CD4⁺ T-Helper Cells Stimulated in Response to Placental Ischemia Mediate Hypertension During Pregnancy

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Abstract—We have shown that hypertension in response to chronic placental ischemia is associated with elevated inflammatory cytokines and CD4⁺ T cells. However, it is unknown whether these cells play an important role in mediating hypertension in response to placental ischemia. Therefore, we hypothesize that reduced uterine perfusion pressure (RUPP)–induced CD4⁺ T cells increase blood pressure during pregnancy. To answer this question, CD4⁺ T cells were isolated from spleens at day 19 of gestation from control normal pregnant (NP) and pregnant RUPP rats, cultured, and adjusted to 10⁶ cells per 100 μL of saline for intraperitoneal injection into NP rats at day 13 of gestation. On day 18, in the experimental groups of rats, arterial catheters were inserted, and on day 19 mean arterial pressure was analyzed. Inflammatory cytokines and antiangiogenic factor soluble fms-like tyrosine kinase 1 were determined via ELISA. Mean arterial pressure increased from 104±2 mm Hg in NP rats to 124±2 mm Hg in RUPP rats (P<0.001) and to 118±1 mm Hg in rats receiving RUPP CD4⁺ T cells (P<0.001). Circulating tumor necrosis factor-α and soluble fms-like tyrosine kinase 1 were elevated in recipients of RUPP CD4⁺ T cells to levels similar to control RUPP rats. In contrast, virgin rats injected with NP or RUPP CD4⁺ T cells exhibited no blood pressure changes compared with control virgin rats. Importantly, mean arterial pressure did not change in recipients of NP CD4⁺ T cells (109±3 mm Hg). These data support the hypothesis that RUPP-induced CD4⁺ T cells play an important role in the pathophysiology of hypertension in response to placental ischemia. (Hypertension. 2011;57:949-955.)

Key Words: hypertension ■ inflammation ■ cytokines ■ pregnancy ■ angiogenesis

Preeclampsia is an immune-based disease that affects 5% to 7% of pregnancies in the United States¹ and has been increased by 40% within the last decade.² Preeclampsia is characterized by new-onset hypertension with proteinuria, immune activation, and imbalances between proangiogenic (vascular endothelial growth factor/platelet derived growth factor) and antiangiogenic factor soluble fms-like tyrosine kinase 1 (sFlt-1) during pregnancy. The pathophysiologic mechanism(s) underlying this disease have yet to be elucidated.³⁻⁵ Previous studies have demonstrated that women with preeclampsia have increased placental and plasma levels of inflammatory cytokines compared with women with normal pregnancies.⁶⁻⁷ Further evidence implicates an imbalance between regulatory T cells (Treg) and effector T cells, subclasses of CD4⁺ T lymphocytes, in preeclamptic women.⁸⁻⁹

There is considerable evidence for the importance of a subclass of CD4⁺ T lymphocytes known as T helper (Th) 17 cells in the development and progression of chronic inflammatory and autoimmune diseases.⁸⁻¹² Th17 cells secrete interleukin 17 (IL-17), which is a proinflammatory cytokine strongly associated with allergic phenomena.¹¹⁻¹² IL-17–producing CD4⁺ T cells (Th17 cells) are the dominant pathogenic cellular component in autoimmune inflammatory diseases such as lupus, allograft rejection, autoimmune arthritis, psoriasis, and multiple sclerosis and are associated with airway remodeling and potential progression to asthma.¹¹⁻¹² Furthermore, IL-17 induces the synthesis of other cytokines such as interleukin 6 (IL-6), granulocyte colony stimulating factor, tumor necrosis factor-α (TNF-α), interleukin 8, and macrophage colony stimulating factor, all of which are associated with preeclampsia and have been shown previously by us to be associated with hypertension in response to reductions in uterine placental perfusion (RUPP) in pregnant rats.¹⁻⁹⁻¹³⁻¹⁷

In contrast, Tregs, a subset of CD4⁺ T cells expressing the transcription factor forkhead box P3 (FoxP3), have anti-inflammatory properties and can cause quiescence of autoimmune diseases. Tregs have been shown recently to be decreased in preeclamptic women compared with normal pregnant (NP) women. In a recent study, the frequencies of circulating Tregs (FoxP3⁺ CD4⁺ T cells), interferon-γ,
interleukin 10, and IL-17 were compared at the end of the third trimester of healthy and preeclamptic pregnancies. Th17 cells and Treg cells were detected by flow cytometry, whereas IL-17 was measured by ELISA. Preeclamptic women showed abnormal ratios of FoxP3+ Treg:IL-17–expressing CD4+ T cells. The percentage of CD4+ IL-17–producing T cells was decreased significantly in NP women compared with preeclamptic pregnancies. In addition, IL-17 and interferon-γ were increased, whereas interleukin 10 was decreased among women with preeclampsia compared with NP women. Although preeclampsia is associated with increased IL-17 and decreased Tregs, it is unknown whether placental ischemia is a stimulus for the imbalance between Th17 versus Tregs or if there are other maternal or paternal factors leading to the dysregulation of these immune factors. Furthermore, it remains undetermined whether this dysregulation among CD4+ Th subtypes lends to the development of hypertension in response to placental ischemia. Therefore, this study was designed to test the hypothesis that placental ischemia stimulates a CD4+ Th-cell imbalance which contributes to excess proinflammatory cytokine production, a shift in angiogenic factors, and leads to the development of hypertension during pregnancy.

**Methods**

All of the studies were performed in 250-g timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN). Animals were housed in a temperature-controlled room with a 12:12 light:dark cycle. All of the experimental procedures in this study were in accordance with the National Institutes of Health guidelines for use and care of animals and were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

**Protocol 1: Effect of Placental Ischemia on Blood Pressure and CD4+ T-Cell Dysregulation**

The RUPP procedure reduces the uterine perfusion pressure with the application of a constrictive silver clip (0.203 mm) to the aorta superior to the iliac bifurcation performed, whereas ovarian collateral circulation to the uterus is reduced with restrictive clips (0.100 mm) to the bilateral uterine arcades at the ovarian end. Under isoflurane anesthesia restrictive clips were implanted on day 14 of gestation, whereas on day 18 of gestation carotid arterial catheters were inserted for blood pressure measurements. The catheters inserted were V3 tubing (SCI), which is tunneled to the back of the neck and exteriorized. On day 19 of gestation arterial blood pressure was analyzed after placing the rats in individual restraining cages. Arterial pressure was monitored with a pressure transducer (Cobe III Transducer CDX Sema) and recorded continuously after a 1-hour stabilization period.

**Determination of Circulating T Lymphocytes**

Circulating CD4+ T-cell populations were measured from peripheral blood leukocytes collected at day 19 of gestation from NP rats and from pregnant RUPP rats. We used flow cytometry analysis to detect specific CD4+ T-cell populations, CD4+FoxP3+ and CD4+ retinoic acid receptor-related organ receptor-γ (RORγT) isolated from RUPP and NP rat peripheral blood leukocytes. Intracellular staining of FoxP3 and RORγ transcription factors were also performed to determine the percentage of positive cells belonging to the specific subtypes of either regulatory T cells (FoxP3+) or Th17 cells (RORγT).

At the time of tissue harvest, plasma was collected and peripheral blood leukocytes were isolated from plasma by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical Corp) according to the manufacturer’s directions. For flow-cytometric analysis, equal numbers of leukocytes (1×10⁶) were incubated for 30 minutes at 4°C with antibodies against mouse CD4 (BD Biosciences, San Jose, CA). After washing, cells were labeled with the secondary fluorescein isothiocyanate antibody (Southern Biotech, Birmingham, AL) for 30 minutes at 4°C. Cells were washed and permeabilized according to the manufacturer’s directions using the FoxP3 staining kit (eBioscience, San Diego, CA) followed by intracellular staining with antimitoerat Foxp3 conjugated to phycoerythrin (BD Pharmingen) or antirat RORγ conjugated to phycoerythrin (BD Pharmingen) for 30 minutes at 4°C. As a negative control, for each individual rat, cells were treated exactly as described above except they were incubated with antfluorescein isothiocyanate and antiphycoerythrin secondary antibodies alone. Subsequently, cells were washed and resuspended in 500 μL of RPMI medium and analyzed for single and double staining on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of positive staining cells above the negative control was collected for each individual rat, and mean values for each experimental group (NP and RUPP) were calculated.

**Protocol 2: Effect of Adoptive Transfer of RUPP CD4+ Th Cells on Blood Pressure in NP Rats**

This protocol was designed following that of previous investigators demonstrating an important role for T cells to mediate hypertension in pregnant mice and was therefore performed to determine the importance of the imbalance of CD4+ Th cells stimulated in response to RUPP to mediate hypertension during pregnancy. Spleens from NP and RUPP rats were isolated at the time of euthanization and immediately placed in ice-cold PBS (pH 7.0). Spleens were homogenized in culture dishes with RPMI medium containing 10% FBS and filtered through a 100-μm cell strainer to obtain single cell suspensions. CD4+ T lymphocytes were isolated from the splenocytes via magnetic separation using CD4+ Dynabeads according to the manufacturer’s recommended protocol (Invitrogen, Carlsbad, CA). Once released from the Dynabeads, CD4+ Th1 cells were washed in PBS and cultured in RPMI medium containing HEPES (25 mM/L), glutamine (2 mM/L), penicillin/streptomycin (100 U/mL), interleukin 2 (1.022 ng/mL), and interleukin 12 (4 ng/mL) for 24 hours at 5% CO₂ at 37°C in a humidified atmosphere. After centrifugation, cell pellets were washed with saline and adjusted to 1×10⁶ cells per 100 μL of saline for injection into recipient NP rats. Cell culture medium was retained for cytokine analysis via ELISA.

**Adoptive Transfer**

At gestational day 13, 1×10⁶ CD4+ T cells per 100 μL of saline (obtained as described above) were administered to NP rats or virgin rats via intraperitoneal injection. Blood pressure and circulating inflammatory cytokines were compared between the groups from plasma collected on day 19. The groups examined were as follows: NP rats; NP rats injected with NP CD4+ T cells (NP + NP CD4+ T cells); RUPP rats; NP rats injected with RUPP CD4+ T cells (NP + RUPP CD4+ T cells); virgin rats (V); virgin rats receiving NP CD4+ T cells (V + NP CD4+ T cells); and virgin rats receiving RUPP CD4+ T cells (V + RUPP CD4+ T cells).

**Measurement of Arterial Pressure in Chronically Instrumented Conscious Rats**

Arterial pressure was determined in all of the groups of pregnant rats at day 19 of gestation or 6 days after injection as described previously in protocol 1 and as we have published previously.

**Determination of Cytokine Production**

Supernatants collected from CD4+ T lymphocytes cultured for 24 hours and plasma from all pregnant rats injected with CD4+ T cells were measured for sFlt-1, IL-6, and TNF-α concentrations using commercial ELISA kits available from R&D Systems (Quantikine) according to the manufacturer’s protocol. IL-17 concentrations were measured using commercial ELISA kits available from eBioscience. Sensitivity of ELISAs is reported in the instruction manual provided.
As seen with previous studies,14 animals in the RUPP (n = 10) compared with placental ischemic (reduced uterine perfusion pressure [RUPP]; n = 10) rats. B, Percentage CD4+ T cells in freshly isolated peripheral blood leukocytes (PBL) collected from NP and RUPP rats on day 19 of gestation. (*P<0.05 vs NP). Percentage of CD4+ staining cells above the negative control was collected for each individual rat, and mean values for each experimental group (NP and RUPP) were calculated. Representative NP and RUPP PBL scatter plots and staining profiles are shown in C. PBL aliquots from NP or RUPP were stained with anti-CD4 followed by a fluorescein isothiocyanate-conjugated secondary antibody.

Statistical Analysis
All of the data are expressed as mean±SD. Differences between control and experimental groups were analyzed using the Student t test. Differences between multiple groups were analyzed via 1-way ANOVA, and post hoc analyses were obtained through Bonferroni post hoc test. Values of P<0.05 were considered significant.

Results
Protocol 1: Effect of Placental Ischemia on Blood Pressure and CD4+ T-Cell Dysregulation
As seen with previous studies,14 animals in the RUPP (n = 10) group had significant increases in mean arterial pressure compared with NP rats (n = 10; 125±2 RUPP versus 106±2 mm Hg NP; P<0.001; Figure 1A). Figure 1B and 1C demonstrate that CD4+ T lymphocytes are elevated in the circulation of our preeclamptic RUPP pregnant rat model compared with NP rats (46±4% RUPP versus 32±3% NP). In addition, we demonstrate that FoxP3+ T regulatory cells have a tendency to decrease and were 47% less in RUPP rats compared with NP rats (0.085±1.000% RUPP versus 0.178±0.20% NP); however, this difference did not reach statistical significance, with a P value of 0.06, which may be attributed to difficulty in detection of these cells within the circulation (Figure 2). Furthermore, in Figure 2 we demonstrate that circulating CD4+ Th17 cells, identified by expression of the RORγ transcription factor, are significantly elevated in response to placental ischemia during pregnancy compared with NP rats (22±8% RUPP versus 7±2% NP; P<0.05).

CD4+ T-Cell Secretion of TNF-α, IL-6, IL-17, and sFlt-1
CD4+ T cells isolated from NP and RUPP spleens were cultured overnight, and TNF-α, IL-6, IL-17, and sFlt-1 concentrations were determined from cell culture medium. After 24 hours of incubation, TNF-α secretion from RUPP CD4+ T cells was significantly increased compared with NP CD4+ T cells (250±50 pg/mL RUPP versus 133±23 pg/mL NP; P<0.05; Figure 3A). Likewise, IL-6 secretion from RUPP CD4+ T cells was 778±29 pg/mL compared with 287±12 pg/mL (P<0.05; Figure 3B) from NP CD4+ T cells.
IL-17 secretion from RUPP CD4⁺ T cells was significantly increased compared with NP CD4⁺ T cells (714 ± 30 pg/mL RUPP versus 245 ± 45 pg/mL NP; P < 0.05; Figure 3C), as was sFlt-1 secretion (2500 ± 650 pg/mL RUPP versus 1046 ± 280 pg/mL NP; P < 0.05; Figure 3D).

**Protocol 2: Effect of Adoptive Transfer of RUPP CD4⁺ Th Cells on Blood Pressure and Cytokines in NP Rats**

To determine a role for the CD4⁺ T cells stimulated in response to placental ischemia to mediate an increase in...
Effect of Adoptive Transfer on Circulating TNF-α, IL-6, IL-17, and sFlt-1

Circulating cytokines, TNF-α, IL-6, IL-17, and sFlt-1, were measured in plasma collected from control and experimental pregnant rats. We have shown previously that circulating levels of TNF-α, IL-6, IL-17, and sFlt-1 are all increased in RUPP rats compared with NP rats.17,19–20 TNF-α increased significantly in RUPP rats compared with NP rats and increased significantly with adoptive transfer of RUPP CD4+ T cells to NP rats (Figure 5A). IL-17 increased in RUPP rats compared with NP rats but was not elevated to the same degree in NP + RUPP CD4+ T cells (Figure 5B). In addition, IL-6 increased in RUPP compared with NP but was not significantly elevated in NP + RUPP CD4+ T cell rats (Figure 5C). Circulating sFlt-1 increased significantly in RUPP rats compared with NP rats and increased significantly with adoptive transfer of RUPP CD4+ T cells to NP rats. Importantly there was no significant increase with adoptive transfer of NP + NP CD4+ T cells (P=0.01) compared with NP rats (Figure 5D). Although IL-6 and IL-17 were elevated in NP + RUPP CD4+ T-cell recipient rats compared with NP rats, these cytokines did not reach the level of those seen in control RUPP rats. This may be indicative of the lack of endogenous immune stimulus, such as the original antigen presentation stimulating these cells in response to placental ischemia that is not present in the NP rats that received RUPP T cells. Nonetheless, these data may be indicators that, although placental ischemic T cells play a role in the hypertension in RUPP rats, they are not the sole factor influencing the phenotype seen during preeclampsia. Because adoptive transfer did not increase blood pressure in virgin rats, cytokine analysis was not performed in this study group.
Preeclampsia is associated with immune activation and an imbalance among T-lymphocyte regulatory mechanisms with a tendency toward the Th1/Th2 paradigm to play a role in mediating the pathophysiology associated with the disease. Previous studies demonstrated that adoptive transfer of activated Th1-like splenocytes into pregnant mice elicited high blood pressure, proteinuria, and an inflammatory response similar to that observed in preeclamptic women. However, the stimulus for T lymphocytes during preeclampsia remains unknown. With this study we demonstrated that placental ischemia is a stimulus for elevated circulating CD4+ T lymphocytes during pregnancy (Figure 1). Furthermore, we demonstrated that placental ischemic rats have lower levels of regulatory CD4+FoxP3+ T cells and significantly increased autoimmune-associated CD4+Th17 cells (Figure 2). We report that CD4+ T cells isolated from placental ischemic rats, as expected, secrete elevated levels of inflammatory cytokines but surprisingly also secrete excess antiangiogenic factor sFlt-1 in vitro (Figure 3). To determine whether this imbalance among CD4+ T cells in response to placental ischemia mediates hypertension, angiogenic imbalance, and/or inflammation during pregnancy, we adoptively transferred RUPP CD4+Th cells into NP and virgin rats. We demonstrated that adoptive transfer of RUPP CD4+Th cells caused a significant increase in mean arterial pressure, as well as circulating inflammatory cytokines, and stimulated the release of sFlt-1 (Figures 4 and 5). However, this imbalance in immune cells when injected into virgin rats had no effect on blood pressure.

We have demonstrated recently that TNF-α serves as a stimulus for the antiangiogenic factor sFlt-1 in pregnant rats. However, the role of immune cells stimulated in response to RUPP to secrete these cytokines and antiangiogenic factors or to mediate blood pressure effects during pregnancy were not examined. In this study we clearly demonstrated a role for CD4+ T cells, both in vitro and in vivo, to secrete and/or stimulate sFlt-1 in response to placental ischemia. To our knowledge, this is one of the first studies to demonstrate that T lymphocytes stimulated in response to placental ischemia play an important role in mediating the release of this potent antiangiogenic factor.

Although preeclampsia is associated with altered CD4+ T-cell ratios, it is unknown whether placental ischemia is a stimulus for the imbalance. The percentage of CD4+IL-17-producing T cells (Th17 cells) was increased significantly in preeclamptic pregnancies, whereas Tregs (FoxP3+ T cells) were decreased in women exhibiting preeclampsia compared with those with normal pregnancies. In addition, IL-17 was increased among women with preeclampsia compared with NP women, and IL-17 is a proinflammatory cytokine having many immune regulatory functions critical for Th17 cells and is strongly associated with autoimmune diseases such as asthma, lupus, and allograft rejection. IL-6 is the principal stimulus switching proregulatory signals into a Th17 mediator, thus making IL-6 the most important regulator of self versus nonself immune discrimination. Figure 2 demonstrates that the autoimmune-associated Th17 cells (indicated by intracellular staining of the RORγ transcription factor) are elevated in RUPP placental ischemic rats. Although not reaching statistical significance, we demonstrate that Treg cells (indicated by intracellular staining of the FoxP3 transcription factor) are lower in RUPP rats compared with NP rats. We also show increased TNF-α, IL-6, and IL-17 in RUPP versus NP rats. Collectively, these data support the theory that placental ischemia is a stimulus for the loss of self versus nonself regulatory T-cell responses during pregnancy. This may be simply because of early rises in IL-6, thus leading to altered Treg/Th17, or, alternatively, it may be because of upregulation of a specific antigen stimulated by placental ischemia. However, neither of these questions was addressed in this study. Nevertheless, we demonstrated a role for placental ischemic stimulated CD4+Th cells to increase blood pressure, antiangiogenic factors, and TNF-α during pregnancy. Future studies identifying a role for IL-17 and Th17 cells in mediating the pathophysiology of hypertension in response to placental ischemia and proteinuria in this model, as well as experiments measuring renal hemodynamics, will contribute to further defining the pathophysiological role of activated Th cells in mediating hypertension during pregnancy. In addition studies examining a role for CD4+ T cells to mediate endothelial cell activation and dysfunction will be important to understand the affects of T-cell activation on the maternal vasculature during pregnancy. Future studies are designed to further examine the role of placental ischemic induced IL-17 producing Th17 cells to mediate hypertension, endothelin 1, and agonistic autoantibodies to the angiotensin II type 1 receptor during pregnancy. Furthermore, experiments blocking T-cell activation or suppressing CD4+ T cells in response to placental ischemia are underway in our laboratory. Knowledge gained from these types of studies could lead to better treatment and early diagnostic strategies for women developing preeclampsia.

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Disclosures

None.

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