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

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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 placental development, 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. *The Journal of Immunology*, **2015, 195: 1129–1138.**

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 hypoperfusion 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 pathologic pregnancies, altered placental development 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 placental development 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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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.

abnormal placentation and defects in maternal decidual arteries (38, 39). Previous studies demonstrating that inflammation contributes to APO have used pregnant mice treated with pathogenic Abs (anti-phospholipid Abs or anti–angiotensin receptor Abs) (9–11), LPS (12), or nonsyngeneic matings (CBA/JxDBA/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 subline derived from the BPH/2, and C57BL/6J (C57) mice serve as controls (38–40). BPH/5 and C57 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 IgG2b 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)–Crry (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 CO_2 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 embryo, and at E12.5 by identification of the umbilical cord insertion site in the placenta. Sections were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched with H_2O_2 (Sigma-Aldrich). C3 deposition was identified with a polyclonal goat anti-mouse C3 Ab (ICN Pharmaceuticals) as described previously (43). Neutrophil infiltrate was identified with a monoclonal rat anti-mouse neutrophil Ab (Cedarlane clone 7/4) after sodium citrate Ag retrieval. Biotinylated *Griffonia simplicifolia* isolectin B4 (Vector Laboratories) and smooth muscle actin (SMA) (Abcam) were used to identify basement membrane of fetal endothelial cells in the labyrinth (39) and smooth muscle, respectively. Ab detection was performed with Vectastain ABC peroxidase-conjugated streptavidin reagent (Vector Laboratories) followed by diaminobenzidine (DAB; Dako) detection. Appropriate isotype controls were used to determine Ab specificity. Images were taken with a light microscope equipped with a digital camera. Morphometric analysis was performed as described previously (39). All measurements were made by two investigators who were blinded to the experimental groups. Mean values of the two independently obtained measurements were used.

The immunohistochemical detection of uNK cells with DBA lectin and macrophages with F4/80 was performed using a Discovery XT processor (Ventana Medical Systems). The tissue sections were deparaffinized with EZ Prep buffer (Ventana Medical Systems), Ag retrieval was performed with CC1 buffer (Ventana Medical Systems), and sections were blocked for 30 min with Background Buster solution (Innovex Biosciences) followed by blocking with avidin-biotin blocking reagents (Ventana Medical Systems) for 16 min. To detect uNK cells, biotinylated DBA lectin (Sigma-Aldrich) was applied and sections were incubated for 5 h, followed by detection with a DAB detection kit (Ventana Medical Systems) according to the manufacturer's instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific). To detect macrophages, anti-F4/80 (Abcam) Abs were applied and sections were incubated for 6 h, followed by 60 min incubation with biotinylated goat anti-rat IgG (Vector Laboratories). The detection was performed with a DAB detection kit (Ventana Medical Systems) according to the manufacturer's instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific). The number of DBA lectin+ and F4/80+ cells was determined by counting a midsagittal section at E8.5 or E12.5 from untreated C57 and BPH/5 mice or BPH/5 mice treated with anti-GR1, CR2-Crry, or etanercept.

Flow cytometry to characterize cellular infiltrates

Mice were sacrificed at E8.5, the uterine horn was opened, and individual placentas were removed and vigorously rinsed in three changes of ice-cold PBS to remove maternal blood, then gently dissociated and passed through a 100-μm strainer. Trophoblasts and infiltrating lymphocytes were separated with a 30% Percoll (Sigma-Aldrich) gradient and stained with anti-CD45 (eBioscience), anti-CD11b (eBioscience), and anti-GR1 (eBioscience). Neutrophils were defined as $CD45^+CD11b^+GR1^{hi}$. Neutrophil numbers were determined using counting beads (CountBright absolute counting beads; Invitrogen) according to the manufacturer's instructions. Analysis was performed with FlowJo (Tree Star).

Measurement of angiogenic factor and cytokine levels

Blood was collected in EDTA-containing tubes, spun at 5000 rpm, and plasma was collected and stored at −80°C. Placentas were dissected free from surrounding tissue, rinsed in ice-cold PBS, snap frozen in liquid nitrogen, and thawed on ice. After three freeze/thaw cycles, individual placentas were resuspended in PBS, homogenized, centrifuged at 8000 rpm for 10 min, and supernatants were collected and stored at −80°C. A Bradford assay was performed to normalize for protein levels in individual placentas. Placenta and plasma levels of vascular endothelial growth factor (VEGF) and TNF-α were determined by commercial ELISA (R&D Systems). Other cytokines and chemokines were measured using a Luminex mouse multiplex panel (EMD Millipore)

First trimester human trophoblast cell response to neutrophils

HTR-8/SVneo cells (HTR8), an immortalized human first trimester trophoblast cell line that retains many characteristics of first trimester trophoblasts (44), were provided by Dr. Charles Graham (Queens University, Kingston, ON, Canada). HTR8 cells (5×10^5) were cultured until confluent in RPMI 1640 supplemented with 10 mM HEPES, 0.2% sodium bicarbonate, 10% FBS, 0.1 mM minimal essential medium nonessential amino acids, 1 mM sodium pyruvate, and 100 nm penicillin/streptomycin. Human neutrophils were isolated from freshly drawn venous blood by a two-step discontinuous Ficoll-Hypaque gradient as previously described (45). Mouse neutrophils were isolated from the bone marrow of C57, BPH/5, and $TNF^{-/-}$ mice using the EasySep mouse neutrophil enrichment kit (Stem Cell Technologies) according to the manufacturer's instructions. HTR8 cells were cultured until confluent, and human or murine neutrophils were added at a concentration of 1×10^6 neutrophils/ml. For experiments to determine VEGF concentration, culture supernatants were collected after 24 h and assayed by ELISA (R&D Systems). For experiments to determine TNF-α concentration, supernatants were collected after 2 h and assayed by ELISA for human (Invitrogen) and mouse (eBioscience) TNF-α. After collection of supernatants, cell viability was confirmed by trypan blue exclusion.

Statistical analysis

Data were expressed as means ± SEM and analyzed using Prism version 4 for Macintosh (Graphpad Software). Differences between two groups were analyzed with a two-tailed Student *t* test. Multiple comparisons were performed with an ANOVA followed by the Tukey multiple comparison test. A p value < 0.05 was considered statistically significant.

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 $CD45^+CD11b^+GR1^{hi}$ and percentage of $CD45^+$ that were $CD11b^+GR1^{hi}$ 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 $CD45^+CD11b^+GR1^{hi}$ 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

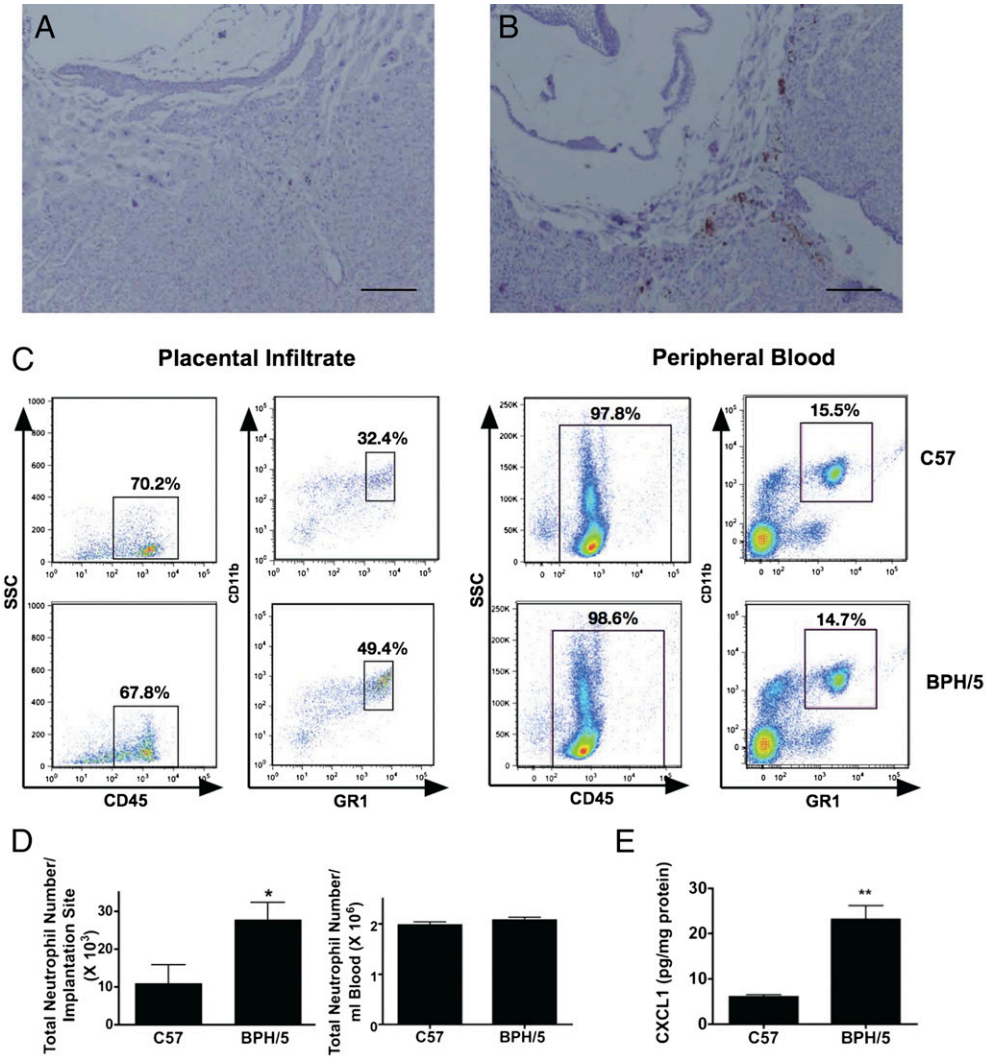

FIGURE 1. Neutrophil infiltrate in placenta of BPH/5 mice. Representative images of the ectoplacental cone at E8.5 stained with an anti-GR1 Ab from C57 (**A**) and BPH/5 mice (**B**). Scale bars, 20 μm. (**C**) Infiltrating neutrophils from implantation sites or peripheral blood of C57 and BPH/5 mice at E8.5 were identified by flow cytometry as $CD45^+CD11b^+GR1^{hi}$. (**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 chemoattactant 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 I). 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 placentation 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. 3E–G, 3J, 3K). 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 $\pm$ 110 versus 990 $\pm$ 120 per midsagittal section, respectively; $n = 4$, p = NS). Similarly, we found no difference in macrophage number in the placentas at E12.5 (14 $\pm$ 4 versus 24 $\pm$ 21 per midsagittal section, respectively; $n = 4$, p = NS). Furthermore, treatment with anti-GR1 did not alter the number of uNK cells (BPH/5, 1000 $\pm$ 110 versus BPH/5 plus anti-GR1, 970 $\pm$ 64 per midsagittal section; $n = 4$, p = NS) or macrophages (BPH/5, 14 $\pm$ 41 versus BPH/5 plus anti-GR1, 6 $\pm$ 1 per midsagittal 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 $\pm$ 4 pg/mg protein; BPH/5, 18 $\pm$ 4 pg/mg protein; BPH/5 plus anti-GR1, 33 $\pm$ 4 pg/mg protein) is associated with normal placentation 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 recruitment, 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 mice; 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 placentation and APOs, we treated BPH/5 mice with targeted complement inhibitors, that is, CR2-Crry or CR2-FH. These agents are fusion proteins of either Crry, 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-Crry 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-Crry and CR2-FH have minimal effects on systemic complement activity. They target cell-bound products of complement activation, accu-

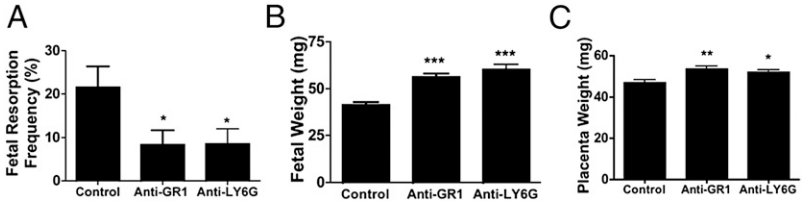

FIGURE 2. Neutrophil depletion normalizes pregnancy phenotype of BPH/5 mice. BPH/5 mice were treated with anti-GR1 or anti-Ly6G Ab to deplete neutrophils, or isotype control on E2.5. Mice were sacrificed on E12.5 and evaluated for resorption frequency and fetal and placental weight. Neutrophil depletion (**A**) protects mice from fetal resorption (control, $n = 13$; anti-GR1, $n = 13$; anti-Ly6G, $n = 9$), (**B**) increases fetal weight (control, $n = 64$; anti-GR1, $n = 54$; anti-Ly6G, $n = 33$), and (**C**) increases placental weight (control, $n = 28$; anti GR-1, $n = 28$; anti-Ly6G, $n = 33$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

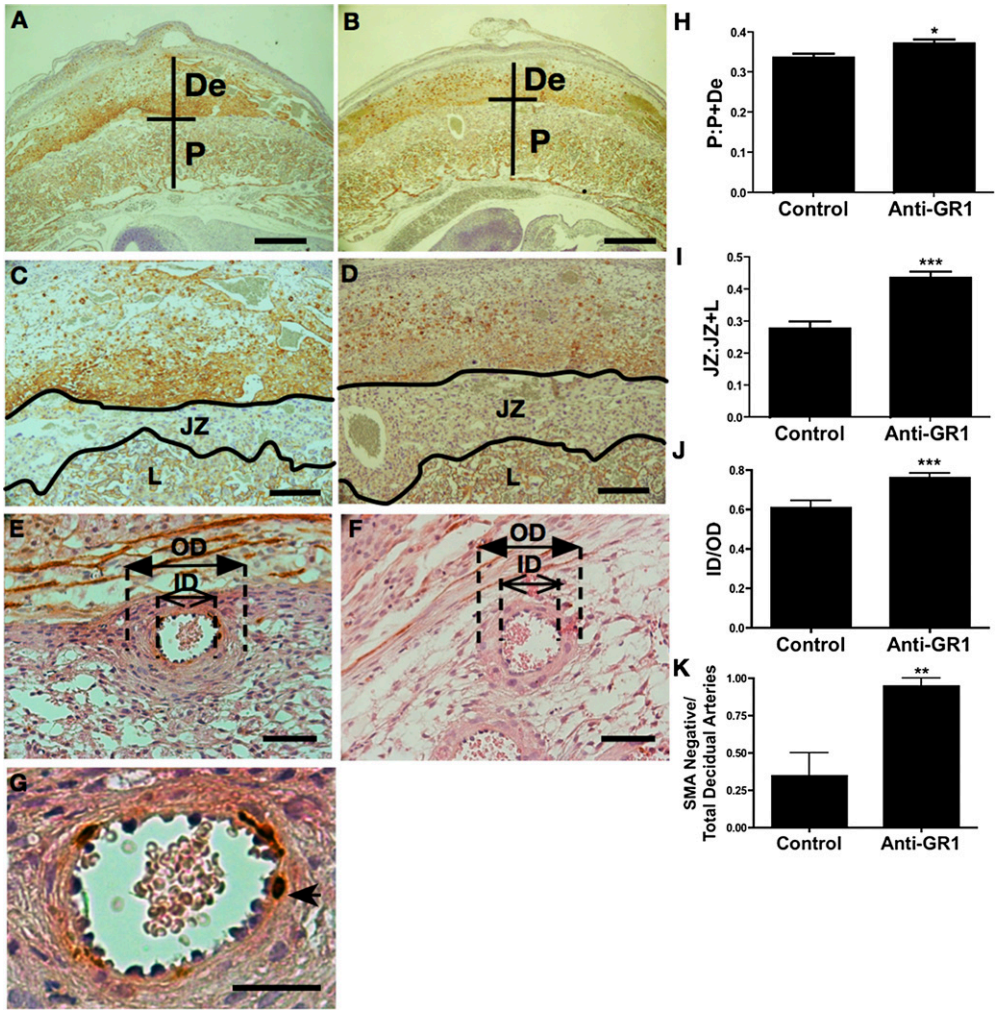

FIGURE 3. Neutrophil depletion improves placental morphology of BPH/5 mice. BPH/5 mice were treated with anti-GR1 Ab to deplete neutrophils or isotype control on E2.5, sacrificed on E12.5, and placental histology was examined. Representative images of intact feto-placental units from isotype control–treated 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.

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

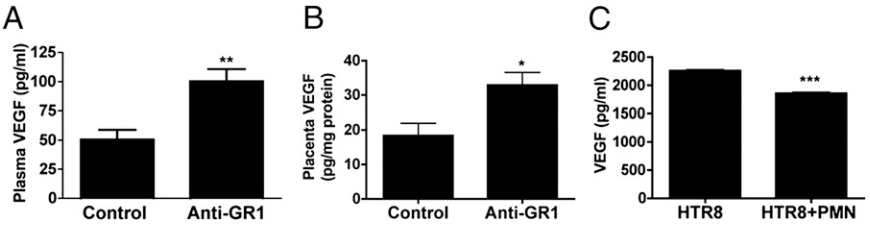

FIGURE 4. Neutrophil depletion is associated with normalization of VEGF levels. BPH/5 mice were treated with anti–GR-1 Ab or isotype control. (**A**) Plasma (n = 6 for each condition) or (**B**) placental homogenates (n = 9 for each condition) were collected on E12.5, and VEGF levels were assayed by ELISA. (**C**) The first trimester trophoblast cells line (HTR8) were cultured alone or with 1×10^6 neutrophils/ml for 24 h. Cell-free supernatants were collected and assayed for VEGF levels by ELISA. Data are pooled from three individual experiments done in duplicate. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

macrophages (control, 14 ± 4 versus CR2-Crry, 10 ± 1 per midsagittal 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, $TNF^{-/-}$, 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 midsagittal section; n = 4; p = NS) or macrophages (control, 14 ± 4 versus etanercept, 4 ± 2 per midsagittal 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 placental 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,

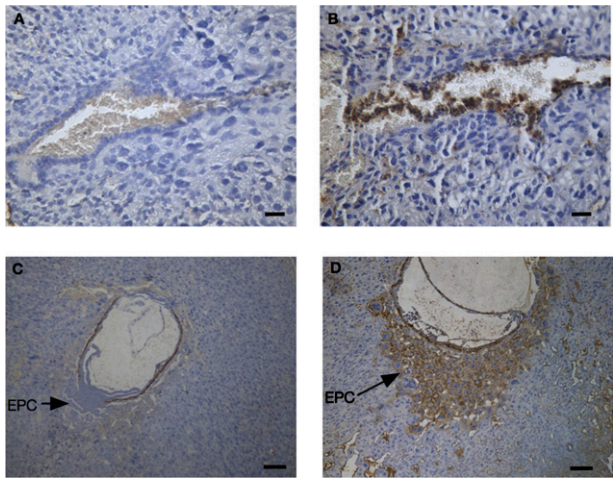

FIGURE 5. Complement deposition in developing BPH/5 mice. Uteri from C57 (**A** and **C**) and BPH/5 (**B** and **D**) mice were stained with an anti-C3 mAb. Representative images of the ectoplacental cone at E6.5 (A and B) and E8.5 (C and D) demonstrate increased complement deposition in the ectoplacental cone (EPC, arrows) of the BPH/5 mice but not the C57 mice. Scale bars, 20 μm (A and B), 100 μm (C and D).

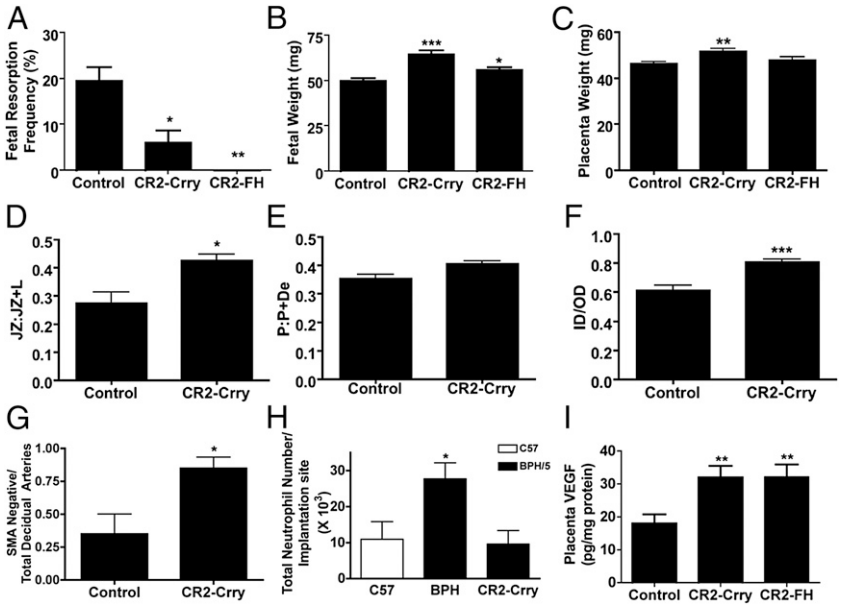

FIGURE 6. Complement inhibition rescues BPH/5 pregnancies. BPH/5 mice were treated with CR2-Crry or CR2-FH to inhibit complement or control (PBS) on E5.5. Mice were sacrificed on E12.5 and evaluated for resorption frequency, fetal and placental weight, and placental histology. The effects of complement 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). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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

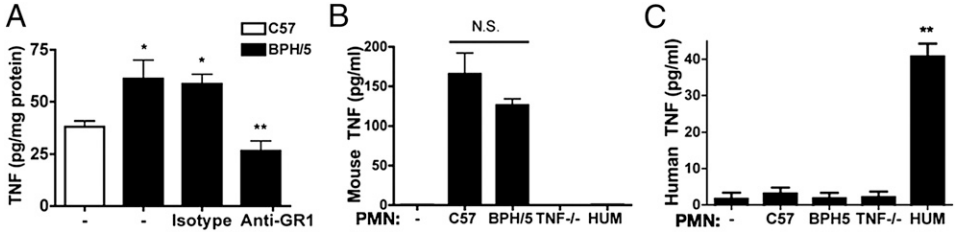

FIGURE 7. Elevated TNF-α in placentas from BPH/5 mice and supernatants from neutrophils cultured with HTR8 cells. (**A**) TNF-α levels were assayed from placental homogenates at E8.5 from untreated C57 and BPH/5 mice, and from BPH/5 mice treated at E2.5 with anti-GR1 Ab to deplete neutrophils or isotype 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.

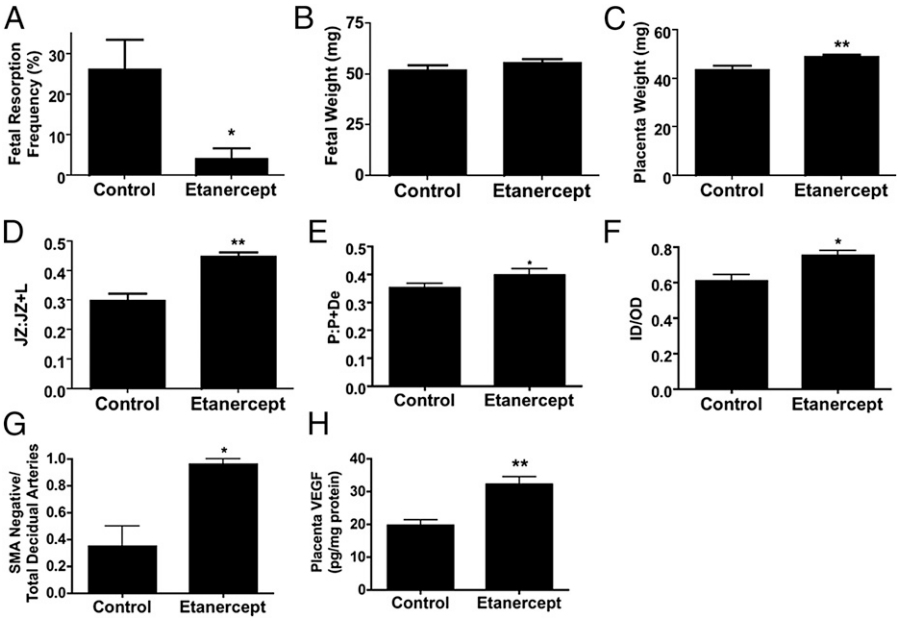

FIGURE 8. TNF-α blockade prevents APO and normalizes placental development in BPH/5 mice. The effect of TNF-α blockade on (**A**) fetal resorption (n = 6 mice/group), (**B**) fetal weight (minimum 28 fetuses/group), and (**C**) placental weight (minimum 28 fetuses/group) is shown. Placental morphology 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 mean ± SEM. For histologic studies a minimum of nine feto-placental units were analyzed for each condition (three implantation sites from three separate pregnancies). $*p < 0.05$, $** p < 0.01$.

mice. Although neutrophil depletion is not a clinically viable option, the importance of infiltrating leukocytes in driving fetal loss and growth restriction provides a framework for understanding the pathogenesis of these conditions. In our experiments, 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.

Evidence for elevated levels of the neutrophil chemoattractant CXCL1 in BPH/5 placenta is consistent with our model that early inflammation leads to APO. 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 placentation and growth restriction, and to recruit and activate other effectors of inflammation (34, 58, 59). In our studies, depletion of neutrophils normalized placental TNF-α and VEGF in BPH/5 mice; blockade of TNF-α also normalized placental VEGF. Both animal models and studies in people have identified release of antiangiogenic factors and proinflammatory cytokines to be key mediators of placental insufficiency and the associated fetal and maternal complications, typically preeclampsia (60–62). Our work defines a pathway that precedes this response and offers a target for treatment before manifestation of impaired placentation and maternal end-organ dysfunction. The salutary effects of TNF-α blockade may be sufficient to justify this therapy to prevent placental inflammation associated with fetal hypoperfusion and APO in high-risk 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.

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