Targeted inhibition of complement activation prevents features of preeclampsia in mice

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Preeclampsia is a major cause of maternal and neonatal morbidity and mortality. In mouse models, complement activation in the placenta is associated with abnormal placental development and miscarriage, and inhibiting complement prevents fetal injury. We mated two mouse strains, DBA/2 and CBA/J, expecting that the pregnancies might show features of preeclampsia and of immunologically mediated pregnancy loss. Along with placental dysfunction, these matings resulted in proteinuria, elevated BUN, fibrin deposition, and glomerular endotheliosis. We blocked placental complement activation throughout pregnancy by administering a single dose of the C3 inhibitor CR2-Crry given on day 5 of the pregnancy. This procedure specifically targets the sites of complement activation without inducing any systemic effects. Placental complement inhibition prevented oxidative stress and placental dysfunction, as well as proteinuria and renal pathologic features of preeclampsia. Thus, local blockade of complement activation at the maternal–fetal interface rescues preeclampsia in mice, and identifies new treatments. Hence, complement triggers a feed-forward cycle of placental damage, antiangiogenic factor production, and maternal vascular damage in patients.

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Preeclampsia is a major cause of maternal and neonatal morbidity and mortality.[1] Although typically diagnosed with the onset of clinical manifestations, proteinuria and hypertension after 20 weeks' gestation, the syndrome begins much earlier in pregnancy with abnormal placental development. Uterine spiral arteries fail to remodel into dilated, flaccid vessels, which results in underperfusion of the intervillous space and placental hypoxia.[2] Placental oxidative stress and inflammation lead to the release of antiangiogenic factors into the maternal circulation.[3–5] Clinical manifestations, widespread endothelial cell dysfunction presenting as proteinuria, hypertension, hemolysis, elevated liver enzymes, low platelet counts (HELLP syndrome), and/or seizures, represent the maternal response to this excess of antiangiogenic factors.[6] One such antiangiogenic factor is soluble fms-like tyrosine kinase 1 (sFlt-1), a secreted splice variant of vascular endothelial growth factor (VEGF) receptor-1 that sequesters circulating VEGF and placental growth factor and prevents their interaction with endogengous receptors.[7] Elevated levels of sFlt-1 are found in the circulation of pregnant women destined for preeclampsia.[8] Patients with cancer, who are treated with inhibitors of VEGF often develop proteinuria (21–64%) and may develop hypertension (3–36%)[9] supports this pathogenic mechanism.

We and others have suggested a relationship between the activation of the complement system and angiogenic factor imbalance linked to the placental dysfunction.[10,11] In mouse models, complement deposition in the placenta is associated with the abnormal placental development and miscarriage, while inhibiting complement rescues pregnancies.[12,13] In patients, complement activation localizes to villous trophoblast injury *in vivo* and modulates trophoblast function *in vitro*.[14] Preeclamptic placentas are characterized by the marked deposition of terminal complement complex (C5b-9).[12] Our finding, that women with evidence of activation of the alternative pathway early in pregnancy are at increased risk for preeclampsia, suggests that complement contributes to the disease pathogenesis.[11] Finally, recent reports of severe preeclampsia in patients with genetic defects

in complement regulation, taken together with observation that complement regulatory proteins are highly expressed on trophoblasts, emphasize the importance of complement to this syndrome.[15,16]

We considered the potential of complement inhibitor therapy to prevent preeclampsia in those at high risk, but were concerned that the prolonged systemic inhibition of complement interferes with host defense. Novel complement therapeutics that are targeted to the sites of complement activation provide an alternative approach[17] with limited immunosuppression, improved bioavailability, and enhanced efficacy in the experimental models of ischemia-reperfusion injury, arthritis, and lupus.[18–20] Whether this strategy could be successful in preeclampsia depends on how important complement activation at the maternal–fetal interface early in pregnancy is to the placental dysfunction, angiogenic dysregulation, and consequent maternal syndrome later in pregnancy. We address this question in an experimental model of preeclampsia triggered by immune and inflammatory mediators.

DBA/2-mated CBA/J mice are well studied as a model of immunologically mediated pregnancy loss that shares features with human recurrent miscarriage.[10,21] We have shown that these matings are characterized by angiogenic dysregulation manifested as elevated levels of circulating sFlt-1, abnormal placental development, and fetal growth restriction, and that systemic complement inhibition reverses angiogenic imbalance, histological changes in placenta, and prevents fetal injury.[10] Because the maternal manifestations of preeclampsia, most notably proteinuria and hypertension, are mediated in part by antagonism of VEGF signaling by sFlt-1 produced by the placenta as a consequence of ischemia and inflammation,[4,7] we hypothesized that CBA/J × DBA/2 matings would demonstrate features of preeclampsia and reveal mediators and mechanisms that activate the vicious cycle of placental damage, antiangiogenic factor production, and maternal vascular damage in patients.

In this report, we provide the first evidence that DBA/2-mated CBA/J pregnancies have characteristics of human preeclampsia, including placental and renal manifestations. We show that targeted complement inhibition early in pregnancy in this mouse model prevents the placental and later maternal syndrome, and that such a therapy is as effective as administering VEGF to restore angiogenic balance.

RESULTS

Features of preeclampsia in CBA/J × DBA/2 matings

In initial studies, we compared placental and maternal phenotypes of DBA/2-mated CBA/J mice with control matings, BALB/c-mated CBA/J (Table 1). In CBA/J × DBA/2 mice, weight of surviving fetuses was decreased, fetal/placental weight ratios were reduced, and fetal resorptions were increased, consistent with the alterations in placental development we previously described.[10] These abortion-prone matings had elevated levels of circulating sFlt-1 as early as day 7 of pregnancy (Table 1), which continued to increase throughout the pregnancy and remained higher than CBA/J × BALB/c ($P<0.05$) until day 15 when mice were killed. CBA/J × DBA/2 mice had increased levels of placental isoprostane 8-iso-prostaglandin F2a (STAT-8, a marker of oxidative stress associated with impaired trophoblast invasion) and elevated circulating thrombin–antithrombin III complexes (TAT, a marker of activation of coagulation cascade increased in microvascular injury) confirming our previous studies (Table 1). Findings similar to those detailed in Table 1 have been described in patients with preeclampsia.[22,23]

Table 1 | Features of preeclampsia in CBA/J × DBA/2 matings[a]

	Mice/ group	CBA/ J × DBA/2	CBA/ J × BALB/c	*P*-value
Placental function				
Fetal weight (mg)	20–26	288 ± 3	314 ± 5	<0.0001
Fetal/placenta ratio (mg/mg)	20–26	2.4 ± 0.05	2.7 ± 0.05	<0.0001
Fetal resorption frequency (%)	20–26	23 ± 4	7 ± 4	<0.0025
STAT-8 (pg/ml)	21–22	171 ± 24	121 ± 14	<0.04
Maternal syndrome				
sFlt-1 (pg/ml)	4	1454 ± 137	425 ± 56	<0.0004
TAT (μg/l)	5	93 ± 27	32 ± 14	<0.05
Urine albumin/creatinine (μg/mg)	10	359 ± 102	166 ± 24	<0.05
Blood BUN (mg/dl)	14–16	73.2 ± 6.9	57.1 ± 5.3	<0.04

Abbreviations: BUN, blood urea nitrogen; sFlt-1, fms-like tyrosine kinase 1; TAT, thrombin–anti-thrombin III.
[a]All assessments were performed on samples obtained on day 15 of pregnancy, except sFlt-1, which was measured on day 7 of pregnancy.

To determine whether DBA/2-mated CBA/J mice develop the maternal features of preeclampia, we assessed renal function and kidney histopathology. Urinary protein and blood urea nitrogen (BUN) on day 15 of pregnancy (mid trimester) were higher in CBA/J × DBA/2 mice compared with CBA/J × BALB/c mice (urine albumin/creatinine ratio: 359 ± 102 versus 166 ± 24 μg/mg, $n=10$/group, $P<0.05$; BUN: 73 ± 7 versus 57 ± 5 mg/dl, $n=14$–16, $P<0.05$; Table 1 and Figure 1a and b). In grouped studies, 50% of CBA/J × DBA/2 mice had albumin/creatinine ratios greater than the mean level of CBA/J × BALB/c matings. Proteinuria tended to be related to the placental function, as defined by mean fetal weight at day 15 (Pearson $r=-0.6$, $n=10$ mice; $P=0.068$). Immunofluorescence studies of kidneys showed diffuse fibrin deposition within glomerular capillary walls and lumina in CBA/J × DBA/2 mice (mean fibrin score 1.5 + range 1–2 +) compared with CBA/J × BALB/c (mean fibrin score 0.25 + ; range 0–0.5 +), a finding described in patients with preeclampsia (Figure 1c). No glomerular intracapillary fibrin thrombi were detected by light microscopy. Electron microscopy revealed focal glomerular endothelial cell body swelling consistent with 'endotheliosis' associated with segmental foot process effacement (Figure 1d). No glomerular intracapillary fibrin tactoids were identified. This constellation of renal pathological findings was not present in CBA/2 × BALB/c matings and represents the classic findings in human preeclampsia.[24–26]

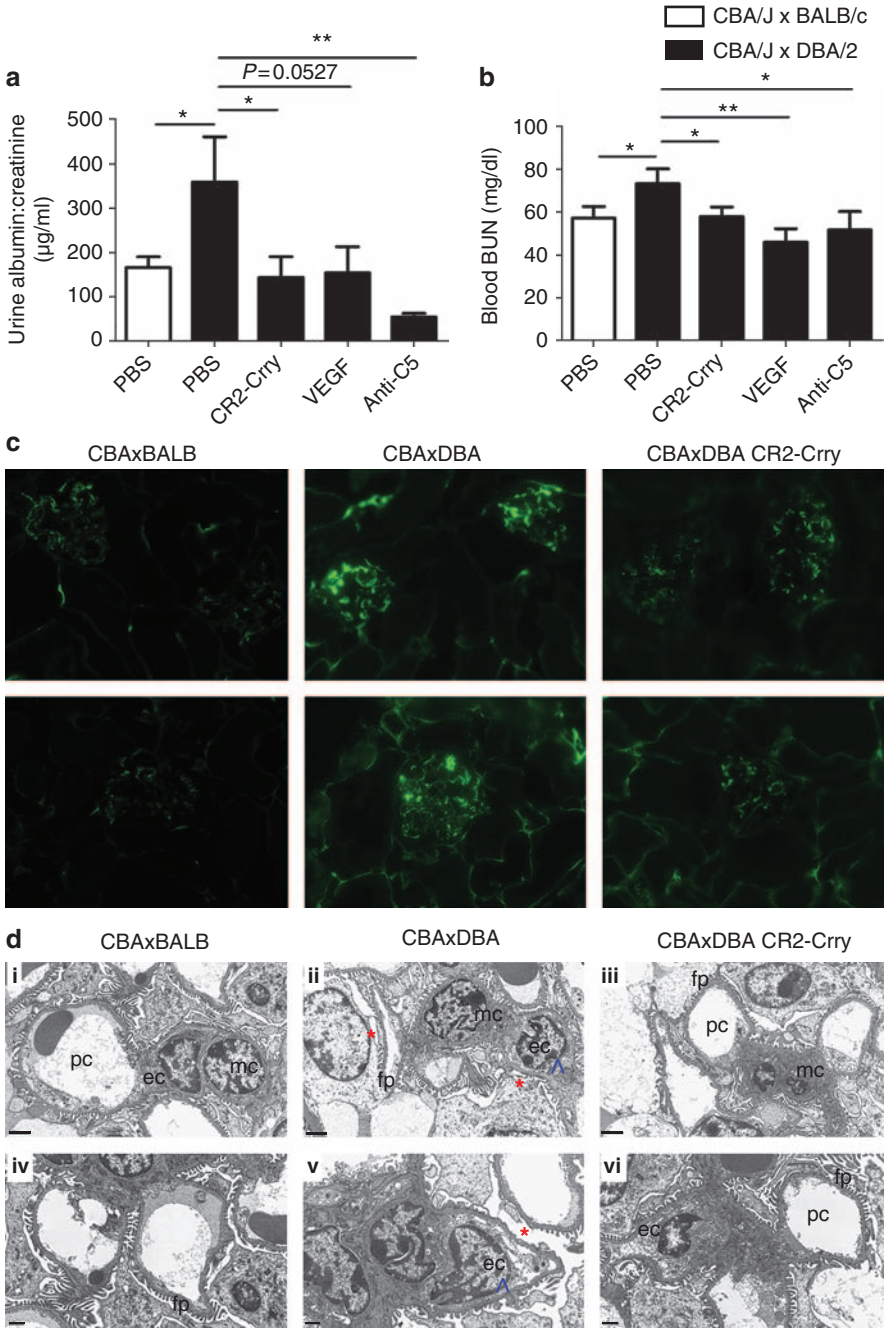

Figure 1 | CBA/J × DBA/2 mice develop kidney damage, which is attenuated by targeted inhibition of complement. Pregnant CBA/J × DBA/2 mice were treated with CR2-Crry (200 μg i.v. on day 5 of pregnancy), VEGF121 (4 μg s.c. daily from day 3 to day 10 of pregnancy), anti-mouse C5 mAb (1 mg i.p. on day 4 and day 6 of pregnancy) or PBS as control. On day 15 of pregnancy, urine and blood was collected to measure albumin/creatinine ratios and BUN, mice were killed and kidneys removed. CBA/J × DBA/2 matings resulted in proteinuria (**a**) and elevated BUN levels (**b**), both of which were prevented by CR2-Crry, anti-C5 mAb, and restoration of VEGF ($n = 4$–16 mice per group). Mean and s.e.m. are shown. $^{*}P < 0.05$, $^{**}P < 0.01$. (**c**) Deposition of fibrin in glomeruli was assessed by immunofluorescence staining and graded from 0–3 +. There was diffuse glomerular capillary wall and luminal staining in CBA/2 × DBA/J mice. Mean fibrin scores (range): CBA/J × BALB/c = 0.25 + (0–0.5 +), CBA/J × DBA/2 = 1.5 + (1–2 +), and CBA/J × DBA/2 with CR2-Crry = 0.75 + (0.5–1 +). Two fields from each condition are shown (magnification × 400 upper panel and × 600 lower panel). Staining outside the glomeruli is in the distribution of interstitial capillaries and was not significantly different between the samples. (**d**) Electron microscopic analysis of kidneys from CBA/J × DBA/2 mice showed endothelial cell swelling (blue arrowhead) with marked narrowing of the capillary lumen (ii and v) and segmental areas of foot process effacement (red asterisk), which was not present in CBA/J × BALB/c mice and which was ameliorated in mice treated with CR2-Crry. i–iii, Magnification × 8000; iv–vi, magnification × 10,000. BUN, blood urea nitrogen; CR2, complement receptor type 2; Crry, CR2-related gene/protein y; ec, endothelial cell; fp, foot process; mAb, monoclonal antibody; mc, mesangial cell; PBS, phosphate-buffered saline; pc, patent capillary; VEGF, vascular endothelial growth factor.

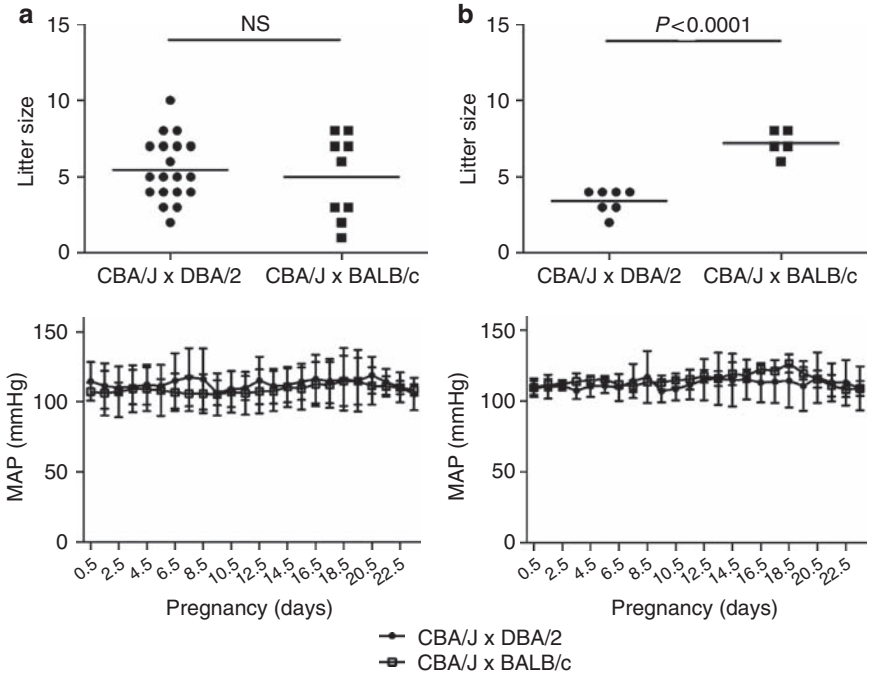

Figure 2 | Blood pressure during pregnancy of CBA/J × DBA/2 and CBA/J × BALB/c mice. Female CBA/J mice implanted with telemeters were mated with male DBA/2 or BALB/c mice. The 24 h MAP was monitored throughout pregnancy. Baseline reflects MAP over 3 days before the pregnancy. The number of pups was recorded upon delivery. (**a**) Litter size and MAP for all pregnancies studied (CBA/J × DBA/2, $n = 19$ and CBA/J × BALB/c, $n = 9$). (**b**) Results of pregnancies for selected mice based on litter size (CBA/J × DBA/2 with <5 pups, $n = 7$ and CBA/J × BALB/c with >7 pups, $n = 5$; $P < 0.0001$). MAP, mean arterial pressure; NS, not significant.

To determine whether pregnancy causes hypertension in the CBA/J × DBA/2 crosses, arterial pressures of CBA/J mice were recorded with surgically implanted radiotelemeters 24 h/day beginning 3 days before mating and continuing through delivery. Mean arterial blood pressure calculated for 19 CBA/J × DBA/2 matings and 9 CBA/J × BALB/c matings were not different throughout pregnancy (Figure 2a). We considered the possibility that mice with severe placental dysfunction would become hypertensive and performed a subset analysis comparing blood pressure in the most divergent groups of mice defined by litter size, a proxy for placental function, because blood pressure was monitored. We compared mean arterial blood pressure in DBA/2 mated-CBA/J mice with fewer than five offspring to that of BALB/c-mated CBA/J mice with greater than seven offspring and again found no differences between the groups (Figure 2b). Although a hallmark of human preeclampsia, other informative genetic mouse models with reduced spiral artery modification and placental dysfunction also fail to develop hypertension[27,28] and 10–15% of patients with the most severe forms of preeclampsia are not hypertensive.[29] The strong evidence for placental dysfunction, taken together with our new findings of renal manifestations characteristic of preeclampsia, identify CBA/J × DBA/2 mice as a new model to explore underlying events that trigger some, but not all, cardinal features of preeclampsia.

Targeted inhibitor of complement activation prevents oxidative stress and placental dysfunction in CBA/J × DBA/2 matings

To determine whether there was systemic evidence for complement activation early in pregnancy, we measured blood levels of C3a, a marker of classical or alternative pathway activation, between days 3 and 7. Circulating C3a was significantly higher in CBA/2 × DBA/J compared with CBA/2 × BALB/c matings (422 ± 43 versus 315 ± 34, $n = 9$–10 mice/group, $P < 0.05$), at a time when there was no evidence of complement activation in the kidney (Figure 3, right panels).

We have previously shown that excessive complement activation occurs at the maternal–fetal interface of the developing placenta by day 8 of pregnancy[10] and confirmed these findings in the current study (Figure 3, left panel). To determine whether blocking complement activation in the developing placenta early in pregnancy would prevent features of preeclampsia in DBA/2-mated CBA/J mice, we treated mice with CR2-Crry, a complement inhibitor specifically targeted to sites of complement activation that provides highly effective local protection without significant systemic inhibition.[17,18,30] CR2-Crry is composed of the complement receptor type 2 (CR2)-binding site for cell-bound C3 degradation products (iC3b/C3d) linked to the complement regulatory protein domain of the murine protein CR2-related gene/protein y (Crry), a murine C3

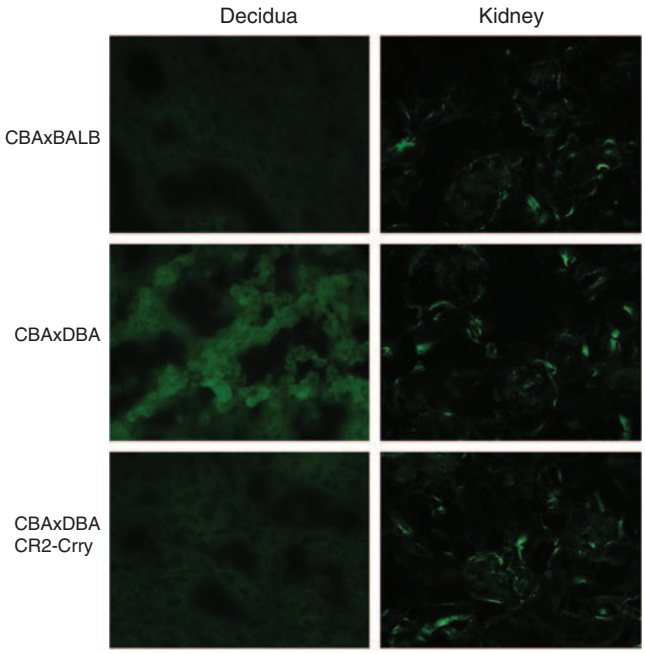

Figure 3 | Complement is activated in the deciduas but not in the kidneys of CBA/J × DBA/2 matings. Deciduas (left panel) and kidneys (right panel) were isolated from pregnant mice on day 8 or day 15, respectively. Deposition of C3 was examined by immunofluorescence staining. In CBA/J × DBA/2 mice, there was extensive C3 deposition on trophoblasts in the developing placenta, whereas only trace C3 staining was present in the interstitium in all three groups. Mean C3 scores (range): Deciduas: CBA/J × BALB/c = 0.25 + (0–0.5 +), CBA/J × DBA/2 = 2 + (1–2.5 +), and CBA/J × DBA/2 with CR2-Crry = 0.5 + (0–1 +); kidneys, CBA/J × BALB/c = 0.5 + (0–1 +), CBA/J × DBA/2 = 0.5 + (0–1 +), and CBA/J × DBA/2 with CR2-Crry = 0.75 + (0.5–1 +; n = 4–6 mice per group). Magnification × 400. CR2, complement receptor type 2; Crry, CR2-related gene/protein y.

convertase inhibitor that blocks all complement activation pathways.[31] A single dose of CR2-Crry (0.2 mg) administered i.v. on day 5 of pregnancy prevented complement deposition (Figure 3, left panels) and placental dysfunction characteristic of CBA/2 × DBA/J matings (Figure 4). In mice treated with CR2-Crry, there was no increase in placental STAT-8, a measure of oxidative stress associated with pregnancy complications[23,32,33] (Figure 4a), and no increase in the circulating levels of the antiangiogenic factor sFlt-1 (Figure 4b). Levels of sFlt-1 in CBA/2 × DBA/J mice treated with CR2-Crry on day 5 were comparable with CBA/2 × BALB/c matings and remained lower than untreated CBA/2 × DBA/J matings through day 15, when the mice were killed ($P < 0.03$). Similarly, in CR2-Crry-treated mice there was no elevation in plasma TAT levels on day 15 of pregnancy (Figure 4c).

As predicted by the measures of improved placental function in CBA/2 × DBA/J mice, CR2-Crry prevented increased fetal resorption characteristic of these matings (Figure 4d). Fetal weights in CR2-Crry-treated pregnancies increased slightly (control versus CR2-Crry: 288 ± 3 versus 294 ± 4 mg, n = 14–28, P = not significant). Studies of CR2-Crry in other experimental models show accumulation of the inhibitor at sites of tissue iC3b/C3d deposition and prolonged tissue half-life consistent with CR2 domain-mediated binding to tissue ligands.[17–19] Taken together with our previous report of C3 degradation fragments in the decidua and ectoplacental cone of DBA/J-mated CBA/2 mice,[10] the current findings indicate that early in pregnancy complement inhibition localized exclusively to the maternal–fetal interface is sufficient to prevent placental dysfunction and poor pregnancy outcomes associated with preeclampsia.

Blockade of complement activation prevents proteinuria and maternal features of preeclampsia

Given that maternal manifestations of preeclampsia are considered to result from placental oxidative stress and vascular damage because of the dysregulation of angiogenic factors,[33–36] we hypothesized that targeting inhibitors of complement to the placenta would not only prevent placental dysfunction but would also attenuate other features of preeclampsia in CBA/J × DBA/2 pregnancies. To investigate this possibility, we administered CR2-Crry on day 5 of pregnancy and measured urinary protein, BUN, and examined renal tissue on day 15. Targeted complement inhibition prevented renal lesions in CBA/J × DBA/2 mice; albumin/creatinine ratios, and BUN at day 15 were comparable with CBA/J × BALB/c mice (Figure 1a and b). Immunofluorescence staining of kidneys showed that the increase in glomerular capillary wall and luminal deposits of fibrin in CBA/J × DBA/2 mice (mean fibrin score 1.5 +; range 1–2 +) was markedly attenuated in mice treated with CR2-Crry (fibrin score 0.75 +; range 0.5–1 +; Figure 1c). Similarly, the endothelial cell swelling and areas of segmental foot process evident by electron microscopy in kidneys of CBA/J × DBA/2 mice, were averted in pregnancies treated with CR2-Crry (Figure 1d). The histologic changes in glomeruli are not related to local activation of complement. Immunofluorescence studies demonstrated minimal C3 (mainly in the interstitium) in kidneys of CBA/J × DBA/2, which did not differ from that in CBA/J × BALB/c mice (Figure 3, right panels). Taken together, our findings demonstrate that blockade of complement activation targeted exclusively to areas of complement activation is sufficient to prevent maternal features of preeclampsia.

To confirm that complement inhibition prevents preeclamptic renal injury and proteinuria, we used a second strategy. We treated CBA/2 × DBA/J mice with a non-targeted systemic complement inhibitor, anti-C5 monoclonal antibody (mAb), that blocks cleavage of C5, a pivotal complement component that generates two effectors, C5a, a potent anaphylatoxin, and C5b, which initiates formation of the C5b-9 membrane attack complex. We have previously shown that C5a–C5a receptor interactions initiate angiogenic dysregulation and placental dysfunction in abortion-prone mice.[10,37] Consistent with our findings using CR2-Crry, anti-C5 mAb prevented proteinuria and decreased BUN in DBA/J-mated CBA/2 mice (Figure 1a and b). Placental oxidative

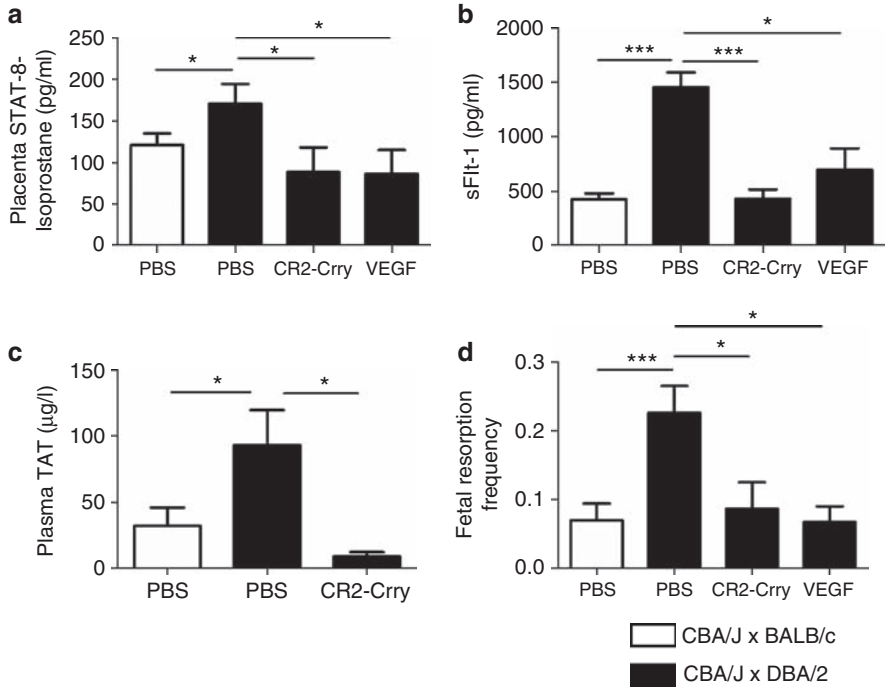

Figure 4 | Targeted inhibition of complement activation prevents oxidative stress and placental dysfunction in CBA/J × DBA/2 matings. Female CBA/J mice were mated with male DBA/2 or BALB/c mice. Some pregnant DBA/2-mated CBA/J mice were treated with CR2-Crry (200 µg i.v. on day 5 of pregnancy), VEGF121 (4 µg s.c. daily from day 3 to day 10), or PBS as control. (**a**) Placental isoprostane 8-iso-prostaglandin F2a (STAT-8) levels measured on day 15 were higher in CBA/J × DBA/2 mice. In mice treated with CR2-Crry, placental STAT-8 levels were decreased to levels similar to CBA/2 × BALB/c mice ($n = 7$–22 mice/group). Treatment with VEGF early in pregnancy also prevented the increase in placental STAT-8. (**b**) Plasma sFlt-1 was increased in CBA/J × DBA/2 mice by day 7 of pregnancy. CR2-Crry or VEGF attenuated the increase in sFlt-1 ($n = 4$ mice/group). (**c**) Plasma TAT levels measured on day 15 of pregnancy were elevated in CBA/2 × DBA/J matings and this increase was also prevented by CR2-Crry ($n = 5$–6 mice/group). (**d**) CBA/J × DBA/2 mating are abortion prone. Treatment with CR2-Crry or VEGF prevented fetal loss ($n = 10$–26 mice/group). *$P < 0.05$, ***$P < 0.001$. CR2, complement receptor type 2; Crry, CR2-related gene/protein y; PBS, phosphate-buffered saline; sFlt-1, fms-like tyrosine kinase 1; TAT, thrombin–anti-thrombin III; VEGF, vascular endothelial growth factor.

stress was also reduced in anti-C5 mAb-treated mice (placental STAT-8: control versus anti-C5 mAb 121 ± 14 versus 77 ± 43 pg/ml, $n = 21$ and 4 mice/group, respectively, $P = 0.057$) supporting the concept that complement activation contributes to the placental injury that drives angiogenic dysregulation and promotes a feed-forward loop that culminates in fetal growth restriction or death and maternal vascular damage.

Restoration of circulating VEGF prevents placental dysfunction, pregnancy complications, and proteinuria in CBA/J × DBA/2 matings

Dysregulation of angiogenic factors presages preeclampsia in humans, but interventions to reverse the imbalance are not yet available.[36,38] In DBA/J-mated CBA/2 mice, targeted inhibitors of complement block the increase in sFlt-1. It was not clear, however, whether correction of sFlt-1 excess, in and of itself, is sufficient to prevent the features of preeclampsia in this model. In a rat model of preeclampsia induced by chronic reductions in uteroplacental perfusion pressure, delivery of recombinant VEGF121 late in pregnancy reduced hypertension and improved renal function, generated by chronic elevations of sFlt-1.[39,40] In contrast, CBA/J × DBA/2 mice are not hypertensive and their placental dysfunction is mediated by inflammation. To determine whether restoration of circulating VEGF during the first half of pregnancy could prevent features of preeclampsia, we administered VEGF121, the most soluble of VEGF isoform, on days 3 through 10. Treatment with VEGF121 reduced placental oxidative stress assessed by STAT-8 on day 15 (Figure 4a), and prevented proteinuria, elevated BUN (Figure 1a and b), and pregnancy loss (Figures 4d), although fetal weights were not significantly altered by VEGF121 treatment (control versus VEGF121: 288 ± 3 versus 280 ± 5 mg, $n = 14$–28, $P =$ not significant). Treatment with VEFG121 seemed to decrease plasma sFlt-1 (Figure 4b), perhaps attributable to decreased capacity of enzyme-linked immunosorbent assay to detect sFlt-1 complexed to VEGF-121. Alternatively, increased available VEGF may lead to enhanced placental perfusion resulting in decreased sFlt-1 expression. That correction of angiogenic imbalance, as well as blockade of complement activation at the maternal–fetal interface, rescues placental and maternal systemic features of preeclampsia supports a relationship between these pathways.

DISCUSSION

DBA/2-mated CBA/J mice have been studied as examples of abortion related to abnormal maternal–fetal tolerance.[41,42] We present the first evidence that these matings show some systemic features of preeclampsia, specifically renal dysfunction. It has been proposed that factors released by the immunologically injured, inflamed, and ischemic placenta cause the clinical syndrome of preeclampsia,[33,35,43] and such factors have been the focus of studies in many experimental animal models. Placental dysfunction has been induced by reduced uterine perfusion, thrombotic diatheses, angiotensin receptor agonistic autoantibodies, and by genetic deficiencies of heme oxygenase, adrenomedullin, and catechol-*O*-methyl transferase.[35,40,44–47] Specific treatments for preeclampsia have been proposed based on these models. CBA/2 × DBA/J matings show similar evidence of placental dysfunction, oxidative stress, activation of coagulation, elevated anti-angiogenic factors, and fetal loss;[10,23] and they manifest the expected phenotype, maternal renal dysfunction, presenting as proteinuria, endothelial cell swelling (endotheliosis), and fibrin deposition in glomeruli. The current work is unique in that it presents a treatment strategy directed at inflammation in the developing placenta, rather than at the downstream systemic mediators. We found no evidence of complement activation in kidneys.

Complement activation at the maternal–fetal interface early in abnormal pregnancies has been documented by our group and others.[10,48–51] Placental oxidative stress, characteristic of experimental models of preeclampsia and the human condition, and exaggerated hypoxia promote complement activation and render tissue more susceptible to complement-mediated injury, perpetuating the vicious cycle.[52] Local generation of C5a triggers the release of sFlt-1 from infiltrating inflammatory cells, and sFlt-1 impairs trophoblast proliferation, reduces placental blood flow, and induces ischemia that leads to increased placental sFlt-1 production. We have previously shown that Crry-Ig, a potent inhibitor of all complement activation pathways in mice (administered as 3 mg i.p. on days 4, 6, and 8 of pregnancy), rescues miscarriage in CBA/J × DBA/2 mice.[10] However, the effects of complement inhibition on placental oxidative stress or renal dysfunction associated with angiogenic dysregulation have not been examined. CR2-Crry had no significant effect on serum complement activity and does not increase susceptibility to infection,[18] but we expected enhanced bioavailability and therapeutic efficacy.[17–19] That was indeed the case, as administration of 0.2 mg i.v. on day 5 of pregnancy (15-fold lower than the Crry-Ig dose and 45-fold less total protein throughout pregnancy) provided equivalent protection from fetal loss to that imparted by Crry-Ig, despite a markedly shorter half-life.[18] The single dose of CR2-Crry prevented the increase in circulating sFlt-1 and the consequent glomerular damage, fibrin deposition, and proteinuria.

Release of sFlt-1 into the maternal circulation blocks VEGF and placental growth factor, the trophic signals required to maintain the renal filtration barrier, and leads to a loss of integrity of this barrier and development of proteinuria.[4,38,40,53] Deletion of VEGF from podocytes in mice promotes microvascular injury, microthrombotic angiopathy, and proteinuria and is followed by hypertension.[53] In patients, VEGF inhibitors have similar effects, mild proteinuria and, less commonly, hypertension; both are transient and reversible, as in preeclampsia.[9] In CBA/J × DBA/2 mice, elevated levels of sFlt-1 precede proteinuria, but hypertension is not present. We speculated that the lack of hypertension in this model is because of the relative magnitude of elevation in sFlt-1 in CBA/J × DBA/2 (nearly threefold increase) is less than that described in animal models of preeclampsia with hypertension which show at least fivefold increase in sFlt-1.[4,47] The phenocopy of gestational proteinuria without hypertension, considered a milder variant of human preeclampsia, was recently shown to be associated with modest angiogenic imbalance, detectable as early as 10–12 weeks of gestation.[54] Administration of excess VEGF121 to CBA/2 × DBA/J mice early in pregnancy prevented placental oxidative stress, microthrombotic angiopathy, proteinuria, and pregnancy loss underscoring the importance of angiogenic dysregulation in this model and its relevance to the proposed pathophysiology of human preeclampsia. That blockade of complement is as effective as VEGF121 administration in averting the preeclampsia-like phenotype and protecting pregnancy argues that local complement activation is an early trigger of placental injury that produces angiogenic dysregulation and ultimately drives preeclampsia.

A role for complement activation in preeclampsia was postulated nearly 20 years ago,[11,55] but whether it was a cause or consequence was unclear. Our findings in a new mouse model prove that local complement activation is sufficient to trigger placental and some maternal features of preeclampsia. Evidence of alternative pathway activation in early pregnancy in women more likely to develop preeclampsia argues that our findings in CBA/J × DBA/2 mice are relevant to patients.[11,55] That low molecular weight heparin blocks activation of complement *in vivo* and in mouse models and prevents preeclampsia in some patients at high risk[56] emphasizes the importance of developing targeted complement inhibitors for this disease.[57] Our studies in CBA/J × DBA/2 mice identify an exciting new approach to limit morbidity and mortality in human pregnancies at risk for preeclampsia.

METHODS

Timed breeding and treatment protocols

Female CBA/J ($H2^k$), male DBA/2 ($H2^d$), and BALB/c ($H2^d$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal studies were approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery or Weill Medical College of Cornell University. Virgin female CBA/J mice (8- to 10-week old) were mated with 10- to 12-week-old male DBA/2 or BALB/c mice as previously described.[10]

To block complement activation during pregnancy, we used a targeted complement inhibitor CR2-Crry. Recombinant CR2-Crry

fusion protein prepared as previously described[18] was injected on day 5 of pregnancy (0.2 mg i.v.). To block C5 cleavage, a group of mice was treated with an anti-C5 mAb (mouse immunoglobulin G1, clone BB5.1, 1 mg/day i.p.) on days 4 and 6 of pregnancy as previously described.[10] To reverse the angiogenic dysregulation that is characteristic of DBA/2-mated CBA/J mice, a group of mice was treated with human VEGF121 (kindly provided by SA Karumanchi, Beth Israel Deaconess Hospital; 4 μg s.c.) on days 3 to day 10 of pregnancy.

Blood was collected to measure soluble Flt1, TAT, BUN, and C3a. Urine was collected on day 15. Pregnant mice were killed on day 15. Fetal resorption frequency was calculated as number of resorption/total number of formed fetuses. Weights of viable fetuses and placentas were recorded. Placentas collected to measure isoprostane 8-iso-prostaglandin F2a (STAT-8), using a commercial EIA kit (Cayman Chemical, Ann Arbor, MI).[58] Kidneys were harvested for immunohistochemical studies and light and electron microscopy.

Plasma levels of sFlt-1 were determined by ELISA following manufacturer's instructions (R&D systems, Minneapolis, MN). TAT levels were measured in sodium citrate plasma by ELISA (Enzygnost TAT, Dade Behring, Deerfield, IL). To measure C3a, plasma samples were collected in EDTA, stored at −80 °C and assayed by ELISA. Plates coated with rat-anti-mouse C3a mAb (clone I87-1162, BD Bioscience (San Jose, CA); 2 μg/ml) were incubated with samples diluted in 1% bovine serum albumin/phosphate-buffered saline. Biotin conjugated anti-C3a mAb was used to detect plate-bound C3a, followed by incubations with streptavidin–horseradish peroxidase and tetramethylbenzidine. Urine albumin/creatinine ratio was determined with an Albuwell M test kit and Creatinine Companion kit (Exocell, Philadelphia, PA). BUN was examined with a Blood Urea Nitrogen kit (Pointe Scientific, Canton, MI).

Morphologic studies

Kidneys from day 15 pregnant mice were harvested for morphologic studies. For immunofluorescence studies, snap frozen deciduas or kidneys were sectioned at 3 μm, washed with phosphate-buffered saline and stained with fluorescein isothiocyanate-conjugated polyclonal antifibrin antibody (Dako, Carpinteria, CA), or fluorescein isothiocyanate-conjugated anti-mouse C3 mAb (Cedarline, Burlington, NC). Intensity of immunofluorescence was scored semiquantitatively (on a scale of 0–3+: 0 negative, 0.5 trace, 1 mild, 2 moderate, 3 marked). There were two to six mice studied for each condition. For kidney studies, a total of 100 glomeruli per mouse were scored and intensity was expressed as a mean (range).

Histology of kidneys was assessed by staining with hematoxylin and eosin, as well as periodic acid-Schiff and Masson's trichrome. In each condition, two to five mice were studied.

Electron microscopy was examined under a JEOL 1011 electron microscope (JEOL USA Inc, Peabody, MA) equipped with digital imaging system. There were two to five mice studied for each condition and a minimum of 10 glomeruli sampled per mouse.

Radiotelemetric measurement of blood pressure

Continuous measurement of blood pressure before, during and after pregnancy was carried out as described previously.[59] Briefly, a cohort of CBA female mice were anesthetized and instrumented with TA11PA-C10 radiotelemeters (Data Sciences International, Arden Hills, MN). Mice were allowed to recover for 5 days before baseline measurements were taken over 3 days. DBA/2 or BALB/c males were placed in the cages for mating. After plug detection, blood pressure was recorded in a 24 h scheduled mode as described previously..[59,60]

Statistical analysis

Data were first tested for normal distribution using Kolmogorov–Smirnov test. Student's *t*-test was used to compare differences in means. Blood pressure data were analyzed using repeated measures analysis of variance. Data are expressed as means ± s.e.m. Statistical analysis was performed using Graphpad Prism 5.0 statistical software. $P < 0.05$ was considered as statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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