Autoantibody-Mediated Complement C3a Receptor Activation Contributes to the Pathogenesis of Preeclampsia

Wei Wang, Roxanna A. Irani, Yujin Zhang, Susan M. Ramin, Sean C. Blackwell, Lijian Tao, Rodney E. Kellems, Yang Xia

Abstract—Preeclampsia (PE) is a prevalent life-threatening hypertensive disorder of pregnancy associated with increased complement activation. However, the causative factors and pathogenic role of increased complement activation in PE are largely unidentified. Here we report that a circulating maternal autoantibody, the angiotensin II type 1 receptor agonistic autoantibody, which emerged recently as a potential pathogenic contributor to PE, stimulates deposition of complement C3 in placentas and kidneys of pregnant mice via angiotensin II type 1 receptor activation. Next, we provide in vivo evidence that selectively interfering with C3a signaling by a complement C3a receptor–specific antagonist significantly reduces hypertension from 167±7 to 143±5 mm Hg and proteinuria from 223.5±7.5 to 78.8±14.0 μg of albumin per milligram creatinine (both $P<0.05$) in angiotensin II type 1 receptor agonistic autoantibody–injected pregnant mice. In addition, we demonstrated that complement C3a receptor antagonist significantly inhibited autoantibody-induced circulating soluble fms-like tyrosine kinase 1, a known antiangiogenic protein associated with PE, and reduced small placental size with impaired angiogenesis and intrauterine growth restriction. Similarly, in humans, we demonstrate that C3 deposition is significantly elevated in the placentas of preeclamptic patients compared with normotensive controls. Lastly, we show that complement C3a receptor activation is a key mechanism underlying autoantibody-induced soluble fms-like tyrosine kinase 1 secretion and decreased angiogenesis in cultured human villous explants. Overall, we provide mouse and human evidence that angiotensin II type 1 receptor agonistic autoantibody–mediated activation contributes to elevated C3 and that complement C3a receptor signaling is a key mechanism underlying the pathogenesis of the disease. These studies are the first to link angiotensin II type 1 receptor agonistic autoantibody with complement activation and to provide important new opportunities for therapeutic intervention in PE. (Hypertension. 2012;60:712-721.) ● Online Data Supplement

Key Words: hypertension ▪ preeclampsia ▪ autoantibody ▪ complement C3a ▪ C3a receptor ▪ AT$_1$ receptor ▪ placenta

Preeclampsia (PE) is a serious and common complication of pregnancy and is a leading cause of maternal and neonatal morbidity and mortality. It is a multisystem disorder generally appearing after the 20th week of gestation and is characterized by hypertension, proteinuria, inflammation, and vascular defects. Despite intensive research efforts and several large clinical trials, the underlying cause of PE remains a mystery, and treatment options continue to be unsatisfactory. Recently, a maternal autoantibody, the angiotensin II type 1 (AT$_1$) receptor agonistic autoantibody (AT$_1$-AA), has been reported as a potential contributor to the pathogenesis of PE.2–7 Early studies showed that these autoantibodies can activate AT$_1$ receptors (AT$_1$Rs) on a variety of cells and provoke cellular responses relevant to PE.8–13 More recently, we and others demonstrated that AT$_1$-AA contributes to key features of PE, including hypertension, proteinuria, and renal and placental injury, in several animal studies using adoptive transfer and immunization experiments.14–17 Thus, these in vivo studies offered direct evidence of the pathophysiological role of AT$_1$-AAs in PE. Extending animal studies to humans, we and others demonstrated recently that AT$_1$-AAs are highly prevalent in PE, and antibody titers correlate with disease severity.18,19 Thus, both human and mouse studies support a novel view that PE contains an autoimmune component associated with AT$_1$-AA, and excessive AT$_1$R activation by these autoantibodies is detrimental and underlies pathophysiology of PE. The complement system consists of a series of >30 proteins that provide one of the first lines of defense against pathogens.20–22 The activation of complement component C3 is a point of convergence for all 3 of the major complement activation pathways, including classic, alternative, and lectin activated.21 The complement activation pathways result in the
production of C3 convertases that cleave C3 into 2 components, C3a and C3b. C3a is a small molecular weight split product that functions as an anaphylatoxin that provokes a strong inflammatory response by activation of complement C3a receptors (C3aRs) on multiple target cells, including inflammatory cells, endothelial cells, vascular smooth muscle cells, and epithelial cells. Although the initial function of complement activation is protective and beneficial in getting rid of pathogens, a growing body of evidence supports the claims that autoimmune conditions or tissue injury can lead to inappropriate activation of the complement cascade resulting in organ damage. Of note, recent studies show that PE is characterized by a pronounced maternal inflammatory response that is coupled with activation of innate immunity, including the complement cascade. Some hypothesize that the activation of complement propagates a state of chronic tissue injury in PE and other pregnancy complications. For example, complement activation and deposition in the placenta and kidneys are associated with abnormal placentation development, miscarriage, and kidney injury in several animal models, whereas inhibiting complement activation rescues pregnancies and prevents kidney damage. Recent clinical studies show that elevated C3a is an independent predictive factor for adverse pregnancy outcomes, suggesting that complement-related inflammatory events in pregnancy contribute to the subsequent development of poor outcomes at later stages of pregnancy. However, why this heightened complement activation occurs in PE is unknown, and the exact contribution of complement to the disease features remains undefined.

Because excessive complement activation is associated with autoimmune diseases and tissue injuries and PE is associated with the presence of pathogenic autoantibodies that activate the AT1R, it is possible that AT1-AA is a causative factor responsible for the increased complement activation in PE. Here, we used both human and mouse studies to explore whether AT1-AA promotes elevated complement activation in PE. Moreover, we investigated the contributory role of the increased complement activation in the pathogenesis of PE and identified potential therapeutic strategies.

Materials and Methods
An expanded Methods section is available in the online-only Data Supplement.

Patients
Patients admitted to Memorial Hermann Hospital were identified by the obstetrics faculty of the University of Texas-Houston Medical School. Preeclamptic patients (n=16) were diagnosed with severe disease on the basis of the definition set by the National High Blood Pressure Education Program Working Group Report. The criteria of inclusion, including no previous history of hypertension, are reported previously. Control pregnant women were selected on the basis of having an uncomplicated, normotensive (NT) pregnancy with a normal term delivery (n=20). The research protocol was approved by the Institutional Committee for the Protection of Human Subjects. The detailed information of human subjects is summarized in the Table.

<table>
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<th>Variable</th>
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<tr>
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<td>32.57±5.6</td>
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<tr>
<td>Systolic BP, mm Hg</td>
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<td>171±6*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>75±6</td>
<td>96±12*</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>&lt;300</td>
<td>1769±438*</td>
</tr>
</tbody>
</table>

This table illustrates that the blood pressure and proteinuria are elevated in severe preeclamptic (PE) women vs normotensive (NT) pregnant women. The category mean or median is indicated (±SEM, where applicable). BMI indicates body mass index; BP, blood pressure.

*P<0.001 vs NT pregnant women.

Statistical Analysis
Results are expressed as mean±SEM. All of the data were subjected to statistical analysis using 1-way ANOVA followed by the Newman-Keuls post hoc test or Student t test to determine the significance between groups. Statistical programs were run by GraphPad Prism 5, statistical software (GraphPad, San Diego, CA). Statistical significance was set at P<0.05.

Results
PE-IgG Induces C3 Deposition in the Placentas and Kidneys of Pregnant Mice via AT1R Activation
C3 is reported to be elevated in the circulation of preeclamptic women. However, the causative factors for its elevation remain unknown. Our preeclamptic animal model with adoptive transfer of autoantibody is a valuable investigative tool to determine whether AT1-AA contributes to elevated C3. Specifically, we injected IgG from NT pregnant women (NT-IgG) or IgG from women with PE (PE-IgG) into pregnant mice on gestation days 13 and 14. Injected mice were euthanized on gestational day 18, and the C3 deposition in placentas and kidneys was examined. Immunohistochemical analysis revealed that the C3 was predominantly deposited in the labyrinthine zone of the placentas and the glomeruli of the
kidneys in PE-IgG–injected pregnant mice (Figure 1A and 1B). Image quantification analysis indicated that C3 deposition was significantly enhanced in both placentas and kidneys of PE-IgG–injected pregnant mice as compared with tissues derived from NT-IgG–injected pregnant mice (Figure 1C and 1D). Coinjection with losartan (n=9) or the 7-amino acid (aa) epitope peptide (n=9) resulted in substantial reduction of C3 staining. Image quantification shows that C3 staining in both placentas (C) and kidneys (D) is significantly elevated in PE-IgG–injected pregnant mice vs normotensive (NT)-IgG–injected pregnant mice (n=4). *P<0.05 vs NT-IgG injected mice; **P<0.05 vs PE-IgG–injected mice.

Blocking C3aR Activation Attenuates Autoantibody-Induced Hypertension and Proteinuria in Pregnant Mice

C3aRs are expressed on multiple cell types and are known to play an important role in C3a-mediated tissue injury.21,23 To determine whether C3aRs are expressed in mouse placenta, we examined C3aR expression by immunohistochemistry. We found that C3aRs were highly expressed in trophoblast cells of mouse placenta (Figure S1, available in the online-only Data Supplement). To determine whether elevated C3 activation signaling via C3aR contributes to the pathophysiology of PE, we treated PE-IgG–injected pregnant mice with a C3aR antagonist, SB290157. We found that systolic blood pressure increased significantly in mice injected with IgG isolated from women with PE (PE-IgG, 167±110 mm Hg) relative to that of mice injected with IgG from NT pregnant women (NT-IgG, 126±10 mm Hg; *P<0.05; Figure 2A). In addition, we found that the increased systolic blood pressure seen in PE-IgG–injected pregnant mice was significantly attenuated by infusion of SB290157 (143±5 mm Hg; **P<0.05; Figure 2A). DMSO, the solvent for SB290157, had no effect on PE-IgG–induced hypertension. These findings demonstrate that autoantibody-induced hypertension was significantly inhibited by the infusion of a C3aR antagonist.

Next, we examined injected mice for proteinuria, another key diagnostic feature of PE. The ratio of urinary albumin:creatinine was significantly increased in pregnant mice injected with PE-IgG (223.5±7.5 μg/mg) compared with mice injected with NT-IgG (58.3±5.9 μg/mg; *P<0.05; Figure 2B). Infusion of the C3aR antagonist significantly decreased urinary protein
Autoantibody-Induced Placental Damage and Fetal Intrauterine Growth Restriction Are Prevented by C3aR Antagonist Treatment

Impaired placental development is often associated with PE and has been observed in autoantibody-injected pregnant mice. 37,39 Placentas were harvested for histological analysis on gestation day 18 from PE-IgG- and NT-IgG-injected pregnant mice. We found that placentas of PE-IgG-injected pregnant mice displayed placental calcifications, a hallmark of placental distress, and centers of fibrinoid necrosis similar to that of acute atherosis, a feature observed in human placentas from women with PE. 27 These histological changes, and reductions in placental and fetal weight were inhibited by infusion of a C3aR antagonist, implying an important role for C3aR signaling in autoantibody-induced abnormal placentas and IUGR associated with PE.

C3aR Antagonist Treatment Improves Autoantibody-Mediated Impaired Placental Angiogenesis and Attenuates Soluble Fms-Like Tyrosine Kinase 1 Induction in Pregnant Mice

PE is often associated with small placentas and impaired placental angiogenesis, features also observed in the placentas of autoantibody-injected pregnant mice. 13,14 To determine whether C3aR activation plays an important role in impaired placental angiogenesis, we analyzed the vasculature of isolated mouse placentas by immunostaining with antibody to CD34, an endothelial cell marker. The results show that CD34 staining was decreased in the labyrinth zone of the placentas of mice injected with PE-IgG compared with those injected with NT-IgG (Figure 3E and 3F). Continuous infusion with the C3aR antagonist-attenuated autoantibody-induced reduction in angiogenesis, as evidenced by improved CD34 staining. DMSO had no effect on placental histology.

In an effort to identify the molecular mechanisms responsible for C3a-mediated reduction in placental angiogenesis in PE-IgG-injected pregnant mice, we measured serum levels of soluble fms-like tyrosine kinase 1 (sFlt-1), a well recognized antiangiogenesis molecule that may contribute to impaired placental angiogenesis. 40 Injection of PE-IgG increased the serum levels of sFlt-1 (71.8±6.4 ng/mL) compared with NT-IgG-injected pregnant mice (33.2±5.1 ng/mL; P<0.05). Concomitant infusion with the C3aR antagonist resulted in a
Figure 3. Complement C3a receptor (C3aR) antagonist treatment reduces preeclampsia (PE)-IgG-induced renal and placental damage and intrauterine growth restriction (IUGR) in pregnant mice. A, Placentas assessed by hematoxylin-eosin staining indicate that PE-IgG-injected pregnant mice had damaged placentas: calcifications (thin arrow) and fibrotic areas (thick arrows). The labyrinth zones appear heterogeneous and sometimes have abnormal pools of blood (inset box). Blocking C3aR activation by SB290157 significantly attenuated placental damage (scale bar, 100 μm). B, An arbitrary histological quantification of the number of calcifications obtained per field under ×10 magnification (12 placentas for each group). Placental weight (C) and fetal weight (D) are remarkably reduced in PE-IgG-injected pregnant mice. SB290157 treatment significantly inhibited the reduction in placental and fetal weights in PE-IgG-injected pregnant mice (n=3–5). *P<0.05 vs normotensive (NT)-IgG-injected mice; **P<0.05 vs PE-IgG-injected mice. E, Mouse placental angiogenesis was assessed by CD34 dual immunostaining (scale bar, 100 μm). CD34 staining in the placenta of PE-IgG-injected pregnant mice was significantly decreased. SB290157 treatment significantly improved CD34 staining in the placenta of PE-IgG-injected pregnant mice. F, Quantification of CD34 staining in the mouse placentas. G, sFlt-1 levels in the maternal circulation were significantly elevated in PE-IgG-injected pregnant mice. SB290157 treatment significantly inhibited the induction of serum sFlt-1 level in these mice (n=3–5). *P<0.05 vs NT-IgG-injected mice; **P<0.05 vs PE-IgG-injected mice.
significant reduction in serum levels of sFlt-1 (41.1±1.7 ng/mL; \( P<0.05 \); Figure 3G). These findings demonstrate that C3aR signaling contributes to the induction of sFlt-1 in autoantibody-injected pregnant mice. Overall, our findings provide direct evidence that C3aR activation underlies autoantibody-induced sFlt-1 production and likely leads to impaired placenta angiogenesis.

### C3 Deposition Is Increased in the Placentas of PE Patients

From the mouse work presented here, we have demonstrated that AT1-AAs contribute to the induction of C3 deposition in mouse placentas of autoantibody-injected pregnant mice. To extend mouse studies to humans, we determined whether C3 is also increased in the placentas of PE patients. Specifically, we examined C3 expression in placentas collected from NT pregnant women and PE patients. Consistent with our mouse findings, immunostaining showed that the C3 expression level was relatively low in the placentas of NT pregnant women. However, C3 levels were elevated in placentas of PE patients, and C3 was predominantly deposited in the endothelium of placenta villi (Figure 4A and 4B). Similarly, Western blot analysis further confirmed that C3 levels were increased in the placentas of preeclamptic women compared with NT pregnant women (Figure 4C). Normalization of C3 levels to \( \beta\)-actin indicates that C3 expression in the placentas of PE patients was significantly elevated compared with NT pregnant women (Figure 4D). These studies provide evidence that C3 deposition is significantly elevated in the placentas of preeclamptic women.

### C3aR Activation Contributes to Autoantibody-Induced Impaired Placental Angiogenesis and Increased sFlt-1 Secretion From Human Placental Villous Explants

Because we found that the C3aR is a key player downstream of elevated C3 underlying autoantibody-induced PE features, including impaired placental angiogenesis and increased sFlt-1 secretion in pregnant mice, it is possible that C3aR signaling also contributes to pathophysiology of PE in humans. Similar to mouse placenta (Figure S1), immunohistochemistry demonstrated that C3aR was expressed in the trophoblast cells of human placenta (Figure S2). Next, to elucidate the pathophysiologic consequences of elevated C3-mediated C3aR activation, we took advantage of human placental villous explants to assess the direct role of C3aR in impaired placental angiogenesis and increased sFlt-1 secretion in human placentas. Briefly, placenta explants isolated from NT individuals were incubated with NT-IgG or PE-IgG in the presence or absence of SB290157, a C3aR antagonist. First, CD34 immunostaining and image quantification were used to determine the effects of C3aR signaling on angiogenesis of cultured human villous explants. Similar to our mouse findings, we found that expression levels of CD34 were decreased in placental tissue incubated with PE-IgG compared with NT-IgG and that C3aR antagonism reduced the antiangiogenic effects of PE-IgG treatment (Figure 5A). Quantitative image analysis indicated that PE-IgG treatment significantly reduced CD34 staining, and C3aR antagonism significantly prevented the PE-IgG–induced decrease in CD34 staining (Figure 5B). Thus, this finding provides direct evidence that C3aR plays an important role in autoantibody-induced impaired angiogenesis in the human placenta.

Next, the levels of sFlt-1 in the medium of normal human placental explant cultures were determined by ELISA. Consistent with our mouse findings, human placental villous explants incubated with PE-IgG showed an increase in secreted sFlt-1 relative to explants incubated with NT-IgG (0.28±0.04 versus 0.15±0.01 ng/mL per milligram; \( P<0.05 \); Figure 5C). Coincubation of PE-IgG with SB290157 markedly attenuated the induction of sFlt-1 levels (0.16±0.03, \( P<0.05 \), versus PE-IgG; Figure 5C). These results show that C3aR signaling contributes to autoantibody-induced sFlt-1 secretion from human placenta villous explants. Thus, these findings are consistent with those observed in the mouse
model and suggest that AT1-AA–induced C3aR activation contributes to sFlt-1 induction and results in impaired placental angiogenesis.

Discussion

In this study, we have provided in vivo mouse evidence and in vitro human evidence that AT1-AA is a novel candidate directly inducing C3 deposition in placentas or kidneys via AT1R activation. Blocking C3aR activation significantly ameliorates key features associated with PE seen in autoantibody-injected pregnant mice and impaired placental angiogenesis in cultured human villous explants. Overall, these studies have identified a previously unrecognized role of AT1-AA–induced C3 elevation coupled with C3aR signaling in PE and demonstrated the importance of this complement cascade in the pathogenesis of the disorder (Figure 5D). These findings suggest a novel therapeutic option for the complicated management of this serious condition.

Although complement, in particular C3a, is reportedly increased in the circulation of preeclamptic women, the exact cause of increased C3a is unknown, and its pathogenic role remains unclear. Several animal studies implicated that increased C3 expression is associated with hypertensive and renal disorders. For example, Pratt et al showed that complement component C3 is elevated in animal models of acute renal transplant rejection. Subsequent studies by Lin et al showed that complement protein C3 is elevated in the vascular smooth cells from the spontaneously hypertensive rats. There are few animal models of PE available, and none of them have delineated the cause of increased C3. Here using
a novel autoantibody-induced model of PE in pregnant mice, we demonstrate that autoantibody-mediated AT1R activation induces C3 deposition in multiple tissues, including placentas and kidneys. Because IgG purified from NT pregnant women did not elicit the same increase, the effect can be attributed to the autoantibody itself and not a nonspecific immunologic response. Furthermore, the effects are blocked by coinjection with losartan or a 7aa epitope peptide, thereby providing additional evidence for specificity. Our findings that maternal circulating autoantibody activating AT1R contributes to elevation of C3 is strongly supported by elegant studies reported by Shagdarsuren et al. For their experiments, they used transgenic rats harboring human renin and angiotensinogen genes that are characterized by a progressive increase in blood pressure and proteinuria that results from elevated levels of angiotensin II. They provided evidence showing angiotensin II–mediated AT1R activation leads to increased complement activation in these rats. However, angiotensin II level does not increase in PE compared with normal pregnancy. Thus, our studies provide novel and compelling in vivo evidence that maternal circulating AT1-AA is a detrimental factor causing elevation of C3 by excessively activating AT1R in PE.

Multiple lines of evidence demonstrate that C3a is a critical complement component contributing to tissue injury by activating C3aRs on target cells. Thus, blocking C3aRs effectively attenuates inflammatory response and tissue damage in several animal models, including experimental lupus, arthritis, myocardial ischemia and reperfusion injury, renal tubulointerstitial damage, and brain injury after intracerebral hemorrhage. Here we provide strong evidence that interfering with C3aR activation by its specific antagonist significantly attenuated almost all of the features of PE seen in the autoantibody-induced preeclamptic model, including hypertension, proteinuria, and small placenta featured with impaired angiogenesis and IUGR. Thus, we have revealed that AT1-AA is a key mediator responsible for inducing increased C3 levels in PE and that blockade of C3aR activation can attenuate disease features. Similarly, others have nicely shown that elevated complement activation, in particular, the C3–C5 complement cascade, plays a causative role in pregnancy loss and IUGR in pregnant mice infused with human antiphospholipid antibodies. More recent studies from Qing et al. point out that C3 activation resulted in oxidative stress and placental dysfunction, as well as proteinuria and renal pathological features in a mouse model of spontaneous miscarriage. In contrast, recent studies demonstrated that genetically deficient mice lacking C1q display preeclamptic-like features attributed to abnormal placental development characterized with shallow trophoblast invasion. These latter results suggest a role for complement component C1q in trophoblast invasion and normal placental development. Nevertheless, our current studies and others demonstrate that excessive activation of downstream signaling cascades of the complement pathways is detrimental and contributes to adverse pregnancy outcome, including PE and pregnancy loss.

In an effort to determine what are the key downstream factors underlying C3aR activation and contributing to autoantibody-induced PE, we used our adoptive transfer PE animal model as an available experimental tool. We demonstrate that sFlt-1, an antagonist of vascular endothelial growth factor and placental growth factor, is elevated in PE-IgG–injected pregnant mice and that C3aR antagonist treatment inhibits PE-IgG–induced sFlt-1 elevation in vivo. As such, we demonstrate that C3aR treatment also completely prevents PE-IgG–induced small placentas featured with impaired angiogenesis and prevents IUGR in autoantibody-injected pregnant mice. Because excess circulating sFlt-1 contributes to endothelial dysfunction, hypertension, and proteinuria in animal models of PE, and in view of our early studies showing that antagonizing elevated sFlt-1 by vascular endothelial growth factor 121 infusion significantly decreases autoantibody-induced hypertension and proteinuria in autoantibody-injected pregnant mice, it is likely that the C3aR-mediated sFlt-1 elevation is a key underlying mechanisms for autoantibody-induced PE features in pregnant mice in vivo. Similar to our antibody-injection model of PE in pregnant mice, we found that C3 deposition is significantly elevated in the human placentas from preeclamptic women and blocking C3aR activation significantly attenuates AT1-AA–induced sFlt-1 secretion and subsequent impaired angiogenesis in cultured human placental villous explants. Thus, our human findings not only strongly support our mouse finding but also demonstrate the direct role of C3aR in autoantibody-induced sFlt-1 induction and impaired placental angiogenesis. Our findings are strongly supported by early studies by Girardi et al., who showed that complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. Of note, inhibition of C3aR activation does not completely reduce BP to normal, suggesting that other downstream factors than C3aR activation-mediated sFlt-1 elevation are likely involved in PE-IgG–induced hypertension. Although our present study highlights the role of C3a/C3aR signaling in AT1-AA–induced PE in pregnant mice, other studies have illustrated a potential pathogenic role for C5a/C5aR signaling in PE by stimulating increased production of sFlt-1. In other studies we have shown that antiangiogenic factors and inflammatory cytokines contribute to AT1-AA–induced pathophysiology of PE. Thus, the complement cascade is one of several pathways mediating autoantibody-induced features of PE in pregnant mice. Moreover, it will be very interesting to compare the AT1-AA–injected PE model with angiotensin II–infused pregnant mice. This approach will be critical to determine whether angiotensin II–infused pregnant mice–mediated PE features can also exactly mimic the actions of autoantibodies.

Taken together we provide both human and mouse studies showing that C3aR-mediated sFlt-1 induction is a key mechanism underlying AT1-AA–induced features of PE in pregnant mice. We believe that, without interference, C3aR activation-induced cell damage and inflammation create a detrimental cycle, facilitating further cell damage and inflammation. However, in the presence of a C3aR antagonist, this damage is decreased, slowing the malicious cycle (Figure 5D).

**Perspectives**

In conclusion, our studies identified AT1-AA as a novel candidate causing increased C3 deposition via AT1R activa-
tion in pregnant mice. Of significant importance, C3aR antagonism reduces key features of the disease in an adoptive transfer mouse model of PE. In humans, we demonstrate that C3 deposition is significantly elevated in placentas of pre-eclamptic patients, and AT1-AA–induced placental damage can be alleviated by preventing C3aR activation in human placental villous explants. Finally, both human and mouse studies indicate that increased C3 coupled with C3aR-mediated sFlt-1 elevation is a key mechanism underlying placental damage and subsequent disease symptom development. Thus, our human studies and preclinical mouse studies could be the foundation leading to future human trials and a possible therapy for PE, a life-threatening disorder of pregnancy for which the current treatment is extremely limited.

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Disclosures
None.

References
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Novelty and Significance

What Is New?
- Our study is the first to link AT1-AA with the complement system in the pathogenesis of preeclampsia.

What Is Relevant?
- We demonstrated that C3a can mediate AT1-AA-induced features of PE in pregnant mice by signaling through the C3aR.
Autoantibody-Mediated Complement C3a Receptor Activation Contributes to the Pathogenesis of Preeclampsia
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Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia

By

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Running Title:
AT1-AA, complement and preeclampsia

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Expanded Methods

Blood Pressure Measurement
The systolic blood pressure was non-invasively measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) \(^{1-3}\). Blood pressure was measured at the same time daily (±1 hour) while the mice were kept warm using a warming pad.

Quantification of Proteinuria
We quantified urinary albumin by ELISA (Exocell, Philadelphia, PA) and measured urinary creatinine by a picric acid colorimetric assay kit (Exocell, Philadelphia, PA). We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein.\(^{1-5}\)

Histological Analysis
We harvested placentas and kidneys of mice and fixed them in 4% formaldehyde overnight at 23 °C. Tissues were infiltrated and embedded in paraffin. We cut 4-μm serial sections and stained them with H&E by standard techniques.\(^{6-7}\) Placental histological quantification was carried by quantifying the number of calcifications/field under \(\times 10\) magnification. Placental sections were examined under the microscope and the number of calcifications was counted in each field and then plotted as number of calcifications recorded per field.

C3 and C3aR Immunostaining in Mouse Placentas and Kidneys and Human Placentas
Mouse placentas and kidneys were harvested on GD18 and human placenta explants were collected after 24 hours incubation. Human placenta were isolated immediately after delivery. Tissues were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4μm were cut and stained with anti-mouse C3 (cat. no. LS-C22283, LifeSpan Biosciences, WA), anti-human C3 (cat. no. sc-20137, Santa Cruz, CA), and anti-human & mouse C3aR (cat. no. sc-20138, Santa Cruz, CA) at a dilution of 1:100 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, anti-mouse IgG HRP detection kit (cat. no.551011, BD Pharmingen, San Diego, CA) or anti-rabbit ABC Staining System (cat. no. sc-2018, Santa Cruz) were used to detect the C3 staining. The immunohistochemical staining for C3 (brown) was quantified by Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The intensity of the brown staining (positive for C3) was measured, and the average densities of 6–10 areas per placenta was determined and averaged to get a mean value.

CD34 Dual Immunostaining in Placenta and Quantification
Mouse Placentas were harvested on GD18 and human placenta explants were collected after 24 hours incubation. Tissues were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4μm were cut and stained with anti-mouse CD34 (cat. no. 553731, BD Pharmingen) or anti-human CD34 (cat. no. 555820, BD Pharmingen) at a dilution of 1:100 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, rat IgG ABC Kit (cat. no. AK-5004, Vector Laboratories, Burlingame, CA) or mouse IgG ABC Kit (cat. no.551011, BD Pharmingen) were used to incubate the slides at room temperate for 30
minutes. Then Alkaline Phosphatase Substrate Kit (cat. no. SK-5100, Vector Laboratories, Burlingame) was used to detect the C3 staining. The fluorescence red staining for CD34 was quantified by Image-Pro Plus software (Media Cybernetics) as before.2-4,8 The density of the red staining (positive for C3) was measured, and the average densities of 6–10 areas per placenta was determined to get a mean value.

**Human placental villous explant collection and culture**

Human placentas were obtained from normotensive patients who underwent an elective term cesarean section at Memorial Hermann Hospital in Houston. The explant culture system was conducted as before.2,6 On delivery, the placentas were placed on ice and submerged in phenol red-free DMEM containing 0.2% BSA and 1.0% antibiotics. 5 to 7 chorionic villous explant fragments were carefully dissected from the placenta and transferred to 24-well plates for an overnight equilibration period at 37°C and 5% CO2. All of the initial processing occurred within 30 minutes of delivery. The next day, the explants were incubated with IgG from normotensive pregnant women (NT-IgG; 1:10 dilution), IgG from PE patients (PE-IgG; 1:10 dilution) ± SB290157 (C3aR antagonist, 1μM, cat. no. #559410, Calbiochem) or SB290157 (1μM) alone. The dosage of SB290157 was adapted from experiments described previously.9 After 24 hours, the collection medium was siphoned and stored at -80°C, and the villous explants were lysed or fixed overnight in 10% formalin for embedding in paraffin wax for further analysis.

**Western blot analysis**

C3 protein in human and mice placentas was detected by western blotting analysis. Frozen tissues were used for protein extraction. We ran proteins on 8% SDS-PAGE gels and transferred the gels to a nitrocellulose membrane. We then incubated membranes with commercially available antibody to the C3 (1:200, cat. no. sc-20137, Santa Cruz) as the primary antibody.

**Enzyme-linked immunosorbent assays for sFlt-1**

The concentrations of sFlt1 in mouse plasma and human placenta explants supernatant were determined quantitatively using commercial kits (R&D Systems).1-3
References:


Supplementary Data and Figure Legends

Figure S1. C3aR protein in mouse placenta. C3aR was examined by immunohistochemistry (scale bar=200 μm). Placenta tissue from NT-IgG injected pregnant mice was incubated with C3aR antibody (right) or blocking serum (left, negative control). The brown staining indicates C3aR signal. C3aR protein was evident in trophoblast cells of mouse placenta.

Figure S2. C3aR protein in human placenta. C3aR was examined by immunohistochemistry (scale bar=100 μm). Placenta tissue from normotensive pregnant women was incubated with C3aR antibody (right) or blocking serum (left, negative control). The brown staining indicates C3aR signal. C3aR protein was evident in trophoblast cells of human placenta.