An increased population of regulatory T cells improves the pathophysiology of placental ischemia in a rat model of preeclampsia

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1Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi; and 2Department of Obstetrics and Gynecology, University of Mississippi Medical Center, Jackson, Mississippi; and 3Charité, Campus Buch, Experimental and Clinical Research Center and HELIOS Clinic Berlin-Buch, Berlin, Germany

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Cornelius DC, Amaral LM, Harmon A, Wallace K, Thomas AJ, Campbell N, Scott J, Herse F, Haase N, Moseley J, Wallukat G, Dechend R, LaMarca B. An increased population of regulatory T cells improves the pathophysiology of placental ischemia in a rat model of preeclampsia. Am J Physiol Regul Integr Comp Physiol 309: R884 –R891, 2015. First published August 19, 2015; doi:10.1152/ajpregu.00154.2015.—The reduced uterine perfusion pressure (RUPP) rat model of preeclampsia exhibits much of the pathophysiology characterizing this disease, such as hypertension, inflammation, suppressed regulatory T cells (TRegS), reactive oxygen species (ROS), and autoantibodies to the ANG II type I receptor (AT1-AA) during pregnancy. The objective of this study was to determine whether supplementation of normal pregnant (NP) TRegS into RUPP rats would attenuate the pathophysiology associated with preeclampsia during pregnancy. CD4+CD25+ T cells were isolated from spleens of NP and RUPP rats, cultured, and injected into gestation day (GD) 12 normal pregnant rats that underwent the RUPP procedure on GD 14. On GD 1, mean arterial pressure (MAP) was recorded, and blood and tissues were collected for analysis. One-way ANOVA was used for statistical analysis. MAP increased from 99 ± 2 mmHg in NP (n = 12) to 127 ± 2 mmHg in RUPP (n = 21) but decreased to 118 ± 2 mmHg in RUPP+NP TRegS (n = 17). Circulating IL-6 and IL-10 were not significantly changed, while circulating TNF-α and IL-17 were significantly decreased after supplementation of TRegS. Placental and renal ROS were 339 ± 58.7 and 603 ± 88.1 RLU·min⁻¹·mg⁻¹ in RUPP and significantly decreased to 178 ± 27.8 and 171 ± 55.6 RLU·min⁻¹·mg⁻¹, respectively, in RUPP+NP TRegS; AT1-AA was 17.8 ± 1.1 beats per minute (bpm) in RUPP but was attenuated to 0.5 ± 0.3 bpm with NP TRegS. This study demonstrates that NP TRegS can significantly improve inflammatory mediators, such as IL-17, TNF-α, and AT1-AA, which have been shown to increase blood pressure during pregnancy.

Hypertension; Pregnancy; Inflammation; Oxidative stress; Regulatory T cells

PREECLAMPSIA IS A PREGNANCY-ASSOCIATED disorder that affects 5–8% of pregnancies and is a major cause of maternal, fetal, and neonatal morbidity and mortality (9, 29, 37). Hallmark characteristics of preeclampsia are new-onset hypertension after 20 wk gestation, proteinuria, chronic immune activation, fetal growth restriction, and maternal endothelial dysfunction. The pathophysiological mechanisms that lead to the development of preeclampsia are poorly understood. It is thought that poor invasion of trophoblasts leads to insufficient spiral artery remodeling, resulting in placental ischemia (4, 10, 31). The hypoxic environment that results from this placental ischemia is suggested to be an important factor in the development of oxidative stress and a shift in the balance of antiangiogenic and proangiogenic factors, which plays a role in endothelial dysfunction of the placenta and maternal vasculature (10). Previous studies from our laboratory in the reduced uterine perfusion pressure (RUPP) rat model of preeclampsia demonstrate that the total population of CD4-positive (CD4+) T cells isolated from RUPP spleens and transferred to NP rats causes similar pathology to that seen in RUPP rats (46), demonstrating a role for this population in mediating pathophysiology in response to placental ischemia. Recent data from both clinical and animal model studies demonstrate an imbalance in the subpopulations of CD4+ T cells and a role for these cells as mediators of inflammation and hypertension during pregnancy (5, 36, 41, 43, 46, 47). Specifically, it has been proposed that the imbalance between two CD4+ T-cell subtypes, regulatory T cells (TRegS), and T-helper 17 cells (Th17s), is involved in the pathophysiology of preeclampsia (38, 41, 43).

Regulatory T cells play a major role in the development and maintenance of immune tolerance (8) and are classically identified by surface expression of CD4 and CD25, as well as the transcription factor forkhead box protein 3 (FoxP3) (8, 35). Regulatory T cells regulate the immune system by suppressing autoreactive T cells, dampening inflammation, and inducing tolerance in normal pregnancy. Maternal immune tolerance is dependent on TRegS and uterine natural killer (NK) cells, recognizing and accepting the fetal antigens and facilitating placental growth (44, 45). Studies suggest that failure of the maternal immune tolerance mechanisms precedes the development of placental ischemia and oxidative stress, both of which are known to be involved in the pathophysiology of preeclampsia (18). Clinical studies have shown that there is a decrease in the number of TRegS in preeclamptic women compared with women with normal pregnancies (5, 38, 42, 43). Our data echo these findings in that the TRegS population is a low percentage among total CD4+ T cells isolated from RUPP rats. There is approximately a 47% decrease in TRegS in their peripheral circulation of RUPP rats compared with normal pregnant rats (46).

We hypothesize that this decreased population of TRegS leads to the failure of maternal immune tolerance and the induction of placental ischemia, oxidative stress, AT1-AA, inflammatory cytokines, and other pathophysiological mechanisms of preeclampsia during pregnancy. Therefore, the objective of the study was to determine whether supplementation of TRegS from normal pregnant (NP) rats into RUPP rats before placental insult would attenuate or lessen the pathology associated with placental ischemia. This current study could shed light as to...
whether or not T_{Reg} numbers present in response to placental ischemia plays a role in allowing factors to be stimulated that cause hypertension and are associated with preeclampsia.

**MATERIALS AND METHODS**

Pregnant Sprague-Dawley rats purchased from Harlan Sprague Dawley (Indianapolis, IN) were used in this study. Animals were housed in a temperature-controlled room (23°C) with a 12:12-h light-dark cycle. All experimental procedures executed in this study were in accordance with the National Institutes of Health guidelines for use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

**Regulatory T-cell isolation, culture, and differentiation.** At the time of harvest (gestation day 19), spleens were collected from NP rats, and lymphocytes were isolated from spleens by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical & Scientific, Westbury, NY), according to the manufacturer’s instructions. Anti-CD4 and anti-CD25 antibodies (BD Biosciences, San Jose, CA) were biotinylated using the DSB-X biotin protein labeling kit (Life Technologies, Grand Island, NY), according to the manufacturer’s instructions. Isolated lymphocytes were incubated with biotinylated anti-CD4 antibody. The CD4^+ population was isolated using Flow-Comp Dynabeads (Invitrogen, Oslo, Norway), according to the manufacturer’s protocol. The CD4^+ population of lymphocytes was then incubated with biotinylated anti-CD25 antibody, and the CD4^+CD25^+ population was again isolated using FlowComp Dynabeads. Biotinylated antibodies and FlowComp Dynabeads were separated from cells, according to manufacturer’s protocol, prior to culture and expansion. The CD4^+CD25^+ splenocytes were incubated on anti-CD3 and anti-CD-28 beads in T-helper media (RPMI, 10% FBS, 5% PenStrep, 1% HEPES) in 96-well plates at 10^5 cells/well on day 0. On day 2, cells were removed from magnetic beads and cultured in T-helper specific media (RPMI, 10% FBS, 5% Pen-Strep, 1% HEPES, 20 ng/ml IL-2, 5 ng/ml TGF-β1, 10 ng/ml TNF-α) for 5–6 days, following standard protocols for expansion of the existing T_{Reg} population in culture (14–17). Differentiation of isolated cells into T_{Reg} cells was verified via flow cytometry.

**Determination of circulating cultured T_{Reg} lymphocyte population.** Circulating T_{Reg} lymphocyte population was determined from peripheral blood leukocytes (PBL) collected at day 19 of gestation from NP, RUPP, and RUPP+NP T_{Reg} animals by flow cytometry. At the time of harvest, plasma was collected, and peripheral blood mononuclear cells were isolated from plasma by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical & Scientific, Westbury, NY), according to the instructions of the manufacturer. The cultured CD4^+ T_{Reg} cell population was analyzed for purity of CD4^+CD25^+FoxP3^+ cells. For flow cytometric analysis, 1×10^6 cells were incubated for 30 min at 4°C with antibodies against rat CD4 and rat CD25 (BD Biosciences, San Jose, CA). After washing, cells were labeled with secondary fluorescein isothiocyanate (FITC; Southern Biotech, Birmingham, AL) and phycoerythrin with cyanin-5 (PE-Cy5; Santa Cruz Biotechnology, Santa Cruz, CA) antibody for 30 min at 4°C. Cells were washed, permeabilized, and stained with anti-mouse/rat FoxP3 conjugated to phycoerythrin (PE; eBioscience, San Diego, CA) for 30 min at 4°C. As a negative control for each individual rat, cells were treated exactly as described above, except they were incubated with isotype control antibodies conjugated to FITC, PE-Cy5, or PE secondary antibodies alone. Subsequently, cells were washed, fixed, and resuspended in 500 μl of Roswell Park Memorial Institute medium (RPMI) and analyzed for single, double, and triple staining on a Gallios flow cytometer (Beckman Coulter, Brea, CA) (Fig. 1). The lymphocyte population of cells was gated, and cells were examined for Foxp3-positive staining. The FoxP3-positive-stained cells were then examined for CD4- and CD25-positive staining. The percent of positive stained cells above the negative control was collected for three separate cultures or individual rats, and the mean values for each experimental group were calculated.

**Adaptive transfer of NP and RUPP T_{Reg} cells.** For consistency with previous adaptive transfer studies performed in our laboratory, 500 μl of cultured T_{Reg} cells, in sterile saline, were injected, intraperitoneally, into normal pregnant (NP) rats at 2×10^6 cells/ml on day 12 of gestation (GD12). In addition, GD12 was chosen to examine the effect of T_{Reg} supplementation prior to placental insult on pathology in response to placental ischemia. On GD14, under isoflurane anesthesia, recipients of the NP T_{Reg} cells underwent the RUPP procedure, as described below. The groups of rats examined in this study were NP (n = 12), RUPP (n = 21), and RUPP recipients of NP T_{Reg} cells (RUPP+NPT_{Reg}8, n = 17).

**Reduction of uterine perfusion pressure.** On GD14, under isoflurane anesthesia, a subset of NP rats and all NP recipients of NPT_{Reg}8 underwent RUPP with the application of a constrictive silver clip (0.203 mm) to the aorta superior to the iliac bifurcation, while ovarian collateral circulation to the uterus was reduced with restrictive clips (0.100 mm) to the bilateral uterine arteries at the ovarian end (11, 21, 46). Rats were excluded from the study when the clipping procedure resulted in total reabsorption of all fetuses.

**Measurement of mean arterial pressure in chronically instrumented conscious rats.** Under isoflurane anesthesia, 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 for 30 min after a 30-min stabilization period. Subsequently, blood and urine samples were collected; kidneys, placentas, and spleens were harvested; and litter size and pup weights were recorded under anesthesia.

Determination of cytokine production. Plasma and serum collected from all pregnant rats were measured for IL-4, IL-6, IL-10, IL-17, and IFN-γ concentrations using commercial ELISA kits available from R&D Systems (Quantikine-IL4, IL-6, IL-10, IL-17, TGF-β, IFN-γ) and MyBioSource (IL-35), according to the manufacturer’s protocol.

Determination of placental endothelin-1 levels in pregnant rats. Placentas were weighed and quickly frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the RNeasy Protect mini-kit supplied by Qiagen, as outlined in the instructions provided by the manufacturer. Real-time PCR was used, as previously described, to determine tissue preproendothelin-1 levels (23, 26).

Briefly, cDNA was synthesized from 1 μg of RNA with Bio-Rad iScript cDNA reverse transcriptase, and real-time PCR was performed using the Bio-Rad SYBR Green Supermix and iCycler. The following primer sequences, provided by Life technologies, were used for preproendothelin (PPET), as previously described: forward 1, ctagtgcaaggcttcg, and reverse 1, tctgggtgcttgc (21, 24, 25). Levels of mRNA were calculated using the mathematical formula for $2^{-ΔΔCt}$ ($2^{−ΔΔCt} = 2^{−(Ct_{target}−Ct_{end})−(Ct_{end}−Ct_{end/2})}$) recommended by Applied Biosystems (Applied Biosystems, User Bulletin, No. 2, 1997).

Determination of placental and renal reactive oxygen species. Superoxide production in the placenta and renal cortex was measured by using the lucigenin technique, as we have previously described (21, 34). Rat placentas and cortices from NP, RUPP, and RUPP+NPTRegs rats were snap frozen in liquid nitrogen directly after collection and stored at −80°C until further processing. Placentas and cortices were removed and homogenized in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Santa Cruz, Santa Cruz, CA), as described previously (21, 34). The samples were centrifuged at 16,000 g for 30 min, the supernatant was aspirated, and the remaining cellular debris was discarded. The supernatant was incubated with lucigenin at a final concentration of 5 μmol/L. The samples were allowed to equilibrate for 15 min in the dark, and luminescence was measured every second for 10 s with a luminometer (Berthold, Oak Ridge, TN). Luminescence was recorded as relative light units (RLU) per minute. An assay blank with no homogenate but containing lucigenin was subtracted from the reading before transformation of the data. Each sample was repeated five times, and the average was used for data transformation. The protein concentration was measured using a protein assay with BSA standards (Pierce, Rockford, IL). The data are expressed as RLU per minute per milligram of protein.

Determination of circulating AT1-AA. On day 19 of gestation, blood was collected and immunoglobulin was isolated from 200 μL of serum by protein G Sepharose on a biolune protein purification system (Knauer, Germany). This IgG fraction was used in a bioassay. The AT1-AA activity was measured using spontaneously beating neonatal rat cardiomyocytes and characterized and antagonized specifically using AT1 receptor antagonists. The results express the difference between the basal beating rate of the cardiomyocytes and the beating rate measured after the addition of the AT1-AA (increase in number of beats/min or Δ beats/min) (6, 7, 19, 21, 32, 33). AT1-As were assessed in NP, RUPP controls, and RUPP+NPTRegs rats.

Statistical analysis. All of the data are expressed as means ± SE. Comparisons of control with experimental groups were analyzed by ANOVA with Tukey’s multiple-comparison test as post hoc analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Adoptive transfer of NP TRegs cells significantly decreased blood pressure in RUPP rats. CD4+/CD25+ T cells were isolated from NP spleens and cultured, as described above; flow cytometry was performed to verify differentiation of cells into TREG population as shown in Fig. 1. Ninety-nine percent of the stained cells were double-positive for CD4+ and FoxP3+, and 65% of the stained cells were triple-positive for CD4+, FoxP3+, and CD25+ (Fig. 1). Adoptive transfer of NP TRegS significantly increased the circulating population of TRegs in the RUPP rat to levels similar to what is observed in NP animals [NP (n = 5) 4.65 ± 1.2% gated, RUPP (n = 6) 1.02 ± 0.68% gated (P < 0.05 vs. NP), RUPP+NPTRegs (n = 5) increased to 4.1 ± 0.69% gated (P < 0.05 vs. RUPP)] (Fig. 2). Mean arterial pressure (MAP) was measured on day 19 of gestation in NP, RUPP, and RUPP+NPTRegs rats (Fig. 3). The MAP increased significantly from 99 ± 2 mmHg in NP rats (n = 12) to 127 ± 2 mmHg in RUPP rats (n = 21, P < 0.0001). Supplementation of NP TRegS prior to placental insult into RUPP rats caused a significant decrease in blood pressure to 118 ± 2 mmHg in RUPP+NPTRegs (n = 17, P < 0.05 vs. RUPP). These statistics indicate the increase in blood pressure from NP compared with
RUPPs was much greater than NP compared with RUPP+NPTRegs. In addition, RUPP+NPTRegs had significantly lower blood pressure than control RUPPs, although blood pressure in RUPP+NP TReg remained higher than NP ($P < 0.0001$), thus, indicating that other mechanisms also contribute to the increased blood pressure in response to placental ischemia.

Adaptative transfer of NP TReg cells does not significantly improve intrauterine growth restriction in RUPP rats. In Fig. 4, pup weight of litters from RUPP rats (1.98 ± 0.05 g, $n = 21$) was significantly lower than pup weight from NP rats (2.3 ± 0.04 g, $n = 14$; $P < 0.01$). Adaptative transfer of NP TReg prior to placental insult into RUPP rats did not significantly increase pup weight (2.02 ± 0.08 g, $n = 17$; $P = 0.15$ compared with RUPP; $P < 0.05$ compared with NP). Placental weight and resorption rate were also unchanged. These statistics indicate that the effect of RUPP to decrease pup weight and cause fetal morbidity is not inhibited with the increased number of NP TReg used in this study.

NP TReg cell supplementation normalizes TGF-β in RUPP rats. Circulating levels of the anti-inflammatory cytokines IL-10, IL-35, IL-4, and TGF-β were measured in plasma (IL-4, IL-10) and serum (IL-35, TGFβ1) from all experimental groups. IL-10 was significantly decreased in RUPP rats compared with NP rats (RUPP 9.6 ± 2.7 pg/ml, $n = 11$ vs. NP 17.1 ± 6.4 pg/ml, $n = 11$, Fig. 5A). Adoptive transfer of NP TReg into RUPP rats ($n = 8$) did not alter circulating IL-10 (Fig. 5A). Circulating IL-35 and IL-4 were not different among the three groups. Serum levels of IL-35 were 2.4 ± 1.1 pg/ml in NP, 2.7 ± 2.6 pg/ml in RUPP, and 15.1 ± 11.1 pg/ml in RUPP+NPTRegs ($n = 5$/group). Plasma levels of IL-4 were 22.4 ± 3.7 pg/ml in NP, 18.7 ± 9.6 pg/ml in RUPP, and 42.3 ± 8.2 pg/ml in RUPP+NPTRegs ($n = 5$/group). Additionally, as we have previously published (12), serum TGF-β trended toward a decrease in response to placental ischemia (NP 1.036 ± 0.82 pg/ml vs. RUPP 567 ± 88 pg/ml, $n = 4$/group, $P = 0.06$) and was normalized after adoptive transfer of NP TReg into RUPP rats (965 ± 145 pg/ml, $n = 6$) (Fig. 5B).

NP TReg cell supplementation attenuates increased IL-17 and TNF-α in RUPP rats. Circulating levels of the proinflammatory cytokines IL-6, IFN-γ, IL-17, and TNF-α were measured in plasma from all experimental groups. Plasma IL-6 was significantly higher in RUPPs (NP: 30 ± 7 pg/ml, $n = 10$ vs. RUPP: 74 ± 15 pg/ml, $n = 13$, $P < 0.05$), and adoptive transfer of NP TReg did not alter circulating IL-6 in RUPP recipients ($n = 16$, Fig. 6A). Plasma IFN-γ was nearly doubled in response to placental ischemia (NP 5.0 ± 2.4 pg/ml vs. RUPP 9.9 ± 3.8 pg/ml, $n = 6$/group) and was normalized with adoptive transfer of NP TReg into RUPP rats (3.7 ± 1.6 pg/ml, $n = 6$) (Fig. 6B). Importantly circulating IL-17 and TNF-α levels significantly increased in RUPP rats compared with NP rats, and supplementation of NP TReg into RUPPs attenuated this increase: IL-17: NP ($n = 7$) 0.44 ± 0.24 pg/ml, RUPP ($n = 7$) 17.19 ± 7.42 pg/ml, $P < 0.05$ vs. NP, RUPP+NPTReg ($n = 8$) 1.37 ± 0.43 pg/ml, $P < 0.05$ vs. RUPP (Fig. 6C); TNF-α: NP ($n = 11$) 16.0 ± 6.4 pg/ml, RUPP ($n = 14$) 61.4 ± 12.2 pg/ml, $P < 0.01$ vs. NP; and RUPP+NPTReg ($n = 17$) 18 ± 6, $n = 17$, $P < 0.01$ vs. RUPP (Fig. 6D).

NP TReg decrease placental preproendothelin mRNA levels. Endothelin-1 is a potent vasoconstrictor that is increased in preeclampsia, and we have shown an important role for increased ET-1 to increase blood pressure in the RUPP rat (40). Real-time PCR was used to measure preproendothelin in the placenta of rats from each group. In Fig. 7, preproendothelin decreased in RUPP rats after supplementation with NP TReg. Preproendothelin (PPET-1) mRNA was twofold higher in the placentas of RUPP rats ($n = 6$) compared with NP rats ($n = 6$) and was blunted to 0.26-fold with NP TReg ($n = 6$) supplementation.

Adaptative transfer of NP TReg cells normalizes reactive oxygen species production in RUPP rats. Placental oxidative stress has been shown to be increased in preeclamptic women, and placental and renal oxidative stress is observed in preclinical models of preeclampsia, and it is implicated in contributing to...
the development of vascular dysfunction, proteinuria, and hypertension during pregnancy (9, 13, 27, 28). Importantly reactive oxygen species (ROS) is a byproduct of inflammation and is elevated in response to RUPP CD4+ T cells and IL-17- and AT1-AA-induced hypertension during pregnancy. Therefore, we wanted to determine whether supplementation with NP TRegs into RUPP rats prior to placental ischemia could lower inflammation and ROS. Fig. 8A shows that placental ROS was 240.9 ± 24.1 RLU·min⁻¹·mg⁻¹ in NP rats (n = 8), and 339.3 ± 58.7 RLU·min⁻¹·mg⁻¹ in RUPP (n = 7) rats. Adoptive transfer of NP TRegs into RUPP (n = 8) rats significantly lowered oxidative stress in the RUPP recipient placentas (178.1 ± 27.8 RLU·min⁻¹·mg⁻¹, P < 0.05 compared with RUPP), Fig. 8B shows that renal ROS was 410.5 ± 129.9 RLU·min⁻¹·mg⁻¹ in NP rats (n = 8) compared with 603.3 ± 88.1 RLU·min⁻¹·mg⁻¹ RUPP rats (n = 7). NP TReg supplementation into RUPP rats (n = 8) significantly reduced renal oxidative stress to below that observed in NP controls (171.3 ± 55.6 RLU·min⁻¹·mg⁻¹, P = 0.001 vs. RUPP rats).

Adoptive transfer of NP TReg cells attenuates AT1-AA in RUPP rats. AT1-AA is an important mediator of hypertension during preeclampsia, and we have shown it to be increased in RUPP rats. AT1-AAs in NP rats (n = 8) were 0.08 ± 0.25 bpm. AT1-AAs increased significantly to 17.81 ± 1.1 bpm in RUPP rats (n = 8) (Fig. 9, P < 0.0001) but were attenuated with supplementation of NP TRegS into RUPP rats (n = 8) (0.50 ± 0.3 bpm, P < 0.0001, Fig. 7).

DISCUSSION

The objective of this study was to determine a role for NP TRegS to counteract the pathology observed in response to placental ischemia in the RUPP rat. Data from this study demonstrate that adoptive transfer of NP TRegS into RUPP rats prior to placental insult results in lower blood pressure, blunts inflammation and ET-1 expression, normalizes tissue oxidative stress, and attenuates AT1-AA production in response to placental ischemia. This suggests that the number of TRegs is important to control inflammation and vasoactive factors contributing to hypertension during pregnancy, such as oxidative stress, AT1-AA, and ET-1. Although no improvement in fetal morbidity was observed with adoptive transfer of NP TRegS into RUPP rats, improving pathophysiology in the mother may indirectly benefit the fetus by increasing time to delivery, thereby, giving the fetus more time to develop and mature.

Immunosuppressive factors known to be secreted by TRegs include IL-10, TGFβ, and more recently, IL-35. All of these soluble factors have direct suppressive effects on effector T cells (3). IL-35 directly inhibits effector T-cell expansion and could affect Th17 cells and, thus, IL-17 (3). Although IL-35 was not significantly increased with TReg supplementation in
significantly lowered blood pressure, TNF-α, and AT1-AA, rats. We have recently shown that IL-10 supplementation and the induction of inducible T Regs. In this study, TGF-β and IL-10 are critical for inhibition of spontaneous and induced development of autoimmune diseases, respectively. TGF-β is thought to be important for peripheral tolerance and the induction of inducible TReg8. In this study, circulating TGF-β was normalized in RUPP rats after TReg supplementation. IL-10 and IL-4 also have important immunoregulatory and anti-inflammatory function. Surprisingly, increases in the circulating levels of IL-10 were not detected after adoptive transfer of NP TReg8 into placental ischemic RUPP rats. We have recently shown that IL-10 supplementation significantly lowered blood pressure, TNF-α, and AT1-AA, while increasing Tregs in RUPP rats (12). In that study, elevations in IL-10 were not detected; however, it could be that in each study, IL-10 is bound to its receptors and mediating anti-inflammatory function, such as repressing TNF-α, AT1-AA, and stimulating TReg8. In addition, IL-4, although not statistically significant, was elevated twofold with supplementation of RUPP rats with NP TReg8. Not many studies have been performed with IL-4 during preeclampsia; however, we know that it is important in programming of Th2 cells, which are known to be depressed in preeclampsia. Although not measured in this study, it could be that the twofold increase in IL-4 may have an effect on further influencing the T-cell profile and will be the subject of future investigations from our laboratory.

In this study, we demonstrate that adoptive transfer of TReg8 from NP rats into RUPP rats reduced TNF-α stimulated in response to placental ischemia. We have previously shown an important role for TNF-α to stimulate AT1-AA, oxidative stress, and ET-1 as mechanisms of hypertension when it is infused into NP rats (1, 22, 24). Conversely, TNF-α has no effect to increase blood pressure in virgin rats. Furthermore, administration of the soluble inhibitor of TNF-α to RUPP rats significantly lowered blood pressure, ET-1, and endothelial cell activation (20). Likewise, in the current study, with supplementation of TReg numbers in the RUPP rat, ET-1, placental and renal oxidative stress, and AT1-AA were all attenuated, possibly by lowering TNF-α. These data indicate not only the importance of inhibition of these hypertensive mechanisms in response to placental ischemia but how influential TReg numbers are in controlling TNF-α and important vasoactive factors that are stimulated in response to placental ischemia. Therefore, these data support a role for a loss of TReg numbers in the pathophysiology of preeclampsia, potentially by controlling factors stimulated by TNF-α and/or IL-17. The supplementation of TReg8 prior to the time of placental ischemia (GD 12 vs. GD 14) suggests the importance of TReg8 in immune regulation in maintaining lower levels of oxidative stress, AT1-AA, and PPET-1, factors, which contribute to higher blood pressure, in the face of placental ischemia.

The importance of TReg8 in maintenance of early pregnancy has previously been established (2, 39). Regulatory T cells from NP animals were introduced at the beginning of late pregnancy, but prior to placental ischemia, and were able to prevent much of the pathophysiology that usually occurs in response to placental ischemia. This would suggest that the TReg maintenance of proper immune function may be important in the middle to late pregnancy, as well. From a clinical perspective, preeclampsia can develop in the middle of pregnancy (~24 wk) or in late gestation (~39 wk). Our study demonstrates that maintenance of proper immune regulation, even in middle to late gestation, may be important to blunt the inflammatory mediators leading to vasoactive pathways that cause hypertension and the pathophysiology that accompanies preeclampsia.

Fig. 8. NP TReg8 blunt placental and renal oxidative stress. Oxidative stress is a hallmark of preeclampsia. Oxidative stress was measured in the placentas (A) and renal cortices (B) of NP (n = 8), RUPP (n = 7), and RUPP + NPTReg8 (n = 8) rats using chemiluminescence methods. Tukey’s test was performed as post hoc analysis to generate P values. (*P < 0.05).

Fig. 9. NP TReg8 decrease production of AT1-AA. AT1-AA levels were measured via chronotropic events in cardiomyocytes in culture (beats/min). Tukey test was performed as post hoc analysis to generate P values. (*P < 0.0001).
Inhibition of endogenous effector T-cell activation in RUPP rats could also be the mechanism by which inflammation and oxidative stress are decreased after adoptive transfer of TRegS from NP rats. Without T-cell activation, inflammatory cytokine production would be decreased. This, in turn, would result in fewer inflammatory cells and less production of ROS. Our previous study of T-cell depletion with abatacept indicated that decreasing the overall CD4+ T-cell count improved the pathology in RUPP rats. However, abatacept also inhibits maturation of T helper 2 and TRegS and, thereby, calling into question the maternal benefit of treating preeclampsia with a global T-cell inhibitor such as abatacept. Therefore, we feel it is important to continue looking for safe alternatives to boost Treg during pregnancy. In our current study, we did not quantitate the total CD4+ T-cell population after TReg supplementation, nor did we measure the TReg/T effector ratio in our recipient rats (30). However, the data from this study suggest that supplementation of TRegS had a suppressive effect on immune activation, as supported by significant decreases in plasma TNF-α and IL-17, tissue oxidative stress, and AT1-AA.

Studies investigating the mechanistic pathways modulating TReg inhibition of the pathophysiology in preeclampsia need to be conducted. Supplementation of TReg soluble factors, such as IL-10, TGFβ, and IL-35, or IL-4 into preeclampsia animal models may provide insight into the mechanisms involved in the effect that TRegS have in averting preeclampsia. Future studies investigating TReg modalities or stimulators will be important to determine a role for the TReg cell in the normal development and growth of the fetus, as well as correcting the chronic inflammation known to exist following placental ischemia. These type of studies will lend insight into mechanisms and pathways that could lead to the identification of drug targets and development of more effective treatments for this maternal syndrome.

**Perspectives and Significance**

Importantly, this study demonstrates a role for decreased TRegS in the pathophysiology of preeclampsia. The data presented suggest that regulation of the immune response by TRegS is critical to maintenance of maternal health during pregnancy. Increasing the population of TRegS may help to normalize the altered immune response and regulate inflammation, resulting in decreased blood pressure, endothelial dysfunction, inflammation, oxidative stress, and AT1-AA production. The benefits of restoration in number of the TReg population could lead to healthier, prolonged pregnancies and decreases in the maternal and perinatal morbidity and mortality associated with preeclampsia.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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