Pregnancy/Preeclampsia

Interleukin 10 Deficiency Exacerbates Toll-Like Receptor 3–Induced Preeclampsia-Like Symptoms in Mice

Piyali Chatterjee, Valorie L. Chiasson, Shelley E. Kopriva, Kristina J. Young, Victor Chatterjee, Kathleen A. Jones, Brett M. Mitchell

Abstract—Preeclampsia may result from overactivation of the maternal immune system and is characterized by endothelial dysfunction and excessive inflammation. Given the importance of maternal immune system regulation and anti-inflammatory cytokines in normotensive pregnancies, we hypothesized that maternal immune system activation via Toll-like receptor 3 during pregnancy would cause preeclampsia-like symptoms in mice, which would be made worse by deficiency of the anti-inflammatory cytokine interleukin 10. The Toll-like receptor 3 agonist polyinosine-polycytidylic acid (poly I:C) caused hypertension, endothelial dysfunction, and proteinuria in mice only when pregnant. In the absence of poly I:C, pregnant interleukin 10 knockout mice exhibited a significant increase in systolic blood pressure, endothelial dysfunction, and serum proinflammatory cytokines, as well as aortic and placental platelet-endothelial cell adhesion molecule expression compared with pregnant wild-type mice. Deficiency of interleukin 10 further augmented these measures in poly I:C–treated pregnant mice. In addition, sera from poly I:C-treated pregnant wild-type mice significantly decreased relaxation responses and increased platelet-endothelial cell adhesion molecule expression in isolated aortas from nonpregnant wild-type mice, and these effects were augmented by sera from poly I:C-treated interleukin 10 knockout mice. Coincubation with recombinant interleukin 10 normalized relaxation responses and platelet-endothelial cell adhesion molecule expression in all of the groups. Collectively, Toll-like receptor 3 activation during pregnancy causes preeclampsia-like symptoms, which are exacerbated by the absence of interleukin 10. Exogenous interleukin 10 treatment had beneficial effects on endothelial function and may be beneficial in women with preeclampsia. (Hypertension. 2011;58:489-496.) • Online Data Supplement

Key Words: interleukin 10 • endothelium • hypertension • pregnancy-induced • inflammation • pregnancy • preeclampsia

Hypertensive disorders of pregnancy, such as preeclampsia (PE), affect ~10% of all pregnancies, are one of the leading causes of fetal morbidity and mortality, and cause 15% to 20% of maternal deaths worldwide.1 PE is diagnosed by new-onset hypertension and proteinuria during pregnancy and is associated with endothelial dysfunction, excessive inflammation, and abnormal fetal development.2–4 Although the etiology of PE remains unknown, evidence strongly supports a role for the maternal immune system.5 PE is more common in women with autoimmune diseases and during the first conception, and conversely, the incidence of PE is decreased in women with immune deficiency (ie, HIV).6 Thus, successful pregnancy depends on controlled maternal immunity, and PE represents excessive immune system responses.5,8

The danger model as applied to pregnancy suggests that normal pregnancy does not activate the maternal immune system unless danger signals are present during gestation.9 During excessive cellular stress, molecules known as danger-associated molecular patterns are released by injured cells and activate Toll-like receptors (TLRs).10,11 Recently, certain TLRs have been identified in human uteroplacental tissues, and TLR4, responsive to lipopolysaccharide, is upregulated in the placenta of women with PE, suggesting a link among danger-associated molecular patterns, TLRs, and PE.12–16 TLR3 (Tlr3 is the most abundantly expressed TLR mRNA in uteroplacental tissues of mice) is activated specifically by double-stranded RNA expressed by viruses or released by necrotic tissue.13 Single-stranded RNA viruses, which are associated with placental dysfunction and/or PE, may also activate TLR3 during replication.17–20 In addition, dysfunctional cytotrophoblast invasion and spiral artery remodeling may cause large amounts of tissue necrosis and release of double-stranded RNA at the feto-maternal interface. Therefore, TLR3 may play an important role in maternal immune system modulation during pregnancy.21

Clinical studies have reported increased levels of proinflammatory cytokines and decreased levels of anti-
inflammatory cytokines in women with PE. The anti-inflammatory cytokine interleukin (IL) 10, produced by activated immune cells, inhibits nuclear factor-κB activation, suppresses proinflammatory cytokine/chemokine production, and inhibits costimulatory signals, which are necessary for T-cell proliferation, inflammation, and immunity. Activation of IL-10 receptors, present on endothelial cells, prevents platelet-endothelial cell adhesion molecule (PECAM) expression, monocyte adhesion, and cytokine-mediated endothelial dysfunction. Thus, IL-10 deficiency may contribute to the development of PE. In support, women with PE exhibit decreased placental IL-10 expression and serum IL-10 levels. Although IL-10 knockout (KO) mice exhibit fairly normal pregnancies with respect to fetal development, maternal endothelial function and blood pressure were not measured.

Therefore, we addressed the following 3 questions related to the role of TLR3 and IL-10 in the development of PE: (1) is TLR3 activation during pregnancy sufficient to cause PE-like symptoms in mice; (2) does a deficiency in IL-10 cause PE-like symptoms in mice and does this exacerbate PE-like symptoms induced by TLR3 activation; and (3) can exogenous IL-10 prevent the endothelial dysfunction induced by sera from mice with PE-like symptoms. We hypothesized that TLR3 activation will cause pregnancy-dependent hypertension and endothelial dysfunction, and this will be worsened by the lack of IL-10. We also hypothesized that IL-10 supplementation will prevent the endothelial dysfunction caused by sera from mice with PE-like symptoms.

Methods
An expanded Methods section can be found in an online Data Supplement, available at http://hyper.ahajournals.org.

Animals, Treatment, and Blood Pressure
Male and female C57BL/6J wild-type (WT) and IL-10 KO female mice on C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). All of the procedures were approved by Texas A&M Health Science Center/Scott & White Memorial Hospital Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals. Female WT and IL-10 KO female mice on C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). All of the procedures were approved by Texas A&M Health Science Center/Scott & White Memorial Hospital Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals. Female WT and IL-10 KO mice were grouped as follows: nonpregnant controls treated with vehicle (C), nonpregnant controls treated with poly I:C (C-PIC), pregnant treated with vehicle (P), or pregnant treated with poly I:C (P-PIC). Poly I:C, effective for 48 hours, was injected on days 13, 15, and 17 of pregnancy or corresponding days in C-PIC mice, whereas all of the C and P mice were injected with the same volume of PBS on the same days. Gestational day 13 was chosen to initiate injections based on previous reports and pilot studies, because initiation at gestational days 6.5, 7.5, or 10.0 induces embryo resorption. Tail-cuff systolic blood pressures (ITC, Inc, Woodland Hills, CA) were measured at baseline and on day 13, before poly I:C injections, and day 18, as described previously. All of the mice were euthanized on gestational day 18 or the corresponding day in nonpregnant mice, and the following measures were performed.

Serum Cytokine ELISA Array and Urinary Protein Concentration
The mouse Th1/Th2/Th17 Multi-analyte ELISArray kit (SA Biosciences, Frederick, MD) was used to measure 12 proinflammatory and anti-inflammatory cytokines and was performed according to the manufacturer’s protocol. Protein concentrations in urine obtained on day 18 were measured using the pyrogallol red method (Total Protein kit, Micro Pyrogallol Red Method, Sigma), as described previously.

Vascular Reactivity
Relaxation responses were assessed in isolated endothelium-intact aortic rings (2 mm) obtained from mice on day 18, as described previously.

Immunoblotting
Immunoblotting for TLR3, PECAM, BAX, caspase 3, and β-actin in aortas and placentas obtained from mice on day 18 was performed as described previously.

In Vitro Recombinant IL-10 Treatment
Aortas from female WT mice were incubated overnight with sera obtained at day 18 from WT or IL-10 KO P or P-PIC mice either alone (1:1 ratio with RPMI 1640) or with recombinant IL-10 (rIL-10, 5 to 10 ng/mL) at 37°C.

Statistical Analyses
Results are presented as mean±SEM. The Student t test was used to compare measures between P and P-PIC mice. For measurements examined among C, C-PIC, P, and P-PIC WT and IL-10 KO mice, a 1-way ANOVA was used for comparisons between groups, followed by the Student-Newman-Keuls post hoc test, when necessary. Changes in systolic blood pressure during pregnancy were analyzed by a repeated-measures ANOVA. The relationship between placental TLR3 activation and systolic blood pressure was analyzed using a Pearson product-moment correlation test. The significance level was 0.05.

Results
Systolic Blood Pressure Responses to TLR3 Activation During Pregnancy in WT and IL-10 KO Mice
In nonpregnant mice, neither poly I:C treatment nor IL-10 gene deletion had any significant effect on systolic blood pressure (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). However, in pregnant mice, poly I:C treatment increased systolic blood pressure on day 18 of pregnancy (P: 95±2 mm Hg versus P-PIC: 143±2 mm Hg, P<0.05; Figure 1). Systolic blood pressures in pregnant IL-10 KO mice were increased modestly, but significantly, at day 18 of pregnancy compared with P mice (108±4 mm Hg, P<0.05, versus P; Figure 1). Compared with P-PIC mice, P-PIC IL-10 KO mice exhibited a further increase in systolic blood pressure (154±4 mm Hg, P<0.05, versus P-PIC; Figure 1).

Aortic Relaxation Responses in WT and IL-10 KO Mice After TLR3 Activation During Pregnancy
Poly I:C treatment and/or IL-10 KO gene deletion did not have any effect on acetylcholine-mediated relaxation responses at day 18 in nonpregnant mice (Figure S2). However, maximal acetylcholine-mediated relaxation responses of aortas from P-PIC mice were decreased significantly compared with P mice (P: 85±3% versus P-PIC: 77±4%; P<0.05; Figure 2). Aortas from P IL-10 KO mice exhibited a modest but significant decrease in maximal acetylcholine-mediated relaxation responses compared with P mice (69±1%, P<0.05, versus P; Figure 2). Compared with P-PIC mice, P-PIC IL-10 KO mice exhibited a further decrease in maximal acetylcholine-mediated relaxation responses (22±1%, P<0.05, versus
P-PIC; Figure 2). Endothelium-independent relaxation responses to sodium nitroprusside were not significantly different across any of the groups (Figure S3).

**Fetal Development in WT and IL-10 KO Mice After TLR3 Activation During Pregnancy**

We observed no change in mean litter size among P, P-PIC, and P IL-10 KO mice (P: 7.0 ± 0.6, P-PIC: 7.8 ± 0.5, and P IL-10 KO: 8.3 ± 0.4; P > 0.05). However, P-PIC IL-10 KO mice had a significant decrease in the mean number of pups per litter (5.1 ± 1.1, P < 0.05, versus P). P-PIC mice exhibited a significant increase in fetal demise assessed as the number of malformed or resorbed pups per litter on day 18 of pregnancy (P: 0.0 ± 0.0 versus P-PIC: 0.8 ± 0.2; P < 0.05), and this was further increased in P-PIC IL-10 KO mice (2.7 ± 0.4, P < 0.05, versus P and P-PIC). Consistent with previous reports, P IL-10 KO mice did not have any fetal demise.

**Urinary Protein Concentration in WT and IL-10 KO Mice After TLR3 Activation During Pregnancy**

TLR3 activation in nonpregnant WT mice had no effect on urinary protein concentration (C: 53 ± 4 mg/dL versus C-PIC: 66 ± 9 mg/dL; P > 0.05). However, poly I:C treatment significantly increased day 18 urinary protein concentrations in pregnant WT mice (P: 72 ± 11 mg/dL versus P-PIC: 127 ± 18 mg/dL; P < 0.05 versus P). There were no differences in day 18 urinary protein concentrations between C IL-10 KO mice and C mice. P IL-10 KO and P-PIC IL-10 KO mice exhibited significantly increased urinary protein concentrations compared with P mice (P IL-10 KO: 132 ± 4 mg/dL, P-PIC IL-10 KO: 125 ± 7 mg/dL; P < 0.05 versus P); however, there was not a further increase in P-PIC IL-10 KO mice compared with P-PIC mice (P > 0.05 versus P-PIC).

**Serum Cytokine Levels in WT and IL-10 KO Mice After TLR3 Activation During Pregnancy**

With respect to anti-inflammatory cytokines, day 18 serum levels of IL-4 increased significantly in both P and P IL-10 KO mice but failed to increase in P-PIC and P-PIC IL-10 KO mice (Figure 3A). Serum IL-10 levels increased significantly in P-PIC mice but not in P-PIC IL-10 KO mice, as expected. Serum levels of IL-13, another anti-inflammatory cytokine that works in concert with IL-4 and IL-10, were increased only in P-PIC IL-10 KO mice at day 18. Regarding proinflammatory cytokines, day 18 serum levels of interferon-γ, tumor necrosis factor-α, and IL-12, were increased significantly in P-PIC mice (Figure 3B). In P IL-10 KO mice, these cytokines were increased significantly and further increased in P-PIC IL-10 KO mice (Figure 3B).

**Endothelial Cell Activation in WT and IL-10 KO Mice After TLR3 Activation During Pregnancy**

PECAM, an adhesion molecule expressed by endothelial cells that is increased during inflammation and endothelial cell activation, correlates positively with the severity of PE.29,40,41 Aortic protein expression of PECAM at day 18 was increased significantly in P-PIC mice, whereas P IL-10 KO mice had significantly increased PECAM expression compared with P mice (Figure 4A). In addition, P-PIC IL-10 KO mice had significantly increased aortic PECAM expression compared with P-PIC and P IL-10 KO mice (Figure 4A). Day 18 placental PECAM expression was increased significantly in both P-PIC mice and P IL-10 KO mice compared with P mice, and this was further increased in P-PIC IL-10 KO mice (Figure 4B). TLR3 activation was increased in day 18 placentas from P-PIC and P IL-10 KO mice, and this was further increased in P-PIC IL-10 KO mice (Figure 4A). Placental TLR3 activation correlated positively with systolic blood pressure (r = 0.983; P < 0.05).
During normal pregnancy, inflammation is necessary during several stages of fetal development but is also tightly regulated to prevent tissue damage at the fetomaternal interface. However, excessive inflammation resulting from an increase in proinflammatory cytokines and/or a decrease in anti-inflammatory cytokines occurs in PE. TLR activation may provide an important link between inflammation and hypertensive disorders of pregnancy. Here, we demonstrate that TLR3 activation during pregnancy was sufficient to increase systolic blood pressure and endothelial dysfunction only in pregnant mice. Our study also demonstrates that the lack of IL-10 coupled with TLR3 activation exacerbated PE-like symptoms, that sera from poly I:C-treated pregnant WT mice caused endothelial dysfunction, and that recombinant IL-10 can prevent these effects.

The “danger model” with respect to pregnancy states that activation of the maternal immune system occurs when danger signals are encountered during gestation. Endogenous danger signals released during cellular stress can activate TLRs, of which TLRs 1 to 9 are abundantly expressed in the placenta. Consistent with this model, TLR3 activation in mice caused an increase in systolic blood pressure and endothelial dysfunction, as well as fetal demise, which was likely mediated by decreased placental perfusion. Zhang et al also observed fetal demise after TLR3 activation as well as fetal demise, which was likely mediated by decreased placental perfusion. Zhang et al also observed fetal demise after TLR3 activation. Together, these data fully support a pregnancy-specific danger model, because TLR3 activation in nonpregnant mice had no effect on blood pressure and endothelial function, and suggest that maternal immune system activation during pregnancy, in addition to the danger signal double-stranded RNA, can cause PE-like symptoms.
The induction of PE-like symptoms by TLR3 activation was associated with a proinflammatory state similar to that of women with PE. Activation of TLR3 increases production of type I interferons, such as interferon-α and -β, as well as the activation of nuclear factor κB. This is apparent by an increase in serum proinflammatory cytokines, as well as markers of endothelial cell activation in blood vessels and in the placenta, of P-PIC mice. Although TLRs play a beneficial role in resolving infections at the feto-maternal interface during normal pregnancy, overstimulation may be the source of excessive chemokine and cytokine production and decrease in anti-inflammatory cytokines. We observed augmented levels of IL-4 during normal pregnancy; however, IL-4 failed to increase in mice with PE-like symptoms. The failure of IL-4 levels to increase in P-PIC mice caused a compensatory increase in IL-10 levels that was not sufficient to prevent hypertension, endothelial dysfunction, or fetal demise. Furthermore, the lack of IL-10 availability in IL-10 KO mice led to a decrease in IL-13 that was also not sufficient to reduce PE-like symptoms. These results suggest that, in normal pregnancy, IL-4 is the predominant anti-inflammatory cytokine augmented to control inflammation and that TLR3 activation prevents this response. In the absence of increased IL-4, IL-10, followed by IL-13, plays compensatory roles to futilely limit inflammation. These data demonstrate a possible anti-inflammatory cytokine hierarchy involved in normal and hypertensive pregnancies, but more studies are needed to determine their integrative roles in the development of PE.

IL-10 KO mice had a normal number of pups per litter and lack of fetal demise that is consistent with previous reports; however, pregnant IL-10 KO mice develop mild hypertension and endothelial dysfunction, as well as inflammation. Compared with telemetry measures of blood pressure during pregnancy, which show a gradual, but nonsignificant, increase in blood pressure in the latter part of pregnancy, we saw that systolic blood pressure in pregnant WT mice tended to decrease from day 13 to day 18 of gestation. However, in pregnant IL-10 KO mice, systolic blood pressure failed to decrease and was significantly increased at day 18 of gestation compared with pregnant WT mice. Lai et al. reported that pregnant IL-10 KO mice exposed to hypoxia early in pregnancy not only exhibited PE-like symptoms but increased placental expression of the proapoptotic molecules BAX and caspase 3. However, we found that both P and P-PIC IL-10 KO mice had increased placental BAX expression compared with WT mice, whereas P IL-10 KO mice had the most placental caspase 3 expression (Figure S5). This would suggest that IL-10 plays a somewhat protective role in preventing placental apoptosis through the p53 pathway; however, this did not correlate with blood pressure or endothelial function. We have also treated human cytotrophoblasts with poly I:C for 12, 24, and 48 hours and did not see any changes in BAX and caspase 3 expression (data not shown). Lai et al. also reported that hypoxic, pregnant IL-10 KO mice developed atrophic tubules, interstitial edema, and enlarged glomeruli. However, kidneys from P and P-PIC WT and IL-10 KO mice did not exhibit any tubular atrophy or interstitial or glomerular changes (data not shown). PE-like symptoms induced by TLR3 activation were further augmented in IL-10 KO mice, and this was associated with a decrease in the number of pups per litter, as well as fetal cell activation in wild-type (WT) and interleukin (IL) 10 knockout (KO) mice after Toll-like receptor (TLR) 3 activation during pregnancy. A, Aortic platelet-endothelial cell adhesion molecule (PECAM) expression at day 18. B, Placental PECAM expression at day 18. Representative Western blots showing PECAM and β-actin, as well as densitometry. C indicates nonpregnant controls; C-PIC, nonpregnant controls treated with poly I:C; P, pregnant; P-PIC, pregnant treated with poly I:C. n=3 independent experiments. Comparisons of protein expression between groups were analyzed using a 1-way ANOVA. *P<0.05 vs C and †P<0.05 vs P-PIC in aortas, ‡P<0.05 vs P in placentas and †P<0.05 vs P-PIC in placentas.
demise, likely attributed to a further decrease in placental perfusion. In rats treated with the TLR4 agonist lipopolysaccharide at gestational day 14.5, fetal demise also occurred as a result of decreased placental perfusion.47

Because IL-10 deficiency exacerbated the hypertension and endothelial dysfunction induced by TLR3 activation during pregnancy, we hypothesized that restoring IL-10 would be beneficial. Serum from mice with PE-like symptoms was sufficient to cause endothelial dysfunction in aortas isolated from nonpregnant mice, and this was likely attributed to the increased levels of proinflammatory cytokines present in the blood. Supportive of our hypothesis, rIL-10 prevented the endothelial dysfunction caused by sera from poly I:C-treated pregnant mice. The direct, beneficial vascular effects of rIL-10 may be explained by its effects on both the blood vessel and the immune cells in the sera. Zemse et al48 reported that rIL-10 could prevent the endothelial dysfunction caused by overnight incubation of mouse aortas with tumor necrosis factor-α, which was elevated in the sera of our P-PIC and P-PIC IL-10 KO mice. In addition, rIL-10 was shown to inhibit oxidizing species produced by immune cells in vitro.49

As a potential therapeutic, studies have shown that rIL-10 can reduce blood pressure in pregnant, hypertensive rats and prevent the TLR4-induced decrease in placental perfusion.

Figure 5. Direct vascular effects of sera from wild-type (WT) and interleukin (IL) 10 knockout (KO) mice after Toll-like receptor (TLR) 3 activation during pregnancy on endothelial function and activation. A, Effects of day 18 sera from P and P-PIC mice with or without recombinant IL-10 on acetylcholine-induced relaxation responses in aortas isolated from nonpregnant mice. B, Effects of day 18 sera from P and P-PIC mice with or without recombinant IL-10 on platelet-endothelial cell adhesion molecule (PECAM) expression in aortas isolated from nonpregnant mice. C, Effects of day 18 sera from P and P-PIC IL-10 KO mice with or without recombinant IL-10 on acetylcholine-induced relaxation responses in aortas isolated from nonpregnant mice. D, Effects of day 18 sera from P and P-PIC IL-10 KO mice with or without recombinant IL-10 on PECAM expression in aortas isolated from nonpregnant mice. Results are expressed as mean±SEM (n in each group shown in parentheses). Representative Western blots show aortic PECAM and β-actin and densitometry. Comparisons of relaxation responses and protein expression between groups were analyzed using a 1-way ANOVA. *P<0.05 vs P sera, †P<0.05 vs P-PIC sera.
and fetal demise. In humans, preclinical studies in autoimmune diseases have demonstrated the efficacy of rIL-10 treatment in reducing inflammation. Together, our results demonstrate the important roles of TLR3 and IL-10 in the development of PE-like symptoms in mice and may partially explain the development of PE in women.

Perspectives

The incidence of PE is increasing in women; however, no therapies are available other than delivery of the fetus. Our study demonstrates that TLR3 activation is sufficient to cause PE-like symptoms in mice and may be a possible cause of PE in women. In addition, this study determined the importance of the anti-inflammatory cytokine IL-10, because IL-10 deficiency caused hypertension and endothelial dysfunction and exacerbated PE-like symptoms after TLR3 activation during pregnancy. rIL-10, which is used clinically in other diseases characterized by inflammation and immune system activation, may prove to be effective in preventing PE in women.

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Disclosures

None.

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INTERLEUKIN-10 DEFICIENCY EXACERBATES TOLL-LIKE RECEPTOR 3-INDUCED PREECLAMPSIA-LIKE SYMPTOMS IN MICE

Piyali Chatterjee,¹ Valorie L. Chiasson,¹ Shelley E. Kopriva,¹ Kristina J. Young,¹ Victor Chatterjee,¹ Kathleen A. Jones,² and Brett M. Mitchell¹

¹Department of Internal Medicine, Division of Nephrology & Hypertension; ²Department of Pathology; Texas A&M Health Science Center College of Medicine/Scott & White Memorial Hospital, Temple, TX 76504

Short Title – IL-10, Toll-Like Receptor 3, and Preeclampsia

Correspondence to:
Brett M. Mitchell, PhD
Assistant Professor
Dept. of Internal Medicine, Division of Nephrology & Hypertension
Texas A&M Health Science Center College of Medicine
702 SW HK Dodgen Loop
Temple, TX 76504
Phone: 254.724.6267 Fax: 254.742.7181 Email: bmitchell@tamhsc.edu
EXPANDED METHODS

Animals, Treatment, and Blood Pressure
Male and female C57BL/6J wild type (WT) and IL-10 KO female mice on C57BL/6J background (stock #: 002251) were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice were used for mating purposes only. Animals were housed in a facility with 12 hr dark and 12 hr light cycle at ambient temperature (23°C). All procedures were approved by Texas A&M Health Science Center/Scott and White Memorial Hospital Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals. Female WT and IL-10 KO mice, aged 10-12 weeks, were grouped either as non-pregnant controls treated with vehicle (C), non-pregnant controls treated with poly I:C (C-PIC), pregnant treated with vehicle (P), or pregnant treated with poly I:C (P-PIC). All P-PIC mice were injected with poly I:C (20 mg/kg, i.p.) on days 13, 15, and 17 of pregnancy or corresponding days in C-PIC mice, while all C and P mice were injected with the same volume of PBS on the same days. Tail-cuff systolic blood pressures (IITC, Inc.; Woodland Hills, CA) were measured at baseline and on day 13, prior to poly I:C injections, and day 18 as described previously. All mice were euthanized on gestational day 18 or corresponding day in non-pregnant mice and the following measures were performed.

Serum Cytokine Elisa Array and Urinary Protein Concentration
The mouse Th1/Th2/Th17 Multi-analyte ELISArray™ kit (SA Biosciences; Frederick, MD) was used for detection of serum cytokines related to T helper cells. Serum samples (50 µl) were added to each well on the ELISA plate and measurements for 12 pro-inflammatory and anti-inflammatory cytokines [IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and transforming growth factor-β1 (TGFβ1)] were performed according to the manufacturer’s protocol. The minimal detection level for each cytokine was 10 pg/ml. Protein concentrations in urine obtained at the time of sacrifice were measured using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method, Sigma) as described previously.

Vascular Reactivity
Mice were anesthetized with isoflurane and euthanized by cervical dislocation. Isolated endothelium-intact aortic rings (2 mm) were connected to an isometric force transducer in a myograph (Danish Myotechnology; Atlanta, GA). Concentration-force curves were obtained in a half-log, cumulative fashion to acetylcholine (ACh) and sodium nitroprusside (SNP) following contraction to an EC70 concentration of phenylephrine (PE) as described previously.

Immunoblotting
Aortas and placentas were homogenized in the presence of protease and phosphatase inhibitors in a lysis buffer (Cell Signaling; Danvers, MA). Immunoblotting was performed as previously described. Protein concentration was determined by Bradford assay using bovine serum albumin as the standard. Proteins were separated by electrophoresis on 4-12% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (Millipore; Billerica, MA) at 4°C. Western blot analyses were
performed using the following primary antibodies: TLR3 1:1000 (Imgenex; San Diego, CA), PECAM 1:1000 (Santa Cruz Biotechnology; Santa Cruz, CA), BAX 1:1000 (Cell Signaling; Danvers, MA), Caspase 3 1:1000 (Cell Signaling; Danvers, MA), and β-actin 1:5000 (Sigma; St. Louis, MO). Secondary antibodies consisted of anti-rabbit, anti-goat, or anti-mouse IgGs conjugated to Alexa-Fluor 680 or IR800Dye (LI-COR Biosciences; Lincoln, NE), respectively. The bands were identified using infrared visualization (Odyssey System, LI-COR Biosciences; Lincoln, NE) and densitometry was performed using the Odyssey software.

In Vitro rIL-10 Treatment
To examine the direct effects of recombinant IL-10 (rIL-10) on endothelial function, aortas from female WT mice were incubated overnight with WT or IL10 KO P or P-PIC sera alone (1:1 ratio with RPMI 1640 media) or with rIL-10 (5-10 ng/mL) at 37°C. Endothelium-dependent relaxation responses were assessed as described above. The remaining aortic tissue was used for immunoblotting as described above.

Statistical Analyses
Results are presented as mean ± SEM. The Student’s t-test was used to compare measures between P and P-PIC mice. For measurements examined between C, C-PIC, P, and P-PIC WT and IL-10 KO mice, a one-way analysis of variance was used for comparisons between groups followed by the Student’s-Newman-Keuls post hoc test when necessary. Changes in systolic blood pressure during pregnancy were analyzed by a repeated-measures analysis of variance. The relationship between placental TLR3 activation and systolic blood pressure was analyzed using a Pearson product-moment correlation test. The significance level was 0.05.

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Figure S1. Systolic blood pressure responses to TLR3 activation in non-pregnant WT and IL-10 KO mice. Poly I:C treatment (administered on days 13, 15, and 17) and/or IL-10 gene deletion had no effect on systolic blood pressure in non-pregnant mice. Results are expressed as mean ± SEM (n in each group shown in parentheses). C = non-pregnant controls, C-PIC = non-pregnant controls treated with poly I:C. Changes in systolic blood pressure during gestation were analyzed using a repeated-measures ANOVA while comparisons of systolic blood pressure between groups were analyzed using a one-way ANOVA.
Figure S2. Aortic relaxation responses in non-pregnant WT and IL-10 KO mice following TLR3 activation. Poly I:C treatment (administered on days 13, 15, and 17) and/or IL-10 gene deletion had no effect on aortic acetylcholine-induced relaxation responses in non-pregnant mice. Results are expressed as mean ± SEM (n in each group shown in parentheses). C=non-pregnant controls, C-PIC=non-pregnant controls treated with poly I:C. Comparisons of relaxation responses between groups were analyzed using a one-way ANOVA.
Figure S3. Aortic endothelium-independent relaxation responses in non-pregnant and pregnant WT and IL-10 KO mice following TLR3 activation. Endothelium-independent relaxation responses to sodium nitroprusside were not different between non-pregnant (A) or pregnant (B) mice following poly I:C treatment and/or IL-10 gene deletion. Results are expressed as mean ± SEM (n in each group shown in parentheses). C=non-pregnant controls, C-PIC=non-pregnant controls treated with poly I:C, P=pregnant, P-PIC=pregnant treated with poly I:C. Comparisons of relaxation responses between groups were analyzed using a one-way ANOVA.
Figure S4. Placental TLR3 activation in WT and IL-10 KO mice following TLR3 activation during pregnancy. Placental TLR3 expression was increased in P-PIC mice (left) and in P IL-10 KO (right), and was further increased in P-PIC IL-10 KO mice compared to P-PIC mice. Representative Western blots showing TLR3 and β-actin as well as densitometry. P=pregnant, P-PIC=pregnant treated with poly I:C. n>3 independent experiments. Comparisons of protein expression between groups were analyzed using a one-way ANOVA. *p<0.05 vs. P and †p<0.05 vs. P-PIC.
Figure S5. Placental BAX and Caspase 3 protein expression WT and IL-10 KO mice following TLR3 activation during pregnancy. Placental BAX expression was increased in P and P-PIC IL-10 KO mice (top), while placental Caspase 3 expression was increased in P IL-10 KO mice. Representative Western blots showing BAX, Caspase 3, and β-actin as well as densitometry. P=pregnant, P-PIC=pregnant treated with poly I:C. n>3 independent experiments. Comparisons of protein expression between groups were analyzed using a one-way ANOVA. *p<0.05 vs. P.