those with below-average peak parasitemia (mean birth weight=1.39 [1.32; 1.47] g) (mean difference=0.12 [0.04; 0.23] g, P(t)=0.008). In contrast, the average birth weight of pups from immunized mice with only low-grade parasitemia was not
significantly different from that of the non-immunized mice (mean difference=0.01 g [-0.14; 0.15], P(t)=0.95).

We could not demonstrate that immunized primigravidae produced smaller litters or pups than mice of higher parity, as might have been expected. However, litter size and pup birth weights can only be determined after a successful pregnancy, which renders these markers unreliable among primigravid mice, where pregnancies are often unsuccessful (maternal death, fetal resorption, miscarriage) if high parasitemia develops. Our results show that the level of parasitemia in pregnancy-associated *P. berghei* recrudescence adversely affects litter size and pup weights.
In 1915, Clark noted that it “has long been known that it is possible to find an abundance of 
malaria parasites in the... placenta” but that it was generally regarded as “a curious feature 
sometimes encountered” (5). Later, when it was discovered that pregnancy modulates the 
immune system in order to protect the developing fetus from maternal immune attack (15), 
many malaria researchers started to see PAM as the inevitable consequence of pregnancy-
associated immunosuppression (20). It was known early that primigravidae are particularly 
susceptible to placental infection (3), but this finding, which is at variance with the 
immunosuppression hypothesis, was largely ignored – with some notable exceptions (18). 
Eventually, a coherent understanding of the pathogenesis and immunology of PAM emerged 
when it was found that placenta-sequestering \textit{P. falciparum}-IEs have unique adhesive 
properties (13), and that susceptibility to PAM is related to levels of antibodies recognizing 
particular parasite-encoded inter-clonally variant proteins (the so-called VSA\textsubscript{PAM}) on the IE 
surface (14,34). These insights have spurred the current intensive and worldwide effort to 
develop vaccines against PAM. However, progress is being hampered by the lack of animal 
models that exhibit the characteristic features of PAM. Although the chimpanzee parasite 
\textit{P. reichenowi} possesses a gene homologous to the \textit{P. falciparum} var2csa gene implicated in 
the pathogenesis of PAM (37), higher primates are very impractical experimental models of 
malaria in general, let alone of PAM. The situation is not much better with respect to lower 
primates, and essentially nothing is known about the relationship between immunity and 
susceptibility to infection in pregnant monkeys (8). Rodents are the most accessible and 
therefore best-studied experimental malaria model system, and a number of studies of malaria 
in pregnant animals is available, including a recent study advocating murine \textit{P. berghei} 
infection as a useful model of PAM (24). However, the report by Neres \textit{et al.} (24) was 
exclusively based on data from non-immune animals, and available studies of immunity and
susceptibility to infection in pregnant mice either predate or ignore the apparent importance of VSA-specific immunity in PAM. In the present study we show that most, if not all, previously described features of *P. berghei* infection during pregnancy are consistent with the current, VSA-based understanding of PAM pathogenesis and immunity (29). Thus, in the *P. berghei*-infected mouse model, pregnancy-associated recrudescences are associated with placental and CSA-dependent sequestration of IEs (9,24,36), decrease in frequency and severity with increasing parity (38,39), have adverse consequences for the pregnant mice and their offspring (24), and occur despite immunity acquired before the first pregnancy (38,39).

Furthermore, we show for the first time that pregnancy-associated recrudescence in *P. berghei*-infected mice leads to acquisition of antibodies that are specific for variant antigens expressed only during recrudescence. Acquisition of these antibodies appears to be associated with clinical protection from the consequences of pregnancy-associated recrudescence, such as maternal anemia and low birth weight. However, direct evidence of a causal relationship must await identification of the antigen(s) involved and their use in vaccination studies.

In common with most other malaria parasites, *P. berghei* does not possess genes homologous to the *var* genes encoding the PfEMP1 proteins thought to be the major *P. falciparum* IE adhesion ligand. Nevertheless, IE sequestration, including placental sequestration during pregnancy, is clearly not restricted to *P. falciparum* infection (25-27,36). The capacity for glycosaminoglycan-dependent IE sequestration in the placenta might thus have evolved independently several times. Alternatively, it might be a truly ancient feature, raising the possibility that orthologs to the parasite genes involved in placental IE sequestration in mice exist in *P. falciparum*. In either case, identification of the pregnancy recrudescence-related *P. berghei* genes and characterization of their products are of considerable interest and a current priority in our laboratories.
In conclusion, we have demonstrated many similarities between PAM in *P. falciparum*-exposed women, and pregnancy-related *P. berghei* recrudescence in immune mice. This finding opens new opportunities for research on the pathogenesis and immunity of PAM, which remains a major source of poor mother-infant health in large parts of the world.
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FIGURE LEGENDS

Figure 1. Parasitemias during immunization and after challenge.
Temporal development of peripheral blood parasitemia in female mice immunized by infection (▼) on Day 0 and two rounds of intermittent, suppressive sulfadiazine treatment (—). The immunized mice (○), and non-immunized control mice (●) were challenged by infection on Day 32. Data are shown as medians and 95% confidence intervals for the immunized mice, whereas individual results are shown for the control mice.

Figure 2. Pregnancy-related recrudescence of *P. berghei* in immunized and non-immunized mice.
Development of peripheral blood parasitemia in immunized primigravidae (○) at various time points following conception. Data for non-pregnant, immunized control mice followed in parallel are shown for comparison (●). Data for individual mice are shown. Mice with parasitemias >15% or having signs of severe disease were killed.

Figure 3. Levels of hemoglobin and parasitemia in different groups of mice.
Levels of peripheral blood hemoglobin (A) and parasitemia (B) in different groups of female mice: non-immunized never-pregnant controls (NI); non-immunized primigravidae (NP1); immunized, never-pregnant controls (IF0), immunized mice pregnant for the first (IP1), second (IP2), or third time (IP3); and immunized mice shortly after first (IPP1) or second (IPP2) pregnancy. Data for pregnant mice (NP1, IP1, IP2, and IP3 mice) were obtained around Day 16 of pregnancy, while post-partum data (IPP1 and IPP2 groups) were obtained approximately 10 days after delivery. Data are shown as medians (center line), central 50% of data (box), central 80% of data (whiskers) and outliers (dots). Horizontal lines indicate statistically significant (P<0.01) differences. N-values indicate mice per group.
Figure 4. Proportions of anemia and patent parasitemia in different groups of mice.

Proportions of animals with anemia (hemoglobin levels <12 g/dL) (A) and microscopically detectable peripheral blood parasitemia (B) in different groups of immunized female mice: never-pregnant controls (IC), mice pregnant for the first (IP1), second (IP2), or third time (IP3); and mice shortly after first (IPP1) or second (IPP2) pregnancy. Data presentation as in Fig. 3.

Figure 5. VSA-specific IgG levels in different groups of mice.

Levels of plasma IgG with specificity for variant antigens on the surface of erythrocytes infected either by *P. berghei* obtained from non-pregnant (A) or from pregnant mice (B). IgG levels were measured by flow cytometry in different groups of mice: non-immunized controls (NI); immunized male (IM) mice; and immunized female mice (IF) with (IF>0) or without (IF=0) previous pregnancies. Data presentation as in Fig. 3.

Figure 6. The relationship between levels of VSA-specific and parity.

Parity-dependent acquisition of IgG with specificity for variant antigens on the surface of erythrocytes infected by *P. berghei* obtained from non-pregnant (A) or pregnant (B) mice. Levels of IgG at delivery were measured by flow cytometry in immunized mice pregnant for the first (IP1), second (IP2), third (IP3), or fourth (IP4) time. Data presentation as in Fig. 3.

Figure 7. *P. berghei* parasitemia in different tissues.

Tissue-specific *P. berghei* parasitemia in the placenta (A), peripheral blood (B), spleen (C), and kidney (D) of an immunized, pregnant mouse.
Figure 8. Maternal immune status and pregnancy outcome.

The relationship between litter size and birth weight in non-immunized, non-infected (●) and immunized, infected (○) mice. Individual data points and regression lines (light line: non-immunized mice, heavy line: immunized mice) are shown.
FIGURE 1

![Graph showing parasitemia (%) over time after start of immunization. The graph compares infection (1x10^6 IEs), sulfadiazine treatment, immunized (N=40), and non-immunized mice (N=3). The x-axis represents time after start of immunization (days) from 0 to 40, while the y-axis shows parasitemia (%) from 0 to 20.]
FIGURE 2
FIGURE 3

A

Hemoglobin levels (g/dL)

NI NP1 IF0 IP1 IPP1 IP2 IPP2 IP3

0 5 10 15 20

N=10 N=14 N=34 N=84 N=35 N=46 N=14 N=20

B

Parasitemia (%)

Mouse category

NI NP1 IF0 IP1 IPP1 IP2 IPP2 IP3

0 n.d. n.d.

N=34 N=84 N=35 N=46 N=14 N=20

n.d. n.d.
FIGURE 4

A: Anemia

B: Parasitemia
FIGURE 5

Mouse category

NI IF+IM IM IF IF0 IF>0

IgG levels (MFI units)

N=9 N=45 N=10 N=35 N=14 N=21

A

B

Mouse category

NI IF+IM IM IF IF0 IF>0

IgG levels (MFI units)

N=9 N=44 N=10 N=34 N=13 N=21
FIGURE 6

Mouse category
IP1 IP2 IP3 IP4
IgG levels (MFI units)
70
80
90
100
N=14 N=8 N=11 N=2

Mouse category
IP1 IP2 IP3 IP4
IgG levels (MFI units)
45
50
55
60
N=13 N=8 N=11 N=2