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Prevention of Defective Placentation and Pregnancy Loss by Blocking Innate Immune Pathways in a Syngeneic Model of Placental Insufficiency

Shari E. Gelber,* Elyssa Brent,* Patricia Redecha,† Giorgio Perino,‡ Stephen Tomlinson,§‖ and Jane E. Salmon‡

Defective placentation and subsequent placental insufficiency lead to maternal and fetal adverse pregnancy outcome, but their pathologic mechanisms are unclear, and treatment remains elusive. The mildly hypertensive BPH/5 mouse recapitulates many features of human adverse pregnancy outcome, with pregnancies characterized by fetal loss, growth restriction, abnormal placentation, and defects in maternal decidual arteries. Using this model, we show that recruitment of neutrophils triggered by complement activation at the maternal/fetal interface leads to elevation in local TNF-α levels, reduction of the essential angiogenic factor vascular endothelial growth factor, and, ultimately, abnormal placentation and fetal death. Blockade of complement with inhibitors specifically targeted to sites of complement activation, depletion of neutrophils, or blockade of TNF-α improves spiral artery remodeling and rescues pregnancies. These data underscore the importance of innate immune system activation in the pathogenesis of placental insufficiency and identify novel methods for treatment of pregnancy loss mediated by abnormal placentation.


Abnormal placentation is a leading cause of adverse pregnancy outcomes (APO), including fetal loss, intrauterine growth restriction, and preeclampsia (1, 2). These disorders are characterized by shallow invasion of trophoblasts into the maternal decidua, inadequate spiral artery remodeling, underperfusion of the intervillous space, and placental hypoxia (1). The effects of placental hyperperfusion on the fetus are growth restriction, and in some cases death. For the mother, antiangiogenic factors released by the ischemic placenta lead to endothelial dysfunction and the clinical manifestations of preeclampsia, including hypertension and proteinuria, later in pregnancy.

Inflammation and innate immune system activation have been associated with abnormal placentation in both humans and rodents (3–8). In experimental models of pathogenic pregnancies, altered placentation is attributed to abnormalities in immune responses to the semiallogeneic fetal-placental unit and to exogenous immunologic triggers that initiate inflammation, some Ab-dependent (9–11) and some Ab-independent (5, 8, 12). Both uterine NK cells and regulatory T cells have been shown to be critical for normal placentation and maintenance of normal pregnancies, and their dysregulation, in genetically altered mice, is associated with abnormal placentation and fetal loss (13–16).

Complement activation is a common pathway of injury in many models of APO. The complement system is an integral component of innate immunity, a crucial element of host defense against invading organisms, and a trigger, as well as respondent, to “danger,” such as tissue inflammation, necrosis injury, and ischemia (17–19). Both animal and human studies support the concept that complement activation is associated with APO (5, 10, 20–22). Complement components are produced by human first trimester trophoblasts, and their expression can be upregulated by inflammatory cytokines (23). Inability to regulate activation of complement has been implicated in fetal loss in animal models of disease (24). Complement activation products generated at sites of inflammation, such as placenta, include anaphylatoxins that recruit and stimulate neutrophils (25), which infiltrate the placental tissue and release cytokines and proteases that enhance complement activity and lead to a feed-forward loop of innate immune system activation (26). Neutrophils have been shown to contribute to fetal loss in mouse models (27, 28) and to endothelial damage in preeclampsia (29).

TNF-α produced by the placenta and decidua modulates trophoblast proliferation and invasion, recruits inflammatory cells, including neutrophils, and stimulates those cells to produce more TNF-α (30–32). In rat models of inflammatory fetal loss and growth restriction, blockade of TNF-α activity prevents APOs (7, 33, 34). Elevated levels of TNF-α are present at the fetal/maternal interface in patients with growth-restricted fetuses (35, 36), as well as in maternal blood and amniotic fluid in preeclampsia (37).

To assess the role of inflammation and define specific pathways of damage in a spontaneous mouse model of APO, we studied the BPH/5 mouse, a mildly hypertensive mouse with pregnancies characterized by fetal losses and growth restriction in association with...

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Address correspondence and reprint requests to Dr. Jane E. Salmon, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10065. E-mail address: SalmonJ@hss.edu

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Abbreviations used in this article: APO, adverse pregnancy outcome; C57, C57BL/6; CR2, complement receptor 2; DAB, diaminobenzidine; E, embryonic day; FH, factor H; SMA, smooth muscle actin; VEGF, vascular endothelial growth factor.

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abnormal placentation and defects in maternal decidua arteries (38, 39). Previous studies demonstrating that inflammation contributes to APO have used pregnant mice treated with pathogenic Abs (anti-phospholipid Abs or anti–angiostatin receptor Abs) (9–11), LPS (12), or nonsynonygetic matings (CBA/J DBA/2) (5) to induce APO. The spontaneous development of placental insufficiency in BPH/5 mice allows for study of early mediators of fetal loss that occur at implantation and in early gestation. Notably, vascular disease, specifically chronic hypertension, is a risk factor for APO in humans, and this phenomenon is recapitulated in the BPH/5 mouse, a syngeneic model of APO secondary to placental dysfunction (38, 39). We sought to determine whether pregnancy complications in the BPH/5 mouse are related to innate immune system activation and, specifically, whether blockade of complement activation, neutrophil infiltration, and TNF-α activity could prevent abnormal placentation, fetal loss, and growth restriction.

Materials and Methods

BPH/5 mice pregnancy model

Animal experiments were approved by the Hospital for Special Surgery Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. As described in detail previously, BPH/5 is an inbred subtype derived from the BPH/2, and C57BL/6J (C57) mice serve as controls (38–40). C57BL/6J mice (8–12 wk old) were obtained from in-house colonies. Mice were housed in a barrier animal facility with a climate controlled environment and 10 h light/14 h dark cycle. Timed matings were performed by pairing strain-matched virgin females and males. Mice were examined daily for the presence of a vaginal plug, and the day of plug detection was designated embryonic day (E) 0.5. Except where indicated, mice were sacrificed at E12.5, uteri were dissected, fetuses and placentas were weighed, and fetal resorption rates were calculated (number of resorptions/total number of formed fetuses and resorptions).

In experiments involving neutrophil depletion, mice were treated with rat anti-mouse granulocyte RB6-8C5 mAb (anti-GR1; 100 μg i.p.; BD Pharmingen) or 1A8 (anti-Ly6G; 250 μg i.p.; Bio X Cell) on E2.5. An IgG2a or IgG2a Ab, respectively, was the isotype control. Depletion of neutrophils, confirmed by flow cytometry and peripheral blood smears, occurred by 48 h after injection and persisted through day 10. For complement blockade, recombinant complement receptor 2 (CR2–Cry) (0.200 mg) or CR2–FH (0.250 mg), prepared as previously described (41, 42), was administered i.v. on E5.5. To inhibit TNF-α, etanercept (10 mg/kg; Amgen) was administered s.c. at E4.5.

Immunohistochemistry and morphometric analysis of placentas

Mice were sacrificed with CO2 asphyxiation at E6.5, 8.5, or 12.5. The uterus was cut between implantation sites, briefly rinsed in cold PBS, fixed in 10% neutral buffered formalin, processed by standard cycle, and embedded in paraffin. Sagittal 5-μm serial sections were obtained through the uterus, embryo, and placenta, and midsagittal sections were used for analysis. Midsagittal sections were identified in early gestations (E6.5 and E8.5) by identification of the umbilical cord insertion site in the embryo, and at implantation and in early gestation. Notably, vascular disease, specifically chronic hypertension, is a risk factor for APO in humans, and this phenomenon is recapitulated in the BPH/5 mouse, a syngeneic model of APO secondary to placental dysfunction (38, 39). We sought to determine whether pregnancy complications in the BPH/5 mouse are related to innate immune system activation and, specifically, whether blockade of complement activation, neutrophil infiltration, and TNF-α activity could prevent abnormal placentation, fetal loss, and growth restriction.

Results

Neutrophils infiltrate the placenta in the BPH/5 mouse model of APO

Fetal loss in mice lacking complement regulatory proteins and in mice treated with antiphospholipid Abs show neutrophil infiltration in the placenta, and abnormal neutrophil activation is seen in the
Peripheral blood in patients with preeclampsia (46, 47). To test the hypothesis that infiltrating neutrophils early in pregnancy contribute to placental insufficiency in BPH/5 mice, we compared the number of neutrophils present in the developing BPH/5 placenta with that in C57 mice. Because neutrophils are transiently present in the decidua at the time of implantation on E4–E5 (48), we assessed the extent of neutrophil infiltration in BPH/5 mice beginning at E6.5. Immunohistochemical studies did not demonstrate neutrophils in the ectoplacental cone, myometrium, or decidua of either BPH/5 or C57 mice at E6.5. In contrast, at E8.5 neutrophils were present in the ectoplacental cone of both strains, and they were markedly increased in the BPH/5 mice (Fig. 1A, 1B). There was no difference in the number of uNK cells or macrophages in the decidua of C57 and BPH/5 mice at E8.5 (uNK, 750 ± 110 versus 640 ± 230 per midsagittal section, n = 6; macrophages, 52 ± 5 versus 51 ± 6 per midsagittal section, n = 6, respectively).

We confirmed the immunohistochemical findings of excess neutrophil infiltration with flow cytometry of leukocytes isolated from placentas. Both absolute number of CD45+CD11b+GR1hi and percentage of CD45+ that were CD11b+GR1hi were increased in BPH/5 mice (C57, 32.3 ± 2.4% versus BPH/5, 50.4 ± 5.8%, p < 0.05) (Fig. 1C, 1D). The difference between C57 and BPH/5 mice was due to infiltrating neutrophils and not to peripheral blood neutrophils, as the number and percentage of CD45+CD11b+GR1hi cells in the blood did not differ between strains (Fig. 1C, 1D) (C57, 14.6 ± 0.9% versus BPH/5, 16.2 ± 3.4%, p = NS).

We assessed cytokines involved in neutrophil recruitment in placental lysates at E8.5 by Luminex multiplex assay. CXCL1 was higher in the BPH/5 lysates compared with C57 (Fig. 1E). Levels of IL-17, IL1α, CCL3, CXCL2, or CXCL5 were not different between the two strains.

Neutrophils are required for abnormal placental and fetal development in BPH/5 mice

Neutrophils are effectors of fetal damage, and depletion of neutrophils has been shown to prevent pregnancy complications in Ab-mediated models of pregnancy loss (27). We sought to determine whether fetal loss and growth restriction in BPH/5 mice could be prevented by depletion of neutrophils. We treated mice at E2.5 with anti-GR1 to deplete neutrophils and assessed pregnancy outcome on E12.5. This time period was selected to ensure the

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Neutrophil infiltrate in placenta of BPH/5 mice. Representative images of the ectoplacental cone at E8.5 stained with an anti-GR1 Ab from C57 (A) and BPH/5 mice (B). Scale bars, 20 μm. (C) Infiltrating neutrophils from implantation sites or peripheral blood of C57 and BPH/5 mice at E8.5 were identified by flow cytometry as CD45+CD11b+GR1hi. (D) Mean neutrophil number from implantation sites or peripheral blood of C57 and BPH/5 mice is shown (n = 6/group). (E) Placental homogenates (n = 4 for each group) were collected on E8.5 and assayed for the neutrophil chemoattractant CXCL1 by Luminex technology and normalized to protein concentration by Bradford assay. *p < 0.05, **p < 0.001.
absence of neutrophils prior to the time that we observed deposition of complement in BPH/5 placentas (see below). In BPH/5 mice treated with anti-GR1, there was a dramatic decrease in fetal loss and growth restriction; pregnancy outcomes in anti-GR1-treated mice were similar to those in C57 mice (Fig. 2). Of note, treatment with anti-GR1 did not affect implantation number in BPH/5 or C57 mice (Supplemental Fig. 1) and did not alter pregnancy outcomes in C57 mice (Supplemental Table 1). Because anti-GR1 depletes not only neutrophils, but subpopulations of other myeloid cells (49), we performed experiments to assess the effects of a different neutrophil-specific mAb anti-Ly6G (1A8) on APOs in BPH/5 mice. Treatment with anti-Ly6G rescued pregnancies in BPH/5 mice (Fig. 2), confirming that neutrophils play a crucial role in APO in this mouse model.

Decreased placental weights, altered invasion of the placental disc into the decidua, and defective spiral artery remodeling, as demonstrated by arteries with thick walls and retention of SMA positivity, are characteristics of BPH/5 placentas (39). It has been suggested that abnormal placentaion defined by these anatomical features in BPH/5 mice leads to poor pregnancy outcomes. Consistent with the observed improved fetal outcomes, depletion of neutrophils in pregnant BPH/5 mice was associated with increased placental weight (Fig. 2C) and normalized placental invasion and spiral artery remodeling (Fig. 3). Both the proportional depth of the placental disc (Fig. 3A, 3B, 3H) and relative area of the junctional zone (Fig. 3C, 3D, 3I) were increased. The increased relative width of the decidual spiral arteries and absence of staining for SMA in the neutrophil-depleted mice are consistent with normal spiral artery remodeling (Fig. 3). Taken together, our findings demonstrate that neutrophils play a pivotal role in abnormal placental development and subsequent fetal loss in BPH/5 mice.

Consistent with previous findings, there is no difference in the number of uNK cells in the placentas of BPH/5 mice compared with C57 at E12.5 (39) (1000 ± 110 versus 990 ± 120 per mid-sagittal section, respectively; n = 4, p = NS). Similarly, we found no difference in macrophage number in the placentas at E12.5 (14 ± 4 versus 24 ± 21 per mid-sagittal section, respectively; n = 4, p = NS). Furthermore, treatment with anti-GR1 did not alter the number of uNK cells (BPH/5, 1000 ± 110 versus BPH/5 plus anti-GR1, 970 ± 64 per mid-sagittal section; n = 4, p = NS) or macrophages (BPH/5, 14 ± 41 versus BPH/5 plus anti-GR1, 6 ± 1 per mid-sagittal section; n = 4, p = NS) in the placenta at E12.5.

Neutrophil infiltration is associated with reduced VEGF levels in vivo and in vitro

Placental insufficiency in BPH/5 mice is characterized by angiogenic imbalance in the dams. Circulating levels of VEGF, an angiogenic factor required for normal placental development, are decreased in the BPH/5 mice compared with C57 mice (38). To determine whether angiogenic imbalance occurs in the absence of neutrophils, we measured peripheral and placental levels of VEGF in BPH/5 mice treated with anti-GR1 at E2.5, as described above. Depletion of neutrophils resulted in higher levels of peripheral VEGF, as well as VEGF in the placenta (Fig. 4A, 4B). That restoration of homeostatic levels of placental VEGF (C57, 34 ± 4 pg/mg protein; BPH/5, 18 ± 4 pg/mg protein; BPH/5 plus anti-GR1, 33 ± 4 pg/mg protein) is associated with normal placental and pregnancy outcomes is consistent with the finding that adenoviral VEGF improves placental function in the BPH/5 (50).

To determine whether neutrophils can directly affect levels of VEGF produced by trophoblasts, we performed in vitro studies with HTR8 trophoblasts cultured in the presence and absence of human neutrophils and measured VEGF in supernatants. Incubation with neutrophils decreased release of VEGF (Fig. 4C). These data support our in vivo findings showing that neutrophil depletion increased placental VEGF, and they suggest direct effects on the availability of VEGF.

Complement deposition precedes pathogenic neutrophil infiltration in vivo

Excessive complement activation is associated with APO in animal models and humans (9, 24, 51, 52), and products of the complement cascade recruit and stimulate neutrophils, which, in turn, amplify activation of complement. To test the hypothesis that complement activation precedes neutrophil infiltration, we examined the kinetics of complement deposition in the BPH/5 mouse. C3 deposition was initially observed in the ectoplacental cone at E6.5 in the BPH/5 mouse; at this gestational age there is no C3 seen in the C57 mouse (Fig. 5A, 5B). By E8.5, there was extensive complement deposition in the ectoplacental cone of BPH/5 with minimal staining of C57 (Fig. 5C, 5D). Notably, neutrophil infiltrates were first evident at E8.5 (Fig. 1) and not before. Thus, complement activation precedes infiltration of neutrophils. To exclude the possibility that systemic complement activation occurs in BPH/5 pregnancies, similar to that noted in humans with preeclampsia, we measured hemolytic complement activity and found no differences in levels between BPH/5 and C57 mice (Supplemental Fig. 2). These data argue that complement is activated locally in BPH/5 mice.

Inhibition of complement activation prevents fetal loss and growth restriction in BPH/5 mice

To determine whether blockade of complement activation can prevent abnormal placentalation and APOs, we treated BPH/5 mice with targeted complement inhibitors, that is, CR2-Cry or CR2-FH. These agents are fusion proteins of either Cry, a pan-C3 convertase inhibitor, or factor H (FH), a regulator of the alternative complement pathway, with CR2, which binds C3 degradation products and thus localizes the protein to sites of complement deposition. CR2-Cry inhibits the classical, lectin, and alternative complement pathways at the C3 activation step, whereas CR2-FH is specific for the alternative pathway (41, 42). At the doses used, CR2-Cry and CR2-FH have minimal effects on systemic complement activity. They target cell-bound products of complement activation, accu-
mulate in tissues at sites of complement activation, and remain there for prolonged periods (53). Blockade of complement activation with either targeted inhibitor prevented fetal loss (Fig. 6A) and growth restriction (Fig. 6B). These results were comparable to those seen with neutrophil depletion.

Because abnormal placentation is a cause of fetal loss and growth restriction, we examined the effect of complement inhibition on placental phenotype. Local complement inhibition was associated with increased weight of the placenta in the CR2-Crry–treated animals but not in those treated with CR2-FH (Fig. 6C). We performed histologic analyses of placentas from CR2-Crry–treated animals because this agent was most effective in preserving placental weight. Complement inhibition with CR2-Crry normalized the junctional zone without affecting the ratio of placenta to decidua (Fig. 6D, 6E) and normalized placental spiral artery morphology to that of low-resistance vessels: arterial walls were thinner and fewer were positive for SMA (Fig. 6F, 6G). Targeted inhibitors of complement improved placental architecture in BPH/5 mice to the same extent as depletion of neutrophils.

**Complement inhibition decreases neutrophil recruitment and angiogenic imbalance in vivo**

Given our immunohistochemical evidence that complement activation precedes infiltration of neutrophils into the placenta, we hypothesized that blockade of complement activation would prevent recruitment of neutrophils. We treated BPH/5 mice with the targeted complement inhibitor CR2-Crry at E5.5 and quantified placental neutrophil infiltration by flow cytometry at E8.5, the time point when it was prominent in BPH/5 (Fig. 1C). When complement activation was blocked, neutrophils were not recruited into the placenta; the number of neutrophils in BPH/5 placentas was comparable to that of C57 placentas (Fig. 6H). Importantly, treatment with CR2-Crry did not alter the number of uNK cells (control, 1000 ± 110 versus CR2-Crry, 1000 ± 60 per midsagittal section; n = 4; p = NS) or...

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**FIGURE 3.** Neutrophil depletion improves placental morphology of BPH/5 mice. BPH/5 mice were treated with anti-GR1 Ab to deplete neutrophils or isotype control on E2.5, sacrificed on E12.5, and placental histology was examined. Representative images of intact feto-placental units from isotype control–treated BPH/5 mice (A and C) and anti-GR1–treated BPH/5 mice (B and D) are shown. Neutrophil depletion normalizes the proportional depth of the placental disc (P:P+De) (H) and the fractional area of the junctional zone relative to the placental disc (JZ:JZ+L) (I). Representative images of decidual spiral arteries demonstrating thick walls in the isotype-treated BPH/5 mice (E) and thin arterial walls in the anti-GR1–treated mice (F) (closed arrows indicate outer diameter [OD]; open arrows indicate inner diameter [ID]). (J) Data are summarized as ratios of the inner lumen to outer vessel diameter (ID/OD). Representative images of decidual spiral arteries demonstrating positive staining for SMA in isotype-treated BPH/5 mice (arrowhead) (E and G) and loss of SMA staining in the anti-GR-1–treated mice (F) are shown. (K) Data are summarized as a ratio of remodeled arteries relative to total number of decidual spiral arteries. Data are presented as mean ± SEM. For histologic studies, a minimum of nine feto-placental units were analyzed for each condition (three implantation sites from three separate pregnancies). Scale bars, 500 μm (A and B), 200 μm (C and D), 50 μm (E and F), 25 μm (G). *p < 0.05, **p < 0.01, ***p < 0.001. De, decidua; JZ, junctional zone; L, labyrinth; P, placental disc.
macrophages (control, 14 ± 4 versus CR2-Crry, 10 ± 1 per mid-sagittal section; n = 4; p = NS) in the placenta at E12.5.

Inhibition of complement activation also increased VEGF concentration in the placenta. We observed a nearly 2-fold increase in placental VEGF in BPH/5 mice treated with either CR2-Crry or CR2-FH (Fig. 6I). There was a more modest effect on peripheral blood levels of VEGF (control, 63 ± 3.4 pg/ml versus CR2-Crry, 76 ± 3.1 pg/ml versus CR2-FH, 72 ± 4.2 pg/ml; p = NS). Taken together, these data support a critical role of local complement activation as a proximal mediator in the pathogenesis of APOs in BPH/5.

TNF-α is a mediator of adverse outcomes in pregnant BPH/5 mice

TNF-α has been shown to mediate APO in LPS and anti-phospholipid Ab-treated rodent models (7, 33). Because complement activation products trigger release of TNF-α by neutrophils, and TNF-α stimulates neutrophils in an autocrine and paracrine manner to amplify damage, we performed studies to determine whether TNF-α contributes to inflammation and fetal loss in BPH/5 mice. We measured TNF-α in placental lysates obtained at E8.5, the time when neutrophil infiltration and complement deposition were increased in the BPH/5 compared with C57, and we found markedly higher TNF-α levels in the BPH/5 mice (Fig. 7A). Furthermore, depletion of neutrophils with anti-GR1 decreased TNF-α, implicating neutrophils in the pathway leading to elevations in TNF-α (Fig. 7A). To investigate the source of TNF-α, we cultured HTR8 human trophoblasts with neutrophils from C57, BPH/5, TNF-α−/−, or humans, collected supernatants after 2 h, and assayed for mouse TNF-α (Fig. 7B) and human TNF-α (Fig. 7C). Mouse neutrophils cultured in the presence of HTR8 produced TNF-α (Fig. 7B), and there was no difference in TNF-α production between C57 and BPH/5 mice (Fig. 7B, Supplemental Fig. 3A). Human neutrophils were also stimulated to release TNF-α in response to HTR8 (Fig. 7C). Neither HTR8 alone (Fig. 7C) or stimulated with C5a (Supplemental Fig. 3B) produces TNF-α. Although the triggers for TNF-α production are not clear, these data indicate that neutrophils are a likely source of TNF-α in inflammatory sites such as BPH/5 placentas. We observed no difference in TNF-α production by neutrophils from BPH/5 and C57 mice, suggesting that excess TNF-α in BPH/5 placenta is due to recruitment of greater numbers of neutrophils.

Treatment with etanercept, an available biological therapeutic that blocks TNF-α activity, reduced fetal loss in BPH/5 mice to levels of C57 mice (Fig. 8A), normalized placental weight (Fig. 8C), and restored all studied metrics of placentation: junctional zone ratio (Fig. 8D), placental invasion (Fig. 8E), thinner spiral artery walls (Fig. 8F), and loss of SMA staining (Fig. 8G). There was no significant change in the weight of the surviving fetuses (Fig. 8B).

Similar to treatment with the complement inhibitors, etanercept increased placental VEGF levels (Fig. 8H). Of note, etanercept did not alter numbers of uNK cells (control, 1000 ± 110 versus etanercept, 790 ± 340 per mid-sagittal section; n = 4; p = NS) or macrophages (control, 14 ± 4 versus etanercept, 4 ± 2 per mid-sagittal section; n = 4; p = NS) in the placenta at E12.5.

Discussion

We have shown that complement activation at the maternal/fetal interface leads to recruitment of neutrophils, elevation in local TNF-α levels, reduction of the essential angiogenic factor VEGF, and, ultimately, abnormal placentation. To our knowledge, we provide the first evidence that complement activation and the ensuing infiltration of neutrophils into the placenta lead to abnormal spiral artery remodeling and angiogenic dysregulation. These findings, to our knowledge the first in a syngeneic spontaneous model of abnormal placentental development, support work from our laboratory and others that complement is an essential proximal mediator in Ab-dependent and Ab-independent mouse models of APO (9, 10, 27). In this study, we demonstrate the critical role of local complement activation as an initiator of APO in BPH/5 mice by showing that features of abnormal placental development and its consequences on the fetus are reversed by inhibiting the complement cascade, specifically at the maternal/fetal interface.

Defective placentation is associated with preeclampsia, growth restriction, and other obstetric syndromes (2). The BPH/5 mouse has placental findings, including inadequate spiral artery remodeling, similar to those seen in humans. As in patients with preeclampsia,
not all spiral arteries are inadequately remodeled in BPH/5 (2). Inadequate spiral artery remodeling is thought to contribute to fetal growth restriction and placental ischemia (1, 2). Inhibition of complement, depletion of neutrophils, and blockade of TNF-α improves spiral artery remodeling in BPH/5 pregnancies.

Pregnancy in BPH/5 mice is characterized by a maternal syndrome including hypertension and proteinuria late in gestation. Our studies focused on early changes at the maternal/fetal interface that precede, and perhaps cause, manifestations of preeclampsia. Although we observed complement deposition, neutrophil infiltration, TNF-α elevation, and decreased VEGF in the placenta, there was no evidence of systemic alterations of these mediators in the second trimester, a point before clinically apparent maternal responses to placental insufficiency in BPH/5 mice or humans. That inflammation is restricted to the placenta, before clinically apparent disease, allows for the insidious progression of disorders of placental dysfunction, which, as in humans, variably lead to maternal or fetal abnormalities.

To attenuate complement activation at the maternal/fetal interface, we used CR2-Crry, which blocks all pathways of complement activation (classical, alternative, and lectin), and CR2-FH, which selectively inhibits the alternative pathway (54). The CR2 domain of both compounds binds covalently bound complement activation fragments iC3b, C3dg, and C3d on tissue (55) and thereby localizes the complement regulators, Crry or FH, to the sites of complement activation. Both compounds prevented fetal loss and growth restriction but did so to different extents. The efficacy of CR2-FH underscores the importance of the alternative pathway in this pathway. Such targeted inhibition is especially attractive because it allows for specific inhibition at the site and time of injury without generalized immune suppression of the host (41).

We show that local complement activation triggers neutrophil recruitment, but complement activation in the absence of neutrophils is insufficient to cause APO. Depletion of neutrophils early in pregnancy was able to reduce all studied metrics of APO in BPH/5

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**FIGURE 6.** Complement inhibition rescues BPH/5 pregnancies. BPH/5 mice were treated with CR2-Crry or CR2-FH to inhibit complement or control (PBS) on E5.5. Mice were sacrificed on E12.5 and evaluated for resorption frequency, fetal and placental weight, and placentome histology. The effects of complement inhibition on (A) fetal resorption (minimum of six mice/group), (B) fetal weight (minimum 28 fetuses/group), and (C) placental weight (minimum of 28 fetuses/group) are shown. (D–G) Placental morphology was assessed after treatment with CR2-Crry or control: (D) the fractional area of the junctional zone relative to the placental disc (JZ:JZ+L) and (E) the depth of the placental disc (P+P+De) are shown. Spiral artery remodeling as measured by (F) ratios of the inner lumen to outer vessel diameter (ID/OD) of decidual spiral arteries and (G) the ratio of SMA (remodeled arteries) relative to total number of decidual spiral arteries after treatment with CR2-Crry or control are shown. (H) Pregnant mice were treated with CR2-Crry at E5.5 and infiltrating cells were separated from trophoblasts at E8.5. The numbers of infiltrating GR1+ cells in C57 or BPH/5 with or without CR2-Crry treatment are shown. (I) The effect of complement inhibition on VEGF levels from placental homogenates was determined by ELISA. Data are presented as means ± SEM. For histologic studies a minimum of nine feto-placental units were analyzed for each condition (three implantation sites from three separate pregnancies). *p < 0.05, **p < 0.01, ***p < 0.001.

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**FIGURE 7.** Elevated TNF-α in placentas from BPH/5 mice and supernatants from neutrophils cultured with HTR8 cells. (A) TNF-α levels were assayed from placental homogenates at E8.5 from untreated C57 and BPH/5 mice, and from BPH/5 mice treated at E2.5 with anti-GR1 Ab to deplete neutrophils or isotype control (n = 9 for each condition). (B) Levels of mouse TNF-α were assayed from supernatants of neutrophils from C57, BPH/5, or TNF−/− mice or humans (HUM) cocultured with HTR8 cells. (C) Levels of human TNF-α were assayed from supernatants of neutrophils from C57, BPH/5, or TNF−/− mice or humans cocultured with HTR8 cells. *p < 0.05 versus C57, **p < 0.001 versus isotype treated.
Sem. For histologic studies a minimum of nine fetoplacental units were analyzed for each condition (three implantation sites from three separate pregnancies). *p < 0.05, **p < 0.01.

Evidence for elevated levels of the neutrophil chemoattractant CXCL1 in BPH/5 placenta is consistent with our model that early inflammation leads to apoptosis. CXCL1 recruits neutrophils and is released from neutrophils in response to TNF-α (56). Additionally, CXCL1 is secreted from trophoblast in response to damage (57). Thus, production of CXCL1 by injured trophoblasts or activated neutrophils may initiate a positive feedback loop in which placental injury drives neutrophil infiltration and TNF-α release, which then increases CXCL1 and amplifies inflammation.

Activated neutrophils release mediators of tissue damage, including TNF-α, which has been shown to cause abnormal placental and growth restriction, and to recruit and activate other effectors of inflammation (34, 58, 59). In our studies, mice were treated on day 2.5 with anti-GR1 Ab and neutrophils were depleted by day 5.5 and returned to circulation by day 12.5. Taken together, these data indicate that prevention of the initial inflammatory insult by neutrophils may have long-term benefits on pregnancy outcome and that blockade of inflammation may not be necessary throughout pregnancy.

FIGURE 8. TNF-α blockade prevents APO and normalizes placental development in BPH/5 mice. The effect of TNF-α blockade on (A) fetal resorption (n = 6 mice/group), (B) fetal weight (minimum 28 fetuses/group), and (C) placental weight (minimum 28 fetuses/group) is shown. Placental morphology was assessed after TNF-α blockade or control: (D) the fractional area of the junctional zone relative to the placental disc (JZ:JZ+L) and (E) depth of the placental disc (P+P+De) are shown. Spiral artery remodeling as measured by (F) ratios of the inner lumen to outer vessel diameter (ID/OD) of decidual spiral arteries and (G) the ratio of SMA−remodeled arteries relative to total number of decidual spiral arteries after treatment with etanercept or control is shown. (H) Placental VEGF levels were assayed in the BPH/5 after TNF-α blockade with etanercept. Data are presented as means ± SEM. For histologic studies a minimum of nine feto-placental units were analyzed for each condition (three implantation sites from three separate pregnancies).

The present work focused on mechanisms of placental insufficiency. We examined fetal loss and growth restriction, which are significant APOs. We did not directly examine the contribution of complement, neutrophils, and TNF-α to the maternal features of preeclampsia, because these clinical manifestations occur later in this model. Nonetheless, maternal disease is highly associated with abnormal placental development and it is likely that inhibition of complement or TNF-α also prevents maternal disease. Taken together, our findings provide the rationale for trials with agents that modulate innate pathways early in pregnancy to prevent APOs in those women at high risk.

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Disclosures
The authors have no financial conflicts of interest.

References


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