Cell Host & Microbe
Article

Complement Activation and the Resulting Placental Vascular Insufficiency Drives Fetal Growth Restriction Associated with Placental Malaria

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http://dx.doi.org/10.1016/j.chom.2013.01.010

SUMMARY

Placental malaria (PM) is a major cause of fetal growth restriction, yet the underlying mechanism is unclear. Complement C5a and C5a receptor levels are increased with PM. C5a is implicated in fetal growth restriction in non-infection-based animal models. In a case-control study of 492 pregnant Malawian women, we find that elevated C5a levels are associated with an increased risk of delivering a small-for-gestational-age infant. C5a was significantly increased in PM and was negatively correlated with the angiogenic factor angiopoietin-1 and positively correlated with angiopoietin-2, soluble endoglin, and vascular endothelial growth factor. Genetic or pharmacological blockade of C5a or its receptor in a mouse model of PM resulted in greater fetoplacental vessel development, reduced placental vascular resistance, and improved fetal growth and survival. These data suggest that C5a drives fetal growth restriction in PM through dysregulation of angiogenic factors essential for placental vascular remodeling resulting in placental vascular insufficiency.

INTRODUCTION

Over 125 million pregnant women are at risk of malaria infection every year (Dellicour et al., 2010). A *Plasmodium falciparum* infection in pregnancy doubles the risk of delivering a child that is low birth weight (LBW, <2500 g) (Desai et al., 2007). Malaria-related LBW is responsible for up to 200,000 infant deaths each year (Guyatt and Snow, 2001). Fetal growth restriction resulting in small-for-gestational-age (SGA) infants accounts for approximately half of LBW outcomes associated with placental malaria (PM); the other half results from preterm delivery (Steketee et al., 2001; Umbers et al., 2011a). Sequestration of infected erythrocytes and accumulation of mononuclear cells in the placental intervillous space are histopathological hallmarks of PM associated with LBW. Chemokines that promote monocyte infiltration and inflammatory cytokines secreted by activated macrophages have been associated with malaria-related LBW (Umbers et al., 2011a). However, the precise mechanism by which parasite accumulation and placental inflammation result in fetal growth restriction, preterm delivery and LBW remains poorly understood.

The complement system is a central component of innate host defense and is activated in malaria-infected individuals (Silver et al., 2010a). Multiple complement activation pathways converge upon cleavage of component C5 to C5a, which has recognized roles in induction of inflammation and initiation of acquired immune response (Guo and Ward, 2005). Levels of maternal plasma C5a and placental messenger RNA (mRNA) encoding the C5a receptor, C5aR, are increased with PM infection (Conroy et al., 2009; Muehlenbachs et al., 2007) but have not been associated with adverse outcomes in human malaria infection. C5a has been shown to influence angiogenesis (Langer et al., 2010), is increased in women with preeclampsia (Soto et al., 2010), and has been implicated as a mediator of poor fetal outcomes in non-infection-based animal models of fetal loss and growth restriction (Girardi et al., 2006). Based on these observations, we hypothesized that C5a might cause fetal growth restriction associated with PM by creating an imbalance in the angiogenic factors required for normal placental vascular development and fetal growth (Dunk et al., 2000; Geva et al., 2002; Gougos et al., 1992; Venkatesha et al., 2006).

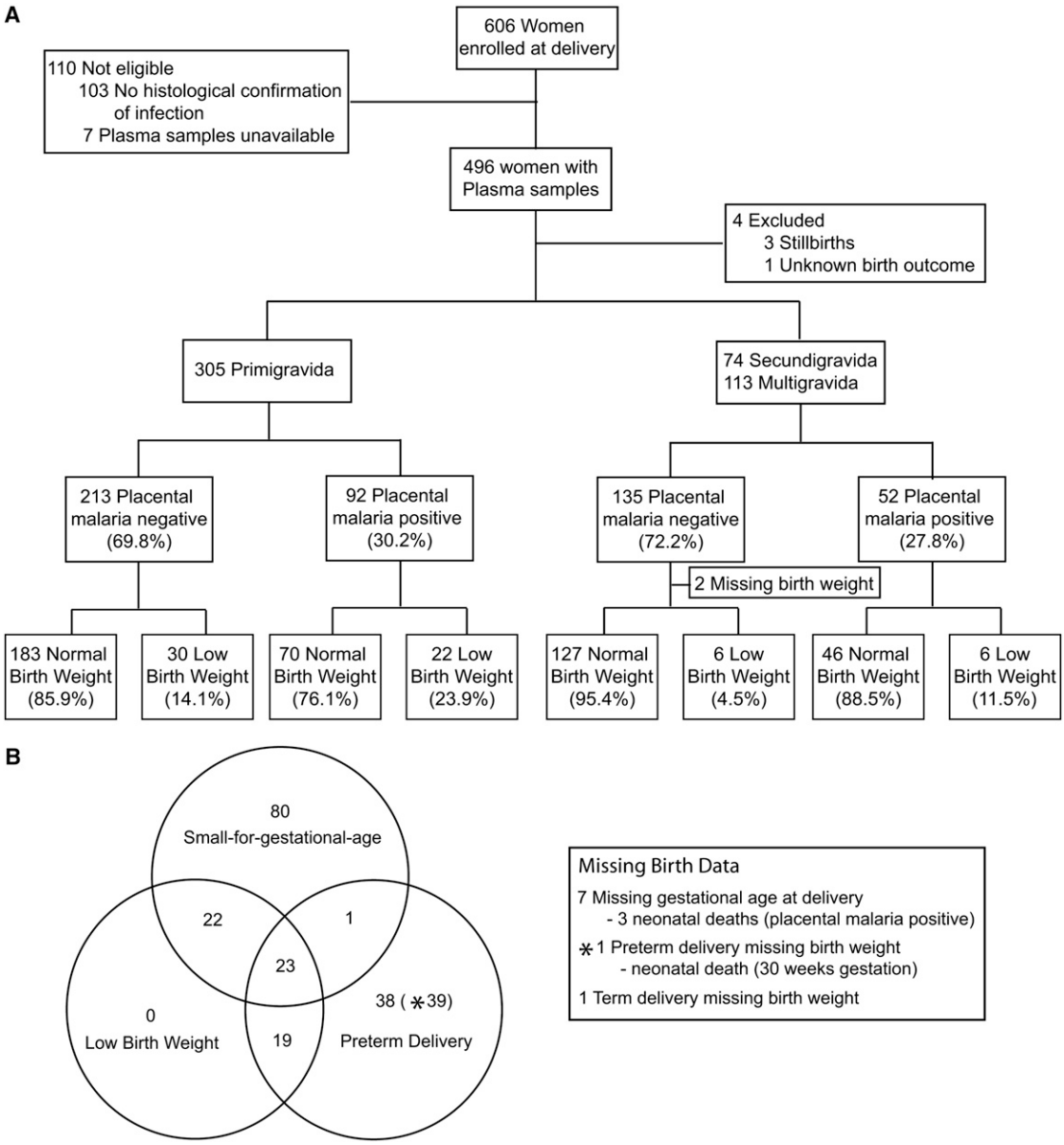

Figure 1. Description of Study Population

(A) Flow chart of samples included as cases (women who were placental malaria positive for *Plasmodium falciparum* parasites by placental blood smear) or controls (negative for malaria parasites by placental and peripheral blood smear).

(B) Breakdown of birth outcomes as low birth weight (<2,500 g), preterm (<37 weeks gestation), or small for gestational age (less than tenth percentile for growth).

RESULTS

Placental Malaria Infection Is Associated with Poor Birth Outcomes

Between 2001 and 2006, we enrolled 606 women who delivered live, singleton infants at Queen Elizabeth Central Hospital, Blantyre, Malawi into a case-control study. Cases were defined by the presence of *P. falciparum* asexual parasites in the placental blood, as assessed by smear microscopy. Of the women enrolled, 492 had live birth outcomes, placental histological examination, and available plasma samples and were eligible for the study (Figure 1). The majority (n = 305) of women were primigravidae. Adverse birth outcomes included LBW, preterm delivery (<37 weeks of gestation), and fetal growth restriction, defined as SGA (less than tenth percentile for growth in sub-Saharan African populations [Landis et al., 2009]) (Figure 1).

Consistent with previous reports (Ismail et al., 2000; Rogerson et al., 2003), women with placental parasitemia were more likely to be febrile and anemic and have histopathological evidence of placental inflammation (Table 1 and Table S1 available online). PM was associated with increased risk of LBW (adjusted odds

Table 1. Characteristics Associated with Placental Malaria and Small-for-Gestational-Age Infants in Primigravidae

	Placental Malaria Status[a]			Fetal Growth Status[b]		
	Positive (n = 92)	Negative (n = 213)	p	SGA (n = 94)	AGA (n = 208)	p
Demographic						
Age (years)	18 (17–20)	19 (18–20)	0.038	18 (17–20)	19 (18–20)	0.127
Height (cm)	154 (150–157)	154 (150–158)	0.646	153 (149–158)	154 (150–158)	0.283
Fetal sex (% female)	60.9	47.1	0.028	58.5	48.8	0.118
Weight of baby (g)	2,700 (2,500–3,000)	2,900 (2,600–3,200)	0.001	2,500 (2,288–2,700)	3,025 (2,800–3,300)	<0.001
Clinical						
Hemoglobin (g/dl)	10.9 (9.6–12.0)	12.5 (11.0–13.7)	<0.001	11.4 (10.0–13.0)	12.2 (10.7–13.5)	0.025
Temperature (°C)	37.0 (36.2–37.4)	36.2 (36.0–36.6)	<0.001	36.5 (36.0–37.0)	36.4 (36.0–37.0)	0.336
Blood pressure > 140/90 (%)	2.2	2.8	1.000	4.3	1.9	0.262
Febrile symptoms[c]	46.7	5.6	<0.001	20.2	16.3	0.971
Histologic Findings						
Monocyte count	30 (12–47)	4 (0–15)	<0.001	11 (0–30)	9 (0–22)	0.458
Monocyte pigment score[d]	2 (1–3)	0 (0–0)	<0.001	0 (0–2)	0 (0–1)	0.092
Fibrin pigment score[d]	3 (1–4)	0 (0–2)	<0.001	1 (0–3)	1 (0–3)	0.152
Biomarkers						
C5a (ng/ml)	85.7 (64.5–111.3)	71.3 (52.0–87.3)	<0.001	78.5 (57.2–106.4)	72.5 (55.2–93.6)	0.059
C3a (μg/ml)[e]	3.3 (2.3–5.5)	3.8 (2.5–6.5)	0.076	3.8 (2.7–5.4)	3.7 (2.4–6.3)	0.701
Angiopoietin-1 (ng/ml)	28.2 (17.4–49.4)	36.6 (22.2–55.7)	0.006	28.7 (19.1–49.9)	36.1 (20.3–54.9)	0.043
Angiopoietin-2 (ng/ml)	15.2 (8.3–45.6)	11.8 (6.5–31.2)	0.039	15.2 (7.9–41.7)	11.6 (6.4–31.8)	0.048
sTie-2 (ng/ml)	38.1 (30.8–45.6)	35.7 (29.5–42.4)	0.167	36.7 (28.7–44.8)	36.0 (30.3–43.0)	0.827
sEndoglin (ng/ml)	64.5 (36.2–122.2)	47.2 (32.2–85.6)	0.057	67.6 (35.9–119.5)	47.1 (32.1–86.7)	0.027
sFlt-1 (ng/ml)	144.2 (46.3–244.4)	115.3 (40.8–213.8)	0.045	149.1 (54.6–247.8)	113.3 (39.0–212.6)	0.044
VEGF (pg/ml)	82.5 (37.8–181.8)	94.0 (43.5–239.5)	0.277	114.5 (47.5–391.8)	86.5 (38.5–190.3)	0.076

Biomarkers measured by ELISA in placental plasma collected at delivery. Values represented as median (interquartile range). See also Table S1.
[a]Defined as a positive placental smear.
[b]Fetal growth assessed with a nomogram generated for sub-Saharan populations (Landis et al., 2009). Small for gestational age (SGA) is defined as birth weight for gestational age less than the tenth percentile. AGA, appropriate for gestational age (Landis et al., 2009).
[c]Any one of the following: self-reported fever, headache, or chills within the week prior to delivery.
[d]Score 0–4: 0, none; 1, little; 2, some; 3, moderate; 4, large amounts in every field at 40× objective as assessed by a single observer.
[e]PM+, n = 81; PM–, n = 174; SGA, n = 81; AGA, n = 171.

ratio [aOR], 2.0; 95% confidence interval [CI], 1.2–3.5; p = 0.011) after adjustment for age and gravidity. PM was associated with increased risk of SGA rather than preterm delivery (Table S2). Thirty-six percent of mothers with PM at delivery had infants SGA, while 27% of mothers of appropriate-for-gestational age (AGA) infants had PM (p = 0.051). Primigravidae with PM had a median 200 g reduction in birth weight as compared to primigravidae without PM (p = 0.001).

Elevated C5a Levels Are Associated with Fetal Growth Restriction

We measured and categorized maternal C5a levels as elevated (>100 ng/ml) or normal (≤100 ng/ml) and compared them to birth outcome (Figures 2A and 2B). A cutoff of 100 ng/ml (~10 nM) was used because above this concentration, C5a can synergistically induce inflammation, cause monocyte release of the antiangiogenic factor soluble Fms-like tyrosine kinase-1 (sFlt-1) in the presence of parasite factors (Conroy et al., 2009), and alter coagulation and white blood cell function (Ward, 2004). Elevated levels of C5a in the peripheral or placental blood were associated with increased risk of delivering a LBW infant after adjustment for maternal and gestational age, gravidity, and PM (aOR [95% CI]: peripheral, 2.7 [1.1–7.0], p = 0.036; placental, 2.1 [0.91–4.6], p = 0.081). Elevated C5a levels were associated with SGA status (aOR [95% CI]: peripheral, 2.4 [1.3–4.4], p = 0.003; placental, 1.7 [1.04–2.8], p = 0.035) more so than preterm delivery (aOR [95% CI]: peripheral, 0.9 (0.4–1.9), p = 0.696; placental, 0.4 [0.2–0.9], p = 0.033).

C5a and Angiogenic Factors Are Altered in Placental Malaria

With the same cutoff of 100 ng/ml, C5a was elevated in both the peripheral and placental blood of women who were positive for *P. falciparum* malaria by placental blood smear microscopy (odds ratio [95% CI]: peripheral, 1.9 [1.1–3.4], p = 0.023; placental, 3.0 [1.9–4.8], p < 0.001; by Pearson chi-square analysis) (Figures 2C and 2D).

Focusing at the site of infection, we also found the median placental levels of C5a to be higher in both primigravidae (p < 0.001; Table 1) and multigravidae (p = 0.002; Table S1) with PM than in those without. We investigated placental levels of angiogenic factors important in forming and remodeling the

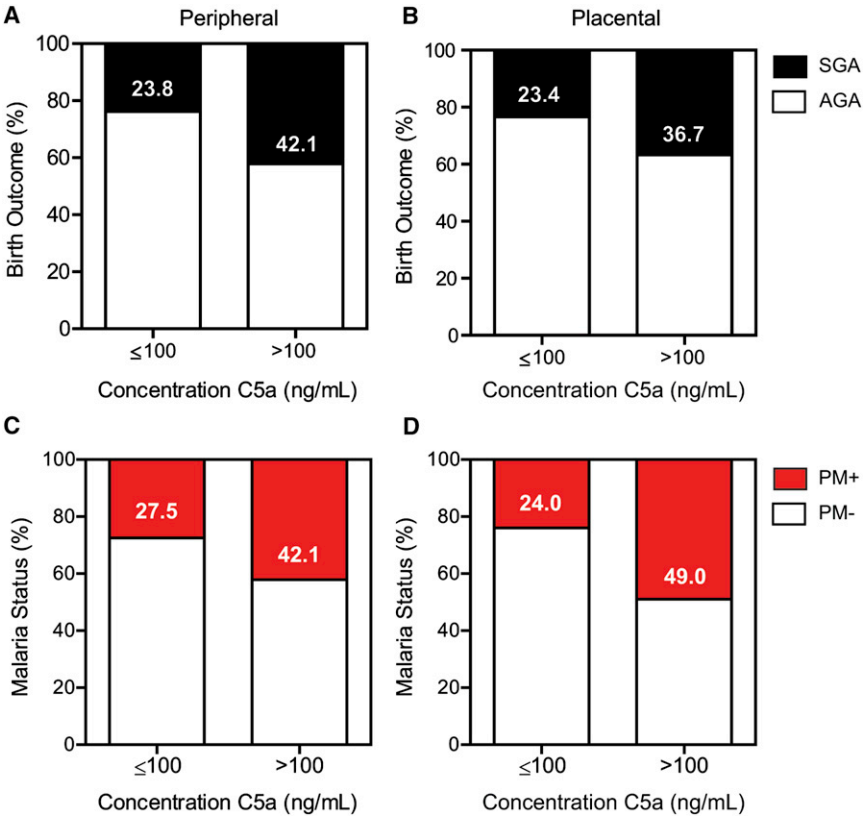

Figure 2. Elevated C5a Levels Are Observed in a Higher Proportion of Women with Fetal Growth Restriction and Placental Malaria

We measured and categorized C5a levels in peripheral and placental blood as elevated (>100 ng/ml) or not elevated (≤100 ng/ml) and compared them to fetal growth status and placental malaria status at delivery. The percentages of adverse outcomes (SGA and placental malaria) are indicated on the graphs.

(A and B) Elevated C5a levels in peripheral or placental plasma were associated with a higher proportion of deliveries of SGA infants versus AGA infants (peripheral, $p = 0.003$; placental, $p = 0.007$ Pearson chi-square test).

(C and D) A higher proportion of women with placental malaria (PM+) than uninfected women (PM–) had elevated C5a levels in peripheral or placental plasma (peripheral, $p = 0.023$; placental, $p < 0.001$; Pearson chi-square test).

See also Table S2.

placental vasculature throughout pregnancy including angiopoietin-1 (Ang-1), Ang-2, vascular endothelial growth factor (VEGF), sFlt-1, and soluble endoglin (sEng) (Dunk et al., 2000; Geva et al., 2002; Gougos et al., 1992; Venkatesha et al., 2006). Median Ang-1 levels in both primigravid ($p = 0.006$; Table 1) and multigravid ($p = 0.024$; Table S1) women with PM were lower than in control women without PM. Conversely, there were higher median levels of Ang-2, sFlt-1, and sEng in primigravidae (Ang-2, $p = 0.039$; sFlt-1, $p = 0.045$; sEng, $p = 0.057$) and multigravidae (Ang-2, $p = 0.011$; sFlt-1, $p = 0.020$; sEng, $p = 0.001$) with PM than in controls.

In primigravidae, C5a levels were negatively correlated with Ang-1 (Spearman's rho, −0.27; $p < 0.001$) and positively correlated with Ang-2 (0.38; $p < 0.001$), sEng (0.46; $p < 0.001$), VEGF (0.32; $p < 0.001$), and sFlt-1 (0.42; $p < 0.001$). Multigravidae showed the same relationships between C5a and angiogenic factors, except for sFlt-1, where the correlation was weaker (Spearman's rho, 0.19; $p = 0.009$).

Dysregulated Placental Angiogenic Factors Are Associated with Fetal Growth Restriction

Ang-1 levels were lower in women who delivered SGA infants than those who delivered AGA infants ($p = 0.043$). Additionally, Ang-2, sEng, and sFlt-1 were significantly increased in women who delivered SGA infants compared to those who delivered AGA infants (Tables 1 and S1). A trend toward increased VEGF was observed in primigravidae and multigravidae delivering SGA infants ($p = 0.076$ and $p = 0.073$, respectively).

Structural Equation Modeling Supports a Role for C5a in Initiating Dysregulated Angiogenesis and Fetal Growth Restriction in Placental Malaria

We applied structural equation modeling to test our hypothesis that C5a mediates fetal growth restriction by altering the expression of angiogenic factors in the placenta within a multivariate framework (Figure 3). We generated our structural equation model using a current understanding of factors involved in PM pathogenesis (Rogerson et al., 2007) including factors known to affect fetal size (e.g., maternal height and fetal sex). Through a process of trimming, we subsequently removed variables that were insignificant ($p > 0.05$) to generate a final model where each path represents a significant relationship after adjustment for all other covariates ($p < 0.05$). We then assessed how well the model as a whole corresponds to the data using standard measures of fit. We generated a latent variable "mononuclear cell infiltrates" composed of mononuclear cell counts and semiquantitative assessments of monocyte and fibrin pigment that indicate the chronicity of infection (Ismail et al., 2000; Rogerson et al., 2003) (Figure 3A). In this model, mononuclear cell infiltration is inversely related to maternal hemoglobin levels, and maternal hemoglobin level is a major determinant of preterm delivery and fetal growth restriction. We also constructed the latent concept "dysregulated angiogenesis" to reflect several measured variables (proangiogenic factor Ang-2 and antiangiogenic factors sFlt-1 and sEng) that change with similar patterns (Figure 3B).

Supporting the validity of the model and in agreement with an accepted understanding of PM, this model indicates that our case-control data fit a sequence of events whereby malaria parasites initially sequester in the placenta and lead to the accumulation of monocytes (Figure 3C). Moreover a unique component of our model is the axis focusing on C5a and fetal growth restriction (Figure 3C). Our human data fit a model in which

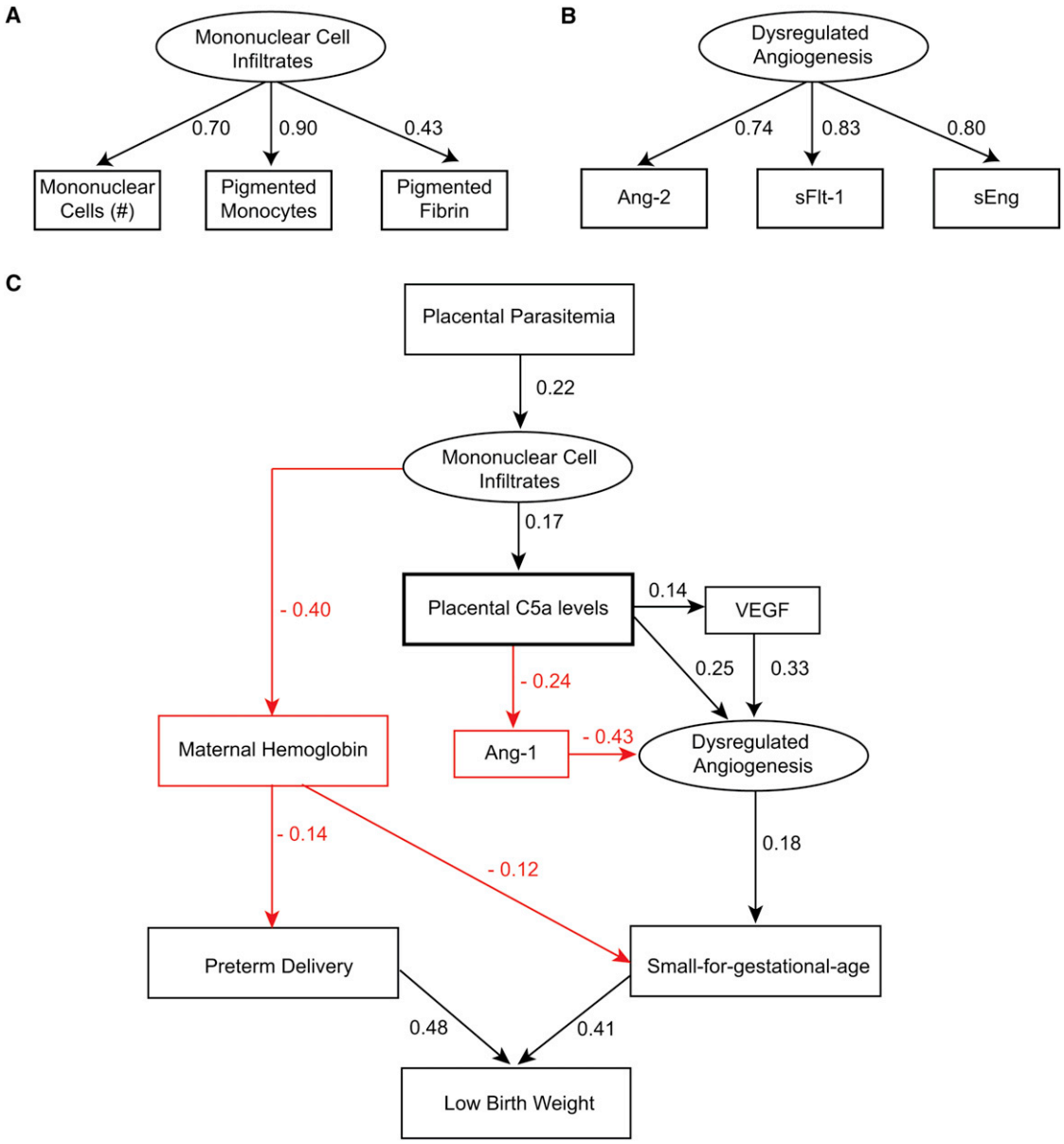

Figure 3. Structural Equation Model for Complement Activation, Dysregulated Placental Angiogenesis, and Birth Outcomes in Primigravid Women with Placental Malaria

Positive associations are indicated by black lines and inverse associations by red lines; lines represent significant relationships ($p < 0.05$) after correcting for all other covariates. Standardized regression coefficients (range, −1.0 to +1.0) of factors associated with placental malaria infection and poor birth outcomes.

(A) The latent variable "mononuclear cell infiltrates" is comprised of the mononuclear cell count, and a semiquantitative score of pigmented mononuclear cells and fibrin pigment (0, none, to 4, large amounts of pigment in every field at 40× objective), as assessed by histology.

(B) The latent variable "dysregulated angiogenesis" is comprised of measures of proangiogenic factor Ang-2, antiangiogenic factor sFlt-1 (which sequesters VEGF), and antiangiogenic factor sEng.

(C) A structural equation model was generated positing how malaria may contribute to adverse birth outcomes through complement activation and dysregulated angiogenesis. This model includes fetal sex, maternal height, and the latent variable "febrile symptoms," composed of self-reported fever, headache, or chills 1 week prior to delivery (omitted here for clarity). The overall fit of the model was good (chi-square = 381.2; df = 282; $p < 0.001$; root-mean-square error of approximation [rmsea] = 0.027 [90% CI: 0.019–0.033]).

See also Figure S1 and Table S3.

mononuclear cells are upstream of, and contribute to, the generation of C5a, and the resultant increase in C5a initiates dysregulated angiogenic responses including increases in Ang-2, sFlt-1, and sEng (Figure 3C). In this model, C5a also modulates angiogenesis by reducing Ang-1 levels and increasing VEGF. Together, the dysregulated angiogenic factors promote fetal growth restriction and SGA infants. We show results for primigravidae since the greatest burden of LBW occurs in this population;

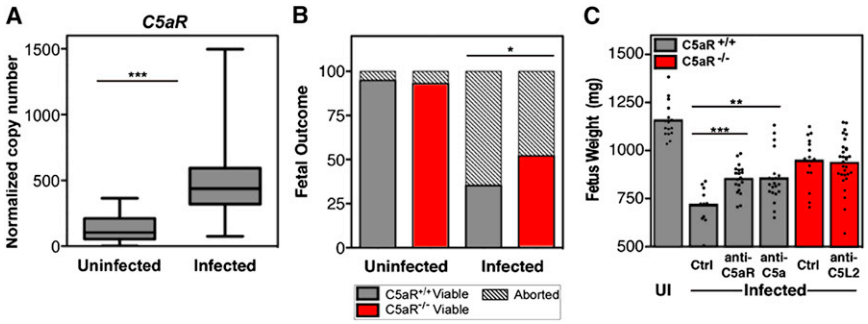

Figure 4. C5a-C5aR Mediates Fetal Growth Restriction and Fetal Loss in a Mouse Model of Placental Malaria

(A) C5aR mRNA transcript levels increase in G19 placentas associated with viable fetuses of wild-type (*C5aR*$^{+/+}$) mice infected with *P. berghei* at G13. Box plots show the median, interquartile range with whiskers denoting the maximum and minimum values. **p < 0.01.

(B) Percent of viable fetuses. Viability of fetuses was increased in *C5aR*-deficient (*C5aR*$^{-/-}$) mice infected with *P. berghei* compared to infected *C5aR*$^{+/+}$ mice. *p = 0.021.

(C) *C5aR* deficiency (*C5aR*$^{-/-}$) and antibody-mediated blockade of C5aR but not C5L2 reduced the fetal growth restriction observed in viable fetuses of wild-type (*C5aR*$^{+/+}$) mice infected with *P. berghei*. Viable fetuses were weighed at G19, 6 days after G13 pregnant mice received an injection of medium alone (UI) or 10^6 *P. berghei* infected-erythrocytes in medium (infected). Bars represent group medians, and dots represent individual viable fetuses. Administration of C5aR (anti-C5aR) or C5a antiserum (anti-C5a) to infected pregnant *C5aR*$^{+/+}$ mice increased G19 fetal weight compared to preimmune control serum (Ctrl), whereas blocking the alternative C5a receptor, C5L2, with anti-C5L2 antiserum did not provide any further protection against fetal growth restriction over that afforded by *C5aR* deficiency alone. **p < 0.01; ***p < 0.0001 by Bonferroni post hoc test of one-way ANOVA. n = 3–15 litters per group.

however, multigravidae yielded a similar model indicating that the same C5a-mediated processes occur in PM in all gravidities (Figure S1 and Table S3). These data are not consistent with a model in which C5a initiates placental monocyte recruitment (p > 0.05). Rather, when taken together, these human data support a role for C5a as a cause of poor fetal growth through altered angiogenesis.

Interruption of C5a-C5aR Signaling Improves Fetal Outcomes

To provide direct experimental evidence of the central role of C5a in malaria-induced fetal growth restriction, we used a mouse model that recapitulates characteristics of PM in primigravidae (Neres et al., 2008). In brief, these include binding of parasitized erythrocytes to placental vascular walls, placental accumulation of monocytes, and decreased fetal viability and growth (Neres et al., 2008). Infection of naturally mated pregnant mice at gestational day 13 (G13; beginning of the third trimester of a normal 20 day mouse gestation) with the rodent malaria parasite, *Plasmodium berghei ANKA*, led to fetal growth restriction as measured by a significant reduction in the weight of viable fetuses at G19 (mean [95% CI]: uninfected, 1,082 mg [1,042 mg–1,090 mg]; infected, 776 mg [744 mg–808 mg]; p < 0.0001). *P. berghei* infection of pregnant mice was associated with an increase in placental levels of mRNA encoding the C5a receptor (C5aR, CD88) (Figure 4A). Genetic deficiency of *C5aR* (*C5aR*$^{-/-}$) significantly increases fetal growth in the presence of PM: the mean weight of fetuses from infected *C5aR*$^{-/-}$ mice was significantly higher than that of fetuses from infected wild-type (*C5aR*$^{+/+}$) mice (mean [95% CI]: *C5aR*$^{+/+}$, 776 mg [744 mg–808 mg]; *C5aR*$^{-/-}$, 913 mg [843 mg–982 mg]; p = 0.0013). Peripheral parasitemia was comparable in both genotypes and with all treatments (data not shown). Additionally, *C5aR* deficiency increased fetal viability at G19 by 50% (p = 0.021; Figure 4B).

A significant improvement in fetal growth was also achieved by blocking C5a-C5aR signaling with antiserum to C5a or C5aR during malaria infection in wild-type (*C5aR*$^{+/+}$) mice (Figure 4C). In contrast, blocking the alternative C5a receptor, C5L2 (Bamberg et al., 2010; Chen et al., 2007; Ward, 2009), did not further improve fetal weight in infected *C5aR*$^{-/-}$ mice (Figure 4C).

Placental Angiogenic Factor Transcription Is Altered with Malaria Infection

Using quantitative real-time PCR, we observed a change in placental angiopoietin transcript levels in favor of Ang-2 in infected murine placentas (Figure 5A): Ang-2 increased (infection: p = 0.026 [two-way ANOVA]) and Ang-1 decreased (infection: p = 0.0004 [two-way ANOVA]) with infection. This increase in Ang-2/Ang-1 was observed regardless of C5aR genotype.

Placental VEGF mRNA expression was increased with malaria (p = 0.031 [two-way ANOVA]; Figure 5B), but this increase was independent of genotype. In *C5aR*$^{+/+}$ mice, the increase was accompanied by a proportional increase in sFlt-1 transcription, which resulted in a consistent VEGF/sFlt-1 ratio. In contrast, *C5aR* deficiency resulted in a significant increase in placental VEGF/sFlt-1 ratio with infection, mainly due to a decrease in sFlt-1 transcription in the absence of C5a-C5aR signaling (p = 0.027 [Mann Whitney]; Figure 5B). The increase in pro-angiogenic VEGF to antiangiogenic sFlt-1 favors a placental environment permissive to placental angiogenesis and vascular remodeling (Maynard et al., 2003).

Placental Malaria-Induced C5a-C5aR Signaling Alters Placental Vascular Development

To examine the effect of malaria-induced changes in complement activation and angiogenic factors on placental vessel structure, we imaged contrast-agent-perfused G18 fetoplacental arterial vasculature with microcomputed tomography (microCT) (Rennie et al., 2011). At G18, all fetuses were viable, and those from infected mice were the same weight as those from uninfected mice (Table S4). Therefore, any changes in the placental vasculature we observed at this point precede the poor fetal outcomes observed at G19.

Three dimensional (3D) analysis of the fetoplacental arterial vasculature revealed a significant increase in placental vessels in the presence of PM (p = 0.017), particularly in vessels of 50–100 μm diameter. Notably, significantly greater placental vessel development occurred with *C5aR* deficiency as compared to

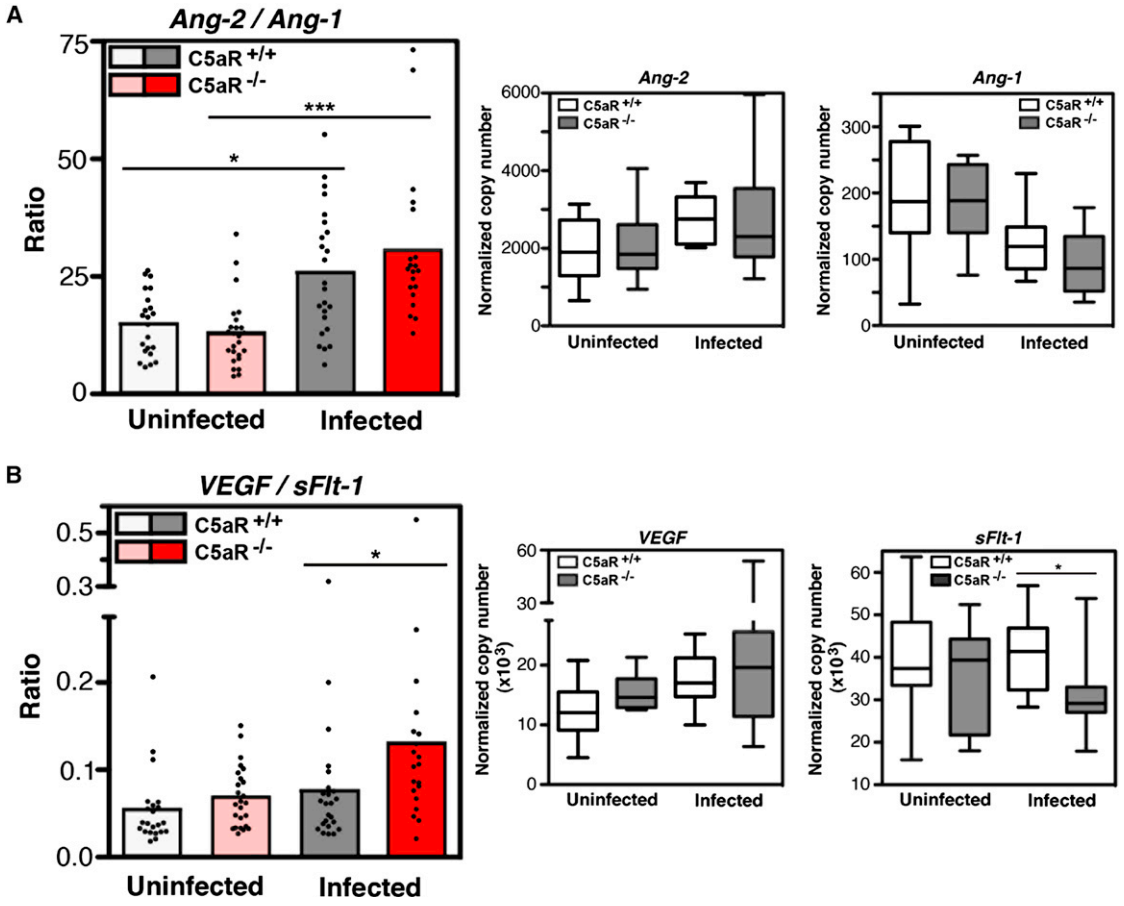

Figure 5. Placental Angiogenic Factor mRNA Expression Is Altered with Infection and *C5aR* Genotype

(A) Infected placentas associated with viable fetuses had increased expression of mRNA encoding Ang-2/Ang-1 regardless of *C5aR* genotype corresponding to an increase in Ang-2 (infection: p = 0.026, two-way ANOVA) and decrease in Ang-1 (infection: p = 0.0004).

(B) The ratio of VEGF/sFlt-1 was unchanged in infected placentas associated with viable $C5aR^{+/+}$ fetuses but significantly elevated in infected $C5aR^{-/-}$ placentas. This appears to be the result of a decrease in sFlt-1 mRNA copy numbers in infected $C5aR^{-/-}$ placentas (p = 0.022 compared to infected $C5aR^{+/+}$ placentas). Overall, VEGF mRNA was increased in infection (p = 0.031, two-way ANOVA), but the increase was independent of genotype.

Bars represent group medians and dots represent individual viable fetuses for ratios. Box plots show the median, interquartile range with whiskers denoting the maximum and minimum values. *p < 0.05; ***p < 0.0001. n = 10 placentas from five to eight litters per group.

wild-type ($C5aR^{+/+}$) counterparts (Figures 6A and 6B and Movie S1). The increased placental vascular development was characterized by an increase in the total length, surface area, volume, and span of the fetoplacental vasculature infected $C5aR^{-/-}$ placentas as compared to infected $C5aR^{+/+}$ placentas (Figure 6B and Table S4). Functionally, PM led to markedly increased placental arterial vascular resistance in $C5aR^{+/+}$ mice. This effect was completely abrogated with *C5aR* deficiency (Figure 6C). In summary, these data support our hypothesis that C5a-C5aR signaling impairs vascular remodeling necessary to compensate for the placental insult induced by the presence of parasitized erythrocytes and/or inflammatory mediators, leading to increased placental vascular resistance.

DISCUSSION

The biological processes that differentiate healthy from pathological pregnancy outcomes remain poorly defined. Our findings provide evidence in humans of a direct connection between innate responses to infection (C5 activation) and altered placental vascular remodeling that result in poor birth outcomes. A critical role for C5a in the pathogenesis of PM and LBW is supported by several observations. First, we show that C5a levels are increased in women of all gravidities with PM. Second, high systemic and placental C5a levels are associated with increased odds of delivering a SGA infant. Third, structural equation modeling of data from our case-control study supports a model in which C5a initiates angiogenic dysregulation and fetal growth restriction. Finally, using a mouse model of PM, we provide direct experimental evidence that C5a could achieve its effect on fetal viability and growth through its effects on placental vascular development.

Remodeling of uterine and placental vasculature is essential for normal placental function and fetal growth. Placental vascular development and remodeling are controlled primarily through the highly regulated actions of angiogenic factors from the

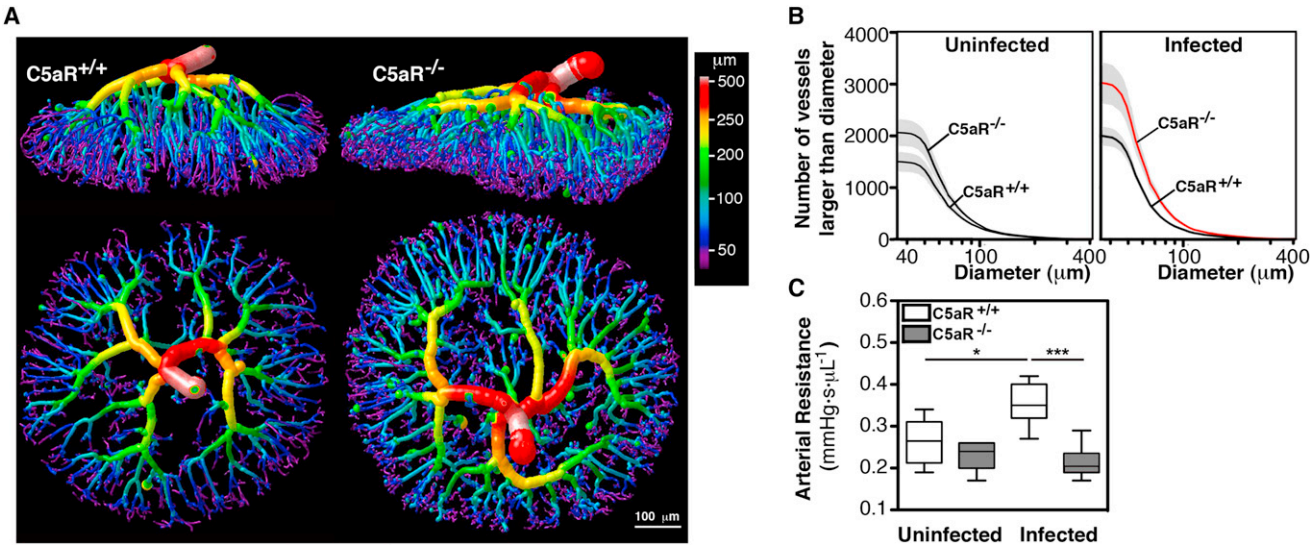

Figure 6. C5a-C5aR Mediates Placental Insufficiency in a Mouse Model of Placental Malaria

(A) Representative microCT imaging of G18 fetoplacental arterial vasculature from infected *C5aR*$^{+/+}$ or *C5aR*$^{-/-}$ mice color-coded by vessel diameter.

(B) Infected *C5aR*$^{-/-}$ placentas had increased cumulative vessel numbers when compared to either uninfected *C5aR*$^{-/-}$ placentas ($p < 0.001$) or infected *C5aR*$^{+/+}$ placentas ($p < 0.001$; post hoc test of repeated-measures ANOVA). Plot depicts the number of fetoplacental arterial vessels larger than the threshold diameter. Lines represent the mean and SEM.

(C) This increase in vessel number in *C5aR*$^{-/-}$ placentas allowed for the arterial resistance to remain low with infection ($p < 0.0001$ by Bonferroni post hoc test of one-way ANOVA; infected *C5aR*$^{+/+}$ versus *C5aR*$^{-/-}$). n = 5–8 placentas from three to four litters per group. Box plot shows the median, interquartile range with whiskers denoting the maximum and minimum values.

See also Table S4 and Movie S1.

VEGF and angiopoietin families (Geva et al., 2002). Ang-1 has a major role in the stabilization and maturation of newly formed vessels, whereas Ang-2 provides the destabilizing effect necessary to initiate vascular remodeling (Jeansson et al., 2011; Yancopoulos et al., 2000). VEGF is necessary for early vessel formation, whereas its soluble receptor, sFlt-1, can inhibit angiogenesis through its ability to bind VEGF and placental growth factor (Levine et al., 2006b). VEGF, which is both proinflammatory and proangiogenic, requires tight regulation during fetal development since either high or low levels result in embryonic lethality (Carmeliet et al., 1996; Hiratsuka et al., 1998; Miquerol et al., 2000). Perturbation of these angiogenic factors have also been reported in preeclampsia and fetal growth restriction associated with inadequate placental vascularization (Kaufmann et al., 2003).

PM is believed to lead to placental insufficiency, a progressive deterioration in placental function and inability to sustain fetal growth, resulting in LBW infants at increased risk of perinatal mortality (Umbers et al., 2011a). Previous reports are consistent with the hypothesis that PM may influence angiogenesis and vascular development. Histological and ultrasound studies of PM suggest that malaria infection alters the placental vascular structure (Arbeille et al., 2003; Dorman et al., 2002; Leke et al., 2002). Altered levels of angiopoietins, sEng, VEGF, sFlt-1, or C5a have recently been shown in several populations of African women with PM at delivery (Conroy et al., 2009; Silver et al., 2011; Silver et al., 2010b). However, it has been unclear whether these changes are a cause or consequence of fetal growth restriction and, if the former, where they are positioned in the sequence of events leading to LBW. We show how alterations in a comprehensive panel of different angiogenic factors were related to fetal growth restriction in PM. Future studies will need to address how changes in these proteins early in pregnancy lead to functional and structural changes in the placenta.

Structural equation modeling provides an opportunity to model putative hierarchal relationships in complex data sets and helps to overcome the limitations of studying mechanisms localized to the placenta, which cannot be ethically tested throughout pregnancy. In our case-control study, we observed a shift in angiopoietin levels favoring the proangiogenic Ang-2, supporting the premise that malaria induces angiogenic remodeling of the placenta. At the same time, C5a-C5aR signaling can inhibit angiogenesis by inducing sFlt-1 (Girardi et al., 2006; Langer et al., 2010). In this study, increased protein levels of antiangiogenic factor sFlt-1 correlated with elevations in C5a in all gravidae with PM and in primigravidae who delivered SGA infants. High sFlt-1 levels may result in less free VEGF available to stimulate placental vessel growth (McKeeman et al., 2004). Application of structural equation modeling to this case-control data set confirmed the relationship of known risk factors for LBW, including maternal anemia, and provided a putative sequence of events linking C5a and the observed changes in placental angiogenic factors. All data supporting the model were collected at delivery, and therefore our ability to model the effect of malaria on angiogenesis throughout pregnancy is limited; but based on the best fit to these data, the model provides evidence for where in the pathological pathway from malaria infection to LBW each factor is most accurately situated. Our data fit a model in which C5 activation is an initiating event

upstream of altered angiogenic factors including angiopoietins, VEGF, and sFlt-1, which then contribute to SGA and LBW infants. The model further illustrates that placental mononuclear cells might be directly cleaving C5 or amplifying C5a production in PM (Huber-Lang et al., 2006), although other factors, which we were unable to measure in this study (e.g., immune complexes), might also contribute to complement activation.

Direct experimental evidence to support these observations was obtained in a murine model of PM. Wild-type mice with PM had reduced fetal viability and lower fetal weight compared to uninfected mice. Functionally, these mice had increased placental resistance despite increased vascular remodeling observed with microCT of placentas. Ablation of C5a-C5aR signaling in this model was characterized by an increase in the placental ratio of VEGF/sFlt-1 mRNA levels and enabled compensatory vascular remodeling and improved fetal growth and survival. Continued production of C5a in wild-type mice was associated with decreased vascular development, mainly though the production of the antiangiogenic protein sFlt-1. These findings are in agreement with our previous report that sFlt-1 secretion in response to in vitro stimulation with malaria bioactive products is decreased when C5a-C5aR signaling is blocked by antibody treatment (Conroy et al., 2009) and with the association of sFlt-1 levels with predicting adverse birth outcomes in PM and preeclampsia (Levine et al., 2006a; Muehlenbachs et al., 2008). Moreover, we show upregulation of C5aR in infected placentas, similar to findings in PM in Tanzanian women (Muehlenbachs et al., 2007). While blocking C5a-C5aR interactions improved fetal growth, blockade of C5L2 did not improve fetal outcome beyond that of *C5aR* deficiency, suggesting that this alternative C5a receptor (Bamberg et al., 2010; Ward, 2009) is not involved in mediating pathogenic responses in the mouse model of PM. Taken together, these data suggest a mechanism of pathogenesis whereby there is compensatory vascular remodeling in response to increased vascular resistance in PM, which is associated with increases in Ang-2 and decreases in Ang-1, favoring angiogenesis. Moreover, increased C5a-C5aR signaling in PM induces sFlt-1 that impairs the vascular remodeling required to compensate for increases in placental vascular resistance. When C5a-C5aR signaling is prevented by genetic or pharmacological strategies, there is enhanced vascular development and a sufficient compensatory response to decrease vascular resistance and improve fetal outcomes.

Although of considerable interest, this model has limitations, including that the mice are immunologically naive to *P. berghei*, which results in a higher peripheral parasitemia than would be expected in primigravidae in malaria endemic regions. However, this model provides an opportunity to define a response to PM that may be anticipated in nonimmune human populations and, in conjunction with data from semiimmune human populations, can establish causal relationships and provide mechanistic insights that would be difficult or unethical to acquire in human studies. Further, while the murine placental structure does differ from that of humans, our understanding of genetic regulation of branching morphogenesis and vascularization of the placenta (Sapin et al., 2001; Watson and Cross, 2005) and of environmental factor effects (Rennie et al., 2011) have been rapidly advanced by the use of mice with targeted mutations, and this approach holds utility in validating hypotheses originating from analysis of clinical data, as we have done here.

This study visualizes the effect of infection on placental vasculature using microCT. The vascular structures derived from the microCT scans enabled the modeling of resistance of the fetoplacental vasculature to blood flow. This study also demonstrates a functional role for C5a-C5aR in impaired vascular remodeling in the placenta. Despite an increase in vessel numbers in malaria-infected placentas, there was an increase in vascular resistance, suggesting that the compensatory vascular remodeling induced by malaria was insufficient in the presence of C5 signaling. *C5aR* deficiency completely reversed the malaria-dependent increase in placental vascular resistance. Maternal vascular resistance is associated with fetal growth restriction (Ghosh et al., 2006). Peripheral malaria parasitemia after 32 weeks of gestation doubled the risk of abnormal uterine artery blood flow velocity waveforms, and this was associated with a doubled risk of low birth weight in a Kenyan population (Dorman et al., 2002). That a single signaling pathway could account for this vascular phenotype induced by malaria is attractive for the development of therapeutic interventions. These findings may also have broad implications for other pregnancy complications associated with immune activation and placental insufficiency (e.g., preeclampsia), where both C5a and sFlt-1 have been associated with adverse birth outcomes (Lynch and Salmon, 2010).

Collectively, our findings suggest that in PM, C5a dysregulates angiogenic factors, counteracts angiopoietin-mediated compensation for PM and inflammation (Umbers et al., 2011a), and contributes to fetal growth restriction through functional placental vascular insufficiency and increased placental resistance to blood flow. In the murine PM model, it is technically challenging to demonstrate a causal relationship between altered angiogenesis and poor fetal outcomes, as manipulation of VEGF signaling or Ang-Tie-2 signaling results in embryonic lethality (Carmeliet et al., 1996; Miquerol et al., 2000; Sato et al., 1995). However, population data from Tanzania demonstrated that a *FLT1* variant associated with increased sFlt-1 production was associated with prior pregnancy loss and LBW in primigravidae (Muehlenbachs et al., 2008). These findings together with the ones described here support the concept that altered angiogenesis occurs in malaria and is associated with poor birth outcomes.

Like other evolutionarily ancient systems, the complement system has diverse effector functions. Through C5a production, the complement system can rapidly amplify an inflammatory response. The association of inflammation with poor birth outcomes has been well described in PM (Rogerson et al., 2003; Umbers et al., 2011a). For example, inflammation can affect the expression of growth regulating hormones (e.g., IGF and leptin), leading to poor fetal growth (Umbers et al., 2011b). Our findings do not exclude a contribution of inflammation in adverse outcomes of PM, but rather suggest that inflammatory and angiogenic pathways may mediate deleterious outcomes through a shared component, C5a. We have previously shown that C5a could amplify inflammatory and antiangiogenic responses in vitro in the presence of malaria toxin *Pf*GPI (Conroy et al., 2009). The relative contribution of (and interaction

between) inflammatory and angiogenic proteins will need to be prospectively assessed.

Our finding that C5a also contributes to increased fetal death in the murine model of PM is consistent with its defined role in non-infection-based animal models of fetal loss (Girardi et al., 2006). We were unable to validate this finding in humans in this study since only women with live births were enrolled, but given that malaria has been shown to increase the risk of stillbirth (Wort et al., 2006; Yatich et al., 2010), future studies should examine the role of C5a in PM-related stillbirth. Likewise, further examination of C5a in malaria-associated preterm birth is warranted.

The link we have established between C5a, angiogenic factor dysregulation, and fetal growth restriction in PM suggests potential biomarkers to predict women at risk of adverse birth outcomes, such as LBW, and potential targets for intervention to prevent these complications.

EXPERIMENTAL PROCEDURES

Participants and Specimens

Between 2001 and 2006, pregnant women delivering a live singleton newborn at Queen Elizabeth Central Hospital, Blantyre, Malawi were recruited into a case-control study. Cases were defined by the presence of *P. falciparum* asexual parasites in the placental blood, as assessed by smear. For each case, two age- (±2 years) and gravidity-matched controls negative for malaria parasites by both peripheral and placental smear were enrolled. The study was approved by the ethics committee of The College of Medicine, Blantyre, Malawi, and written informed consent was obtained from all participants.

At delivery, birth weight was recorded, gestational age was assessed (Ballard et al., 1979), and maternal peripheral and placental EDTA plasma samples were collected and stored at −80°C. The following were considered adverse birth outcomes: low birth weight (< 2,500 g), preterm delivery (<37 weeks of gestation), and fetal growth restriction, defined as SGA (less than tenth percentile for growth in sub-Saharan African populations [Landis et al., 2009]).

Biomarker ELISAs

C5a, Ang-1, Ang-2, soluble Tie-2 (sTie-2), sEng, VEGF, and sFlt-1 levels (DuoSets) and C3a (354113 and purified polyclonal goat IgG) (R&D Systems, Minneapolis, MN) were measured by ELISA (Conroy et al., 2010). All samples were tested consecutively, on the first thaw, with the investigator blinded to group and outcome.

Mouse Model of Placental Malaria

Experiments involving animals were reviewed and approved by the University Health Network Animal Resource Centre and performed in compliance with the Canadian Council for Animal Care. Eight- to ten-week-old BALB/c mice (wild-type [$C5aR^{+/+}$] or $C5aR^{-/-}$) were obtained from Jackson Laboratories (Bar Harbor, ME). Cryopreserved *P. berghei* ANKA (MR4; Manassas, VA) was thawed and passaged through male BALB/c mice. Naturally mated pregnant mice were infected on G13 with 10^6 *P. berghei*-infected erythrocytes in RPMI medium via injection into the lateral tail vein. Control (uninfected) pregnant mice were injected with the same volume of RPMI medium alone. Parasitemia was monitored daily by thin blood smear stained with modified Giemsa stain (Protocol Hema3 Stain Set, Sigma, Oakville, ON). For blocking experiments, polyclonal rabbit antiserum raised against C5aR, polyclonal rabbit antiserum raised against C5L2, polyclonal goat antiserum raised against rat C5a (Riedemann et al., 2002), or preimmune control serum (Sigma) was administered in two 250 μl doses, at 2–3 hr prior to malaria infection and 3 days after infection, via injection into the lateral tail vein.

Pregnant female mice were euthanized by CO_2 on G19 (i.e., 6 days after infection/control injection). Yolk sacs were dissected from uteri, fetuses were removed and weighed, and placentas were snap frozen and stored at −80°C until analyzed. Fetal viability was determined by assessment of pedal withdrawal reflex. Nonviable fetuses (i.e., lacking the pedal withdrawal reflex) were considered aborted.

Placental Transcript Analysis

RNA was extracted from snap-frozen mouse placentas after homogenization in TRIzol (1 ml/100 mg tissue; Invitrogen, Burlington, ON) according to the manufacturer's protocol. Extracted RNA (2 μg per sample) was treated with DNase I (Ambion, Streetsville, ON) and reverse transcribed to complementary DNA (cDNA) with SuperScript III (Invitrogen) in the presence of oligo $(dT)_{18}$ primers (Fermentas, Burlington, ON). Residual RNA was degraded with RNase H (Invitrogen, Burlington, ON). Sample cDNA was amplified in triplicate with SYBR Green master mix (Roche, Laval, QC) in the presence of 1 μM both forward and reverse primers in a Light Cycler 480 (Roche). Transcript number was calculated based on cross-threshold as compared to a standard curve of mouse genomic DNA included on each plate by Light Cycler 480 software (Roche), and normalized to geometric average of GAPDH and HPRT expression levels. See the Supplemental Experimental Procedures for primer sequences.

Fetoplacental Vasculature Analysis

In preparation for microCT scanning a radio-opaque silicone rubber contrast agent (Microfil; Flow Technology, Carver, MA) was perfused via the umbilical artery via methods previously described (Rennie et al., 2011). See the Supplemental Experimental Procedures for details of perfusion protocol and how quantification of vascular measurements was obtained. Vascular resistance was calculated with standard formulas for resistance in parallel and in series (Yang et al., 2010) and the viscosity of blood in small vessels (Pries and Secomb, 2003). The distribution of pressure and flow in the tree was calculated assuming (1) Posieuille's law for flow of fluid through a pipe-like structure, (2) equal pressure at each terminal vessel, and (3) a diameter dependent blood viscosity correction affecting small vessels as previously described (Pries and Secomb, 2003). Analysis was performed on placentas from wild-type ($n_{uninfected}$ = 7 from four litters; $n_{infected}$ = 7 from four litters) and $C5aR^{-/-}$ ($n_{uninfected}$ = 5 from three litters; $n_{infected}$ = 8 from three litters) mice. One data set ($C5aR^{+/+}$ uninfected) for which the umbilical vessel was not present due to the umbilical cord being tied off too close to the chorionic plate during the perfusion process was eliminated from hemodynamic modeling analyses.

Statistical Analysis

Continuous variables were analyzed by Mann-Whitney U test. Categorical data were analyzed by Pearson's chi-square test or Fisher's exact test. Adjusted odds ratios were obtained via logistic regression (SPSS). In order to test our hypothesis within a multivariate framework, we employed structural equation modeling (AMOS) to simultaneously examine the relationships between multiple dependent and independent variables (Calis et al., 2008), as well as latent concepts and their multiple indicators. Risk factors for adverse outcomes in pregnancy were included in exploratory models and, through a process of trimming, were subsequently removed if insignificant ($p > 0.05$; e.g., number of antenatal visits, number of antimalarial doses). Inflammatory cytokines were not measured, so their relative impact on angiogenesis and birth outcomes was not assessed in this structural equation model. Model estimates were generated via maximum-likelihood estimation, and fitness was assessed via the likelihood ratio test statistic (Byrne, 2010), expressed as chi-square and rmsea, respectively. A low rmsea (<0.05) and a narrow confidence interval indicate a good fit. Nonstandardized coefficients for the structural equation model are listed in Table S2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, four tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.01.010.

ACKNOWLEDGMENTS

We thank all the women for participating in this study as well as the study team for their effort. This work was supported by Global Alliance to Prevent Preterm Birth and Still birth and Grand Challenges in Global Health: Preventing Preterm Birth Initiative grant number 12003 (K.C.K.); the Canadian Institutes of Health Research (CIHR) MOP-115160 and 13721 (K.C.K.), Canada Research Chair (K.C.K.), and Doctoral Research Award (A.L.C.); Genome Canada through

the Ontario Genomics Institute (K.C.K.); and a MITACS Elevate Postdoctoral Fellowship (K.L.S.). S.J.R. was supported by a Wellcome Trust Senior Research Fellowship. The funding source had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

Received: August 22, 2012
Revised: October 27, 2012
Accepted: December 7, 2012
Published: February 13, 2013

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