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## Adoptive Transfer of Placental Ischemia Stimulated Natural Killer Cells Causes a Preeclampsia-Like Phenotype in Pregnant Rats

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### Abstract

**PROBLEM**—The Reduced Uterine Perfusion Pressure (RUPP) rat model of placental ischemia recapitulates many characteristics of preeclampsia including maternal hypertension, intrauterine growth restriction (IUGR), and increased cytolytic natural killer cells (cNKs). While we have previously shown a 5-fold higher cytotoxicity of RUPP NKs vs normal pregnant NKs, their role in RUPP pathophysiology remains unclear. In this study, we tested the hypotheses that (1) adoptive transfer of RUPP-stimulated NKs will induce maternal hypertension and IUGR in normal pregnant control (Sham) rats and (2) adoptive transfer of Sham NKs will attenuate maternal hypertension and IUGR in RUPP rats.

**METHOD OF STUDY**—On gestation day (GD)14, vehicle or  $5 \times 10^6$  RUPP NKs were infused i.v. into a subset of Sham rats (Sham+RUPP NK), and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats (RUPP+Sham NK; n=12/group). On GD18, Uterine Artery Resistance Index (UARI) was measured. On GD19 mean arterial pressure (MAP) was measured, animals were sacrificed, and blood and tissues were collected for analysis.

**RESULTS**—Adoptive transfer of RUPP NK cells into Sham rats resulted in elevated NK activation, UARI, placental oxidative stress, and preproendothelin expression as well as reduced circulating nitrate/nitrite. This led to maternal hypertension and IUGR. RUPP recipients of Sham NK cells demonstrated normalized NK activation, sFlt-1, circulating and placental VEGF, and UARI, which led to improved maternal blood pressure and normal fetal growth.

**CONCLUSIONS**—These data suggest a direct role for cNKs in causing preeclampsia pathophysiology and a role for normal NKs to improve maternal outcomes and IUGR during late gestation.

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There are no disclosures

## Keywords

Preeclampsia; Natural Killer Cells; Hypertension; Pregnancy

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## INTRODUCTION

Preeclampsia (PE) is a multisystem obstetric disorder that affects around 2–8% of pregnancies and remains one of the leading causes of maternal and fetal morbidity worldwide.[1] Preeclampsia is characterized by maternal hypertension, intrauterine growth restriction (IUGR) and end organ damage that can manifest as proteinuria, liver dysfunction, or cerebral disturbances.[2] The disorder can also progress to eclampsia, a life-threatening condition characterized by unexplained seizures and multi-organ failure.[3, 4] Together, PE and eclampsia contribute to 10%–15% of maternal deaths.[5] Despite decades of research, the only cure for PE is the delivery, often times early, of the fetus and placenta, which contributes significantly to negative fetal outcomes. [2, 3]

One reason for the lack of treatments is that the pathogenesis of PE is still not completely understood.[6] It is widely believed that PE occurs because of insufficient remodeling of the utero-placental vasculature by cytotrophoblasts, leading to reduced blood flow to the fetus and placenta.[3, 4, 7] The resultant ischemia promotes placental release of pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF)- $\alpha$  and Interleukin (IL)-17 as well as antiangiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) into the maternal circulation.[2–4] This is thought to lead to the maternal endothelial dysfunction, oxidative stress, and increased immune activation that characterize PE.[3, 4]

Natural Killer (NK) cells play an important role in pregnancy, and have been implicated in several pregnancy disorders including PE.[8, 9] In the circulation, these innate lymphocytes primarily function to destroy virally infected or cancerous cells through the release of cytolytic enzymes.[10] However, during normal human pregnancy, a subset of NK cells known as decidual NK cells (dNK) display little cytotoxicity and instead play a supportive role. dNKs make up 70% of decidual cells and function as important immune regulators at the maternal-fetal interface in human pregnancies.[11–13] dNK cells also play important roles in placentation by regulating trophoblast invasion and promoting spiral artery remodeling.[9, 14, 15] Importantly, several clinical studies have documented a Type 1 shift in NK cells towards an activated phenotype in women with pregnancy disorders including recurrent spontaneous abortion (RSA) (early gestation) and PE (late gestation).[7, 8, 16] These cytolytic NK cells (cNKs) display increased secretion of TNF- $\alpha$  and Interferon (IFN)- $\gamma$ , decreased secretion of vascular endothelial growth factor (VEGF), and increased cytotoxicity.[7, 14]

The Reduced Uterine Perfusion Pressure (RUPP) model of placental ischemia has been extensively used to investigate the role of various immune cell types in the development and pathophysiology of PE. [17–19] This well-established animal model recapitulates many of the aforementioned characteristics observed in preeclamptic women including hypertension, IUGR, increased sFlt-1, and chronic immune cell activation.[20] We have previously published that cNKs are significantly increased in RUPP rats compared to their normal

pregnant counterparts.[21, 22] We also showed a 5-fold increase in cytotoxicity of placental NK cells from RUPP compared to those of their normal pregnant counterparts.[22] Additionally, we previously depleted NK cells in RUPP and saw a significant reduction in maternal blood pressure and improved fetal weight.[21] However, because this study depleted all subsets of NK cells, it did not reveal the precise role that cNK cells play in the pathophysiology observed in RUPP animals. Therefore, in this study, we aimed to test the following hypotheses: 1.) RUPP-stimulated NKs play a direct role in causing maternal HTN and IUGR in pregnant rats by inducing exaggerated inflammation, oxidative stress, and endothelin-1 (ET-1), while reducing nitric oxide (NO) to cause endothelial dysfunction and increased vascular resistance; 2.) Normal pregnant control (Sham) NKs attenuate maternal HTN and IUGR in RUPP rats by suppressing inflammation, oxidative stress, and ET-1, while stimulating NO to improve endothelial function and decrease vascular resistance. To test this, we adoptively transferred NK cells from RUPP rats into Sham rats and evaluated changes in NK cell activation, UARI, sFlt-1, eNOS expression and phosphorylation, preproendothelin (PPET) transcription, and oxidative stress as mechanisms to increase MAP and IUGR. Additionally, we transferred placental NK cells from Sham rats into RUPP rats to determine if the normally supportive nature of these cells can improve the pathophysiology observed in RUPP rats.

## METHODS

The authors declare that all supporting data are available within the article and its online supplementary files.

### Animals

12–13 week old, timed pregnant Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN). The animals were delivered to the Center for Comparative Research at the University of Mississippi Medical Center on day 10 or 11 of their gestation and weighed approximately 250–260g upon delivery. The animals were housed in a temperature-controlled room (23°C) with a 12:12-h light-dark cycle and maintained on a normal diet. Rats were randomly assigned to experimental groups. The rats were group housed until after surgery. All experimental procedures were carried out 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.

### Reduction in uterine perfusion pressure.

On Day 14 of gestation (*GD14*), animals underwent either RUPP or Sham surgeries under 2% isoflurane anesthesia delivered by a vaporizer (Ohio Medical Products, Madison, WI). The RUPP surgery was performed to induce placental ischemia as previously described.[23, 24] The animals received carprofen immediately following surgery and 24 hours post-surgery (5 mg/kg) to control for post-operative pain. Sham animals were subjected to the surgery without application of clips. Rats were excluded when the procedure resulted in total reabsorption of all fetuses.

## NK cell isolation

On the day of harvest, placentas from Sham and RUPP rats were collected and homogenized in RPMI (10% FBS). The homogenate was passed through a 70  $\mu\text{m}$  filter and lymphocytes were isolated by centrifugation on a cushion of Ficoll-Hypaque according to the instructions of the manufacturer. The isolated lymphocytes were incubated with the biotin labeled anti-CD3 and anti-biotin microbeads. The CD3<sup>+</sup> population was depleted using (LD) columns (Miltenyi Biotec) according to the manufacturer's instructions. The flow-through containing the CD3<sup>-</sup> population of lymphocytes was then incubated with PE-labeled anti-CD161 antibody and subsequently incubated with anti-PE microbeads (Miltenyi Biotec). CD3<sup>-</sup> CD161<sup>+</sup> NK cells were isolated using a MS column (Miltenyi Biotec) according to the manufacturer's protocol prior to culture and expansion. The collected cells were resuspended in NK cell activation media (RPMI, 10% FBS, 1% Pen/Strep, 2 ng/mL IL-2) [25–27], and allowed to expand in culture for 48 hrs.

## Adoptive Transfer of Natural Killer Cells

On GD14, vehicle or  $5 \times 10^6$  expanded RUPP NK cells were infused i.v. via the femoral vein into a subset of Sham rats (Sham n=12, Sham+RUPP NK, n=12), and vehicle or  $5 \times 10^6$  expanded Sham NK cells were infused i.v. via the femoral vein into a subset of RUPP rats (RUPP n=12, RUPP+Sham NK, n=12).

## Determination of Uterine Artery Resistance Index

On GD 18, rats from all 4 groups were shaved and all abdominal hair was removed with a depilation cream. Power Doppler velocimetry measurements were performed on anesthetized pregnant dams at an imaging station with a Vevo 770 unit (Visual Sonics, Toronto, Canada) using a 30 Hz transducer and an insonating angle  $<30^\circ$  as previously described.[28] The peak systolic flow velocity (PSV) and end diastolic flow velocity (EDV) were measured bilaterally. The uterine artery resistance index was calculated using the following formula:  $\text{UARI} = (\text{PSV} - \text{EDV}) / \text{PSV}$ . Uterine artery resistance index was determined using the mean measurements of three waveforms per side.

## Mean Arterial Pressure measurement and sample collection

Under isoflurane anesthesia, 0.58 mm I.D. x 0.99 mm O.D vinyl catheter tubing (Scientific Commodities Inc., Lake Havasu City, AZ) was implanted into the carotid arteries and tunneled to the back of the neck on GD18 for the measurement of mean arterial pressure. On GD19, rats were placed in individual restrainers and conscious mean arterial pressure (MAP) was monitored with a pressure transducer (Powerlab, AD Instruments, Colorado Springs, CO). The MAP was recorded for 30 minutes following a 30-minute stabilization period. After MAP measurement, the animals were anesthetized for blood and tissue collection. Placental and fetal weights were recorded for each dam and averaged. Randomly selected placentas were processed immediately or snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis.

### **Determination of circulating and placental NK cell populations by flow cytometry**

Single-cell suspensions of placental leukocytes were prepared as previously described [21, 22, 29]. Single-cell suspensions ( $1 \times 10^6$  cells) were stained for flow cytometry after being blocked with 10% goat and mouse serum. Antibodies used for flow cytometry were as follows: VioGreen anti-CD3, anti-ANK61 antibody, anti-mouse FITC, anti-ANK44, and anti-mouse Alexa Fluor 405. Flow cytometry was performed on the MACSQuant Analyzer 10 (Miltenyi Biotec) and analyzed using FlowLogic software (Innovai, Sydney, Australia). Lymphocytes were gated in the forward- and side-scatter plot. CD3- ANK61+ cells identified the total NK cell population and CD3- ANK61+ ANK44+ cells identified cNK cells. After doublet exclusion, additional gates were set using fluorescence-minus-one controls. Results are expressed as percentage of cells in the gated lymphocyte population.

### **Determination of systemic and placental oxidative stress**

Plasma 8-isoprostane (8-isoPGF<sub>2α</sub>) concentrations were measured in duplicate with commercially available enzyme linked immunosorbent (ELISA) assays kits (#516351, Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Superoxide production in the placenta was measured using the lucigenin technique as previously described [22–24, 30]. Each sample was run in triplicate and the average was used for data transformation. The protein concentration was measured using a protein assay with BSA standards (Pierce, Rockford, IL), and the data are expressed as RLU/min/mg.

### **Determination of total Nitrate/Nitrite and cGMP levels**

Blood was collected in EDTA tubes and spun at 3000 x g for 10 minutes at 4° C. The collected plasma was assessed in duplicate using the Nitrate/Nitrite Colorimetric Assay Kit (Catalog# 78001, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Levels of cGMP were measured in plasma and placental homogenates in duplicate using the Cyclic GMP ELISA Kit (# 581021, Cayman Chemical) according to the manufacturer's instructions. The protein concentration of the placental homogenates was measured using a protein assay with BSA standards. All placental data were normalized to protein and are expressed as pmol/mg.

### **Determination of Placental PreProEndothelin (PPET) mRNA levels.**

RNA extraction from the placenta was done via the RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's protocol. 1ug of the extracted RNA was then used to synthesize cDNA, then transformed to cDNA via the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative Real-time PCR (qRT-PCR) was used to express preproendothelin-1 (PPET-1) via the Bio-Rad iQ SYBR Green Supermix and the CFX96 Touch Real-Time PCR Detection System was used for analysis. The following primers were used: Forward primer: CTAGGTCTAAGCGATCCTTG and reverse primer: TCTTTGTCTGCTTGGC [31]. PPET mRNA levels were calculated according to the following formula  $2^{-Ct}$  ( $2^{\text{avg. Ct gene of interest} - \text{avg Ct beta actin}}$ ) as recommended by Applied Biosystems (Foster City, CA).

### Determination of placental and circulating cytokines and angiogenic factors

Circulating and placental IL-17, TNF- $\alpha$ , IFN- $\gamma$ , VEGF, and circulating IL-6 were measured using the Bio-Plex Pro Rat Cytokine Immunoassay Kit (Bio-Rad) according to the manufacturer's instructions. Circulating sFlt-1 levels were measured in collected plasma via ELISA (Catalog# MVR100 R&D systems, Minneapolis, MN) according to the manufacturer's instructions. All sample analyses were performed in duplicate. Protein concentration of the placental homogenates was measured using a protein assay with BSA standards. All placental data were normalized to protein and are expressed as pg/mg.

### Western blot analysis of endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (peNOS).

Placental eNOS and peNOS expression was assessed in placental tissue via Western blot as previously described. [28] Briefly, placentas were homogenized in cold RIPA-buffer and protein extracts, separated by SDS-PAGE using a polyacrylamide gel (4–20%). Protein was transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 5% Blotting-Grade Nonfat Dry milk (Bio-Rad) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody directed against eNOS or eNOS (pS1177) and  $\beta$ -actin, followed by anti-mouse IgG IRDye® 800Dx conjugated secondary antibody for 1 hr at room temperature and scanned using the Odyssey CLx Imager (LI-COR Biosciences, Lincoln, NE). The intensity of specific bands was quantified by densitometry using Image J (National Institutes of Health, USA) and placental eNOS or peNOS expression was normalized to  $\beta$ -actin.

### Antibodies

Specific information for antibodies used in Flow Cytometry, NK isolations, and Western Blot is located in the Major Resources Table.

### Statistical analysis

All of the data are expressed as mean  $\pm$  SEM. Statistical analyses were performed with one-way ANOVA with multiple comparisons followed by Tukey's post-hoc test using GraphPad Prism (Version 8.1.2). A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Effects of NK Adoptive Transfer on Circulating and Placental NK populations

Similar to what we have previously shown [21, 22], total circulating NK cells were elevated in RUPP with a value of  $40 \pm 4\%$  compared to  $13 \pm 5\%$  in Sham ( $p = 0.0008$  vs Sham). Total circulating NK cells were elevated in Sham rats that received RUPP NKs at  $38 \pm 3\%$  ( $p = 0.0032$  vs Sham) and were reduced in RUPP rats that received Sham NKs at  $17 \pm 6\%$  ( $p = 0.005$  vs RUPP; Figure 1A). Similarly, cNKs in the circulation were elevated in RUPP with  $18 \pm 3\%$  gated compared to  $6 \pm 1\%$  in Sham ( $p = 0.005$  vs Sham). Circulating cNKs were also elevated in Sham+RUPP NK with  $17 \pm 3\%$  of gated lymphocytes ( $p = 0.008$  vs Sham). Additionally, circulating cNK were reduced in RUPP+Sham NK compared to RUPP at  $7 \pm 2\%$ . ( $p = 0.01$  vs RUPP; Figure 1B).

Within the placenta, there was a similar trend. Total placental NK cells were elevated in RUPP at 29±4% compared to 9±2% in Sham (p=0.0002 vs Sham). Total NKs were elevated in Sham recipients of RUPP NKs at 22±4% (p=0.02 vs Sham) and were reduced in RUPP recipients of Sham NKs at 8±2% in (p=0.0001 vs RUPP; Figure 1C). Placental cNKs cells were elevated in RUPP at 15±3% compared to 3±1% in Sham (p=0.0006 vs Sham) and were also elevated in Sham+RUPP NK at 14±3% (p=0.004 vs Sham). These cells were reduced in RUPP+Sham NK at 3±1% (p=0.0005 vs RUPP; Figure 1D). The gating strategy for NK cell analysis is shown in Figure S1.

### Effects of NK Adoptive Transfer on UARI and placental PPET

Similar to what has been previously published[17, 28] UARI was elevated in RUPP at 0.75±0.02 compared to 0.56±0.02 in Sham (p=0.001 vs Sham). UARI was elevated in Sham + RUPP NK at 0.76±0.05 (p=0.001 vs Sham) and was reduced in RUPP recipients of Sham NKs at 0.63±0.02 (p=0.049 vs RUPP; Figure 2A). Placental PPET expression was 3 fold higher in Sham+RUPP NK compared to Sham (p= 0.0010 vs Sham). Expression was 1.7±0.3 in RUPP (p=0.48 vs Sham) and 0.67±0.11 in RUPP+Sham NK. (p=0.16 vs RUPP; Figure 2B).

### Effects of NK Adoptive Transfer on MAP and IUGR

MAP was also elevated in RUPP at 127±2mmHg in RUPP compared to 103±2 mmHg in Sham (p<0.0001 vs Sham) and was elevated in Sham recipients of RUPP NKs at 119±2 mmHg (p<0.0001 vs Sham). MAP was reduced in RUPP recipients of Sham NKs at 113±2 mmHg (p<0.0001 vs RUPP), although it remained elevated compared to Sham (p=0.001 vs Sham; Figure 3A). Mean pup weight was reduced in RUPP at 2.0±0.05 g compared to 2.4±0.06 g in Sham (p<0.0001 vs Sham). Similarly, mean pup weight of Sham recipients of RUPP NKs was reduced at 2.1±0.07 g (p=0.0008 vs Sham) and was normalized to 2.3±0.03g in RUPP recipients of Sham NKs. (p=0.001 vs RUPP; Figure 3B). Mean placental weight was also reduced in RUPP at 0.47±0.01 g compared to 0.56±0.02 g in Sham (p=0.0007 vs Sham). Sham+RUPP NK placental weight was reduced at 0.47±0.01 g (p=0.002 vs Sham) and RUPP+Sham NK placental weight was elevated at 0.55±0.02 g (p=0.004 vs RUPP; Figure 3C)

### Effects of NK Adoptive Transfer on Circulating and Placental VEGF and sFlt-1

sFlt-1 was elevated in RUPP at 1391±424 pg/mL compared to 76±15 pg/mL in Sham (p=0.007 vs Sham) and was 780±256 pg/mL in Sham recipients of RUPP NKs (p=0.26 vs Sham). Infusion of Sham NKs into RUPP rats normalized sFlt-1 to 67±8 pg/mL (p=0.005 vs RUPP; Figure 4A). Circulating VEGF was reduced in RUPP at 112±21 pg/mL compared to 506±78 pg/mL in Sham (p=0.02 vs Sham). Circulating VEGF was reduced in Sham+RUPP NK at 92±15 pg/mL (p= 0.01 vs Sham) and was elevated in RUPP+Sham NK at 910±187 pg/mL (p<0.0001 vs RUPP; Figure 4B). Similarly, placental VEGF was reduced in RUPP at 4.6±0.5 pg/mg compared to 40.3±5.9 pg/mg in Sham (p<0.0001 vs Sham). Placental VEGF was also reduced in Sham+RUPP NK at 3.2±0.6 pg/mg (p<0.0001 vs Sham) and was elevated in RUPP+Sham NK at 20.4±4.9 pg/mg (p=0.03 vs RUPP) although the levels were still reduced compared to Sham (p=0.0045 vs Sham; Figure 4C).

### Effects of NK Adoptive Transfer on Placental and Circulating Oxidative Stress, cGMP, and Circulating Nitrate/Nitrite.

Placental ROS was elevated in RUPP at  $63 \pm 6$  RLU/min/mg compared to  $35 \pm 2$  RLU/min/mg in Sham ( $p=0.02$  vs Sham). It was also elevated in Sham+RUPP NKs at  $60 \pm 5$  RLU/min/mg ( $p=0.005$  vs Sham) and remained elevated at  $70 \pm 12$  RLU/min/mg in RUPP+Sham NK ( $p=0.98$  vs RUPP; Figure 5A). Placental levels of cGMP were  $0.01 \pm 0.002$  pmol/mg in Sham,  $0.006 \pm 0.0005$  pmol/mg in RUPP,  $0.006 \pm 0.0009$  pg/mol in Sham+RUPP NK and  $0.01 \pm 0.002$  pmol/mg in RUPP+Sham NK ( $p>0.05$  for all comparisons; Figure 5B).

Circulating levels of Isoprostane-8 were  $54 \pm 7$  pg/mL in Sham,  $122 \pm 37$  pg/mL in RUPP,  $137 \pm 32$  pg/mL in Sham+ RUPP NK, and  $126 \pm 41$  pg/mL in RUPP+Sham NK ( $p>0.05$  for all comparisons; Figure 5C). Total levels of plasma nitrate/nitrite were reduced in RUPP at  $10.6 \pm 2.1$   $\mu$ M/mL compared to  $47.5 \pm 8.9$   $\mu$ M/mL in Sham ( $p=0.0008$  vs Sham). The levels were also reduced in Sham+RUPP NK at  $22.1 \pm 5.9$   $\mu$ M/mL ( $p=0.03$  vs Sham) but were elevated in RUPP+Sham NK at  $63.5 \pm 6.8$   $\mu$ M/mL ( $p<0.0001$  vs RUPP; Figure 5D). Plasma levels of cGMP decreased from  $26 \pm 2$  pmol/mL in Sham to  $16 \pm 3$  pmol/mL in RUPP ( $p=0.04$  vs Sham). cGMP was  $25 \pm 3$  pmol/mL in Sham+RUPP NK ( $p=0.98$  vs Sham) and  $18 \pm 2$  pmol/mL in RUPP+Sham NK ( $p=0.97$  vs RUPP; Figure 5E).

### Effects of NK Adoptive Transfer on Circulating and Placental Cytokines

All circulating and placental cytokine data are summarized in Table 1. Circulating IL-6 was elevated in RUPP at  $426 \pm 56$  pg/mL compared to  $138 \pm 21$  pg/mL in Sham ( $p=0.0004$  vs Sham) and was also elevated in Sham+RUPP NK at  $338 \pm 40$  pg/mL ( $p=0.02$  vs Sham). Compared to RUPP, circulating IL-6 was reduced in RUPP+Sham NK at  $245 \pm 56$  pg/mL ( $p=0.042$  vs RUPP). Circulating IL-17 was elevated in RUPP at  $30.5 \pm 4.9$  pg/mL compared to  $6.8 \pm 1.6$  pg/mL in Sham ( $p=0.008$  vs Sham) and remained elevated in RUPP+Sham NKs at  $45.5 \pm 8.9$  pg/mL ( $p=0.19$  vs RUPP). There was a trend toward elevated circulating IL-17 in Sham+RUPP NK at  $23.8 \pm 3.7$  pg/mL ( $p=0.08$  vs Sham). Circulating TNF- $\alpha$  was elevated in RUPP at  $152 \pm 17$  pg/mL compared to  $30.3 \pm 5.0$  pg/mL in Sham ( $p<0.0001$  vs Sham), was elevated in Sham+RUPP NK at  $109 \pm 14$  pg/mL ( $p=0.002$ ), and remained elevated in RUPP+Sham NK at  $115.4 \pm 17$  pg/mL ( $p=0.27$  vs RUPP). Levels of circulating IFN- $\gamma$  were elevated in RUPP at  $95 \pm 15$  pg/mL compared to  $19 \pm 3$  pg/mL in Sham ( $p<0.0001$  vs Sham) and remained elevated at  $75 \pm 13$  pg/mL in RUPP+Sham NK ( $p=0.53$  vs RUPP). The levels remained normal in Sham+RUPP NK at  $45 \pm 7$  pg/mL ( $p=0.36$  vs Sham).

In the placenta, levels of IL-17 were elevated in RUPP at  $4 \pm 0.7$  pg/mg compared to  $0.6 \pm 0.2$  in Sham ( $p=0.0001$  vs Sham) and remained elevated at  $5 \pm 0.7$  pg/mg in RUPP+Sham NK ( $p=0.53$  vs RUPP). Placental IL-17 remained normal in Sham+RUPP NK at  $0.6 \pm 0.2$  pg/mg ( $p=0.9997$  vs Sham). Similarly, placental TNF- $\alpha$  was elevated in RUPP at  $64 \pm 7$  pg/mg compared to  $36 \pm 4$  pg/mg in Sham ( $p=0.007$  vs Sham) and remained elevated at  $72 \pm 5$  pg/mg in RUPP+Sham NK ( $p=0.77$  vs RUPP). Placental TNF- $\alpha$  remained normal in Sham+RUPP NK at  $46 \pm 5$  pg/mg ( $p=0.63$  vs Sham). Placental IFN- $\gamma$  was elevated in RUPP at  $16 \pm 3$  pg/mg compared to  $5 \pm 1$  pg/mg in Sham ( $p=0.007$  vs Sham) and was also elevated in Sham+RUPP NK at  $15 \pm 2$  pg/mg ( $p=0.02$  vs Sham). In RUPP+Sham NK, levels of placental IFN- $\gamma$  were normalized at  $6 \pm 2$  pg/mg ( $p=0.01$  vs RUPP).



## Effects of NK Adoptive Transfer on Placental eNOS expression and phosphorylation

Placental eNOS expression was reduced by 30% in RUPP ( $0.71 \pm 0.09$ ) compared to Sham, although this was not significantly different ( $p=0.08$  vs Sham). Placental eNOS expression was  $0.86 \pm 0.09$  in Sham+RUPP NK ( $p=0.58$  vs Sham) and  $0.83 \pm 0.10$  in RUPP+Sham NK ( $p=0.77$  vs RUPP; Figure S2). Placental phosphorylated eNOS (pS1177) was not different among the groups (Sham =  $1.0 \pm 0.04$ , Rupp =  $0.97 \pm 0.08$ , Sham+RUPP NK =  $0.87 \pm 0.10$ , RUPP+Sham NK =  $0.99 \pm 0.05$ ;  $p>0.05$  for all comparisons; Figure S3).

## DISCUSSION

Several clinical studies have reported a shift toward activated NK cells in PE. [32–35] However, the precise role of different NK cell phenotypes in PE pathophysiology has not been studied *in vivo*. Therefore, in this study we tested the hypotheses that (1. RUPP stimulated NKs play a direct role in causing maternal HTN and IUGR in pregnant rats and (2. Sham NKs attenuate maternal HTN and IUGR in RUPP rats. As we have previously published, there was a significant increase in both total NK cells and cNKs in the circulation and placenta of RUPP rats compared to Sham rats. We also found that RUPP-stimulated NK cells caused increased NK cell activation in the circulation and placentas of Sham recipient rats, and increased MAP, IUGR, UARI, and placental ROS. Furthermore, we observed that adoptive transfer of Sham NK cells into RUPP rats normalized NK cell activation, attenuated the increased MAP, improved IUGR, and reduced UARI. Thus, we have demonstrated that cNK cells play a direct role in PE pathophysiology, and that the polarization of NK cell phenotypes can influence pregnancy outcomes.

The results of this study not only suggest the direct contribution of placental ischemia-stimulated cNK cells in the pathophysiology of the RUPP model, but also submit for the first time, that normal *placental NK* cells can ameliorate RUPP induced pathophysiology. While the role of placental NK cells in pregnancy has not been thoroughly researched, the beneficial activities of dNK cells during pregnancy have been widely established.[11–13] There have been several *in vitro* studies showing that human dNK assist in trophoblast migration, spiral artery remodeling, and fetal growth via secretion of growth factors.[11, 12, 36, 37] Likewise, experiments in animal models have also shown the importance of NK cells in normal pregnancy with NK cell deficiency or depletion resulting in altered placental structure, incomplete vascular remodeling, and fetal growth restriction.[37, 38] Importantly, there is increasing evidence that pregnancy outcomes can depend greatly on the phenotype of the NK cells present.[36, 39, 40] This current study suggests that this may apply to placental NK cells as well as dNKs.

Activated dNK cells have been implicated in several pregnancy disorders including RSA, fetal/neonatal alloimmune thrombocytopenia (FNAIT), and placental insufficiency.[9, 16, 41] While dNKs normally have little cytotoxic activity, their granules contain large amounts of cytolytic enzymes, and they have the potential to become activated, with enhanced cytotoxic activity. [16, 42, 43] Similarly, in our previous *in vitro* study, we found a 5-fold increase in cytotoxicity of placental NK cells from RUPP compared to those isolated from Sham rats.[22] We hypothesized that the observed shift in NK cell phenotype contributes to the pathophysiology observed in RUPP rats.

While we have previously shown that total NK cell depletion from GD14-GD19 significantly improved the phenotype observed in RUPP rats, that study did not specifically address the role of different NK cell phenotypes. By using adoptive transfer techniques, we are able to distinguish the direct roles of both RUPP and Sham placental NK cells *in vivo*. Additionally, we assessed the resulting NK populations in the circulation and placenta using flow cytometry. With the PE-like phenotype observed in the Sham+RUPP NK rats, the increased NK activation reinforces the possible role of cNKs in PE pathophysiology. Moreover, we observed significant attenuation of NK activation in RUPP recipients of Sham NKs along with marked improvement in their pathophysiology. This suggests that shifting the NK phenotype may be a potential therapeutic strategy for PE treatment.

We observed a significantly elevated UARI in Sham+RUPP NK rats compared to Sham, as well as attenuated UARI in RUPP+Sham NK rats compared to RUPP. Measurement of uterine artery resistance via Doppler ultrasound is a useful screening tool to predict the development of some pregnancy complications including PE and IUGR.[44, 45] Uterine artery resistance typically decreases as pregnancy progresses and this is largely attributed to spiral artery remodeling by trophoblasts.[46–48] Thus, increased UARI observed in preeclamptic women is thought to be due to poorly remodeled vessels, and this has been reflected in rodent models, including the RUPP rat.[37, 45, 49, 50] While the placenta is largely established by GD12 in rats, there is an additional wave of trophoblast invasion that occurs around GD13.5 [51, 52] and introduction of RUPP NK cells into Sham rats on GD14 could inhibit this invasion. This may impair further spiral artery remodeling and be the cause of the increased UARI we observed in this group. Alternatively, the addition of normal NK cells into the RUPP animals may promote the additional wave of trophoblast invasion, therefore causing the decreased UARI observed in RUPP+Sham NK. Another factor that has been shown to influence UARI is nitric oxide (NO) induced vasodilation.[53, 54] Therefore, we evaluated changes in placental eNOS expression as well as total Nitrate/Nitrite as an indication of NO bioavailability. Although we saw no changes in eNOS expression between groups, plasma nitrate/nitrite was reduced in Sham+RUPP NK rats compared to Sham and was elevated in RUPP+Sham NK rats compared to RUPP. These data may explain the respective alterations in UARI observed in each group.

Because increased vascular resistance impedes blood flow to the placenta and placental under-perfusion deprives the fetus of oxygen and nutrients,[55] increased UARI is also associated with IUGR.[55, 56] We observed that changes in fetal and placental weight corresponded with changes in UARI with decreased fetal and placental weight in Sham +RUPP NK which provides additional evidence that cNKs may contribute to IUGR in PE women. Similarly, we observed increased fetal and placental weight in RUPP+Sham NK which suggests that NK mediated reductions in UARI may improve fetal outcomes in response to placental ischemia.

NK cell adoptive transfer altered inflammatory cytokines in the recipient groups. Activated NK cells secrete several cytokines with the main ones being TNF- $\alpha$  and IFN- $\gamma$  [57–59], and these two cytokines have also been observed to be elevated in PE women and RUPP rats.[21, 60–62] In this study, adoptive transfer of RUPP NKs into Sham rats resulted in higher circulating TNF- $\alpha$  and placental IFN- $\gamma$ . There are several mechanisms by which these



known to secrete this angiogenic factor [11, 90, 91], and dNK cells from women with RSA produce less.[92] In the current study, we saw that circulating and placental VEGF levels were reduced in Sham recipients of RUPP NKs and elevated in RUPP recipients of Sham NKs. This suggests that RUPP stimulated NKs secrete fewer pro-angiogenic factors than their normal counterparts. Several investigators have administered recombinant VEGF and PLGF to PE animal models and saw decreased circulating sFlt-1 levels, decreased maternal MAP, and increased fetal weight. [93, 94] Thus, the reduction in circulating sFlt-1 is a possible mechanism by which IUGR was attenuated and MAP was reduced in RUPP+Sham NKs. Furthermore, the reduction in VEGF is possible mechanism by which IUGR was induced and MAP elevated in the Sham+RUPP NK group.

While these results have provided more information concerning NK polarization in PE, there are some limitations to the current study. Although we observe significantly higher placental ROS in Sham+RUPP NK animals, there was no change in placental ROS in RUPP+Sham NK suggesting that the mechanisms responsible are independent of cNKs and warrant further study. Additionally, RUPP recipients of Sham NKs still displayed elevated levels of circulating and placental TNF- $\alpha$  and circulating IFN- $\gamma$ . Since both total and activated NK cells in the circulation and placenta were normalized in RUPP+Sham NK, this points to an additional cellular source of these cytokines in RUPPs. Inflammatory CD4+ T cells are known to also produce these cytokines[95, 96] and these cells are also elevated in RUPPs [97], making them them a possible source. Finally, continued examination into the precise mechanisms by which RUPP NK cells induced a PE like phenotype in Sham rats is needed, particularly the role of cNK associated cytokines TNF-  $\alpha$  and IFN- $\gamma$ . While previous studies in the RUPP have shown that TNF- $\alpha$  inhibition decreases MAP, additional investigation into the mechanism of this improvement is warranted. Furthermore, the role of IFN- $\gamma$  in RUPP pathophysiology has not been explored and further study could reveal the pathways involved. Despite these limitations, the results of this study provide significant insight on the role of NK cell polarization in PE pathophysiology.

In this study, adoptive transfer of RUPP NK cells into Sham pregnant rats increased placental ROS, circulating TNF- $\alpha$ , placental IFN- $\gamma$ , and decreased circulating and placental VEGF. This resulted in a PE like phenotype with significantly elevated NK cell activation, MAP, UARI, and IUGR. Alternatively, transfer of Sham NK cells into RUPP rats normalized circulating and placental NK cell populations, sFlt-1, circulating and placental VEGF, and placental IFN- $\gamma$ . This led to significantly lower MAP, improved IUGR, and decreased UARI compared to RUPPs. Research focused on identifying factors involved in NK cell activation as well as those that can normalize the placental NK populations could provide therapeutic targets to prevent or treat PE in women.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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OKT, CHB, GAT, LMA, CJ, MG, CAG, TI, OTH JMW, and DCC, made substantial contributions to the conception and design of acquisition of data or analysis and interpretation of data. O

OKT and DCC drafted the manuscript and revised it critically for important intellectual content.

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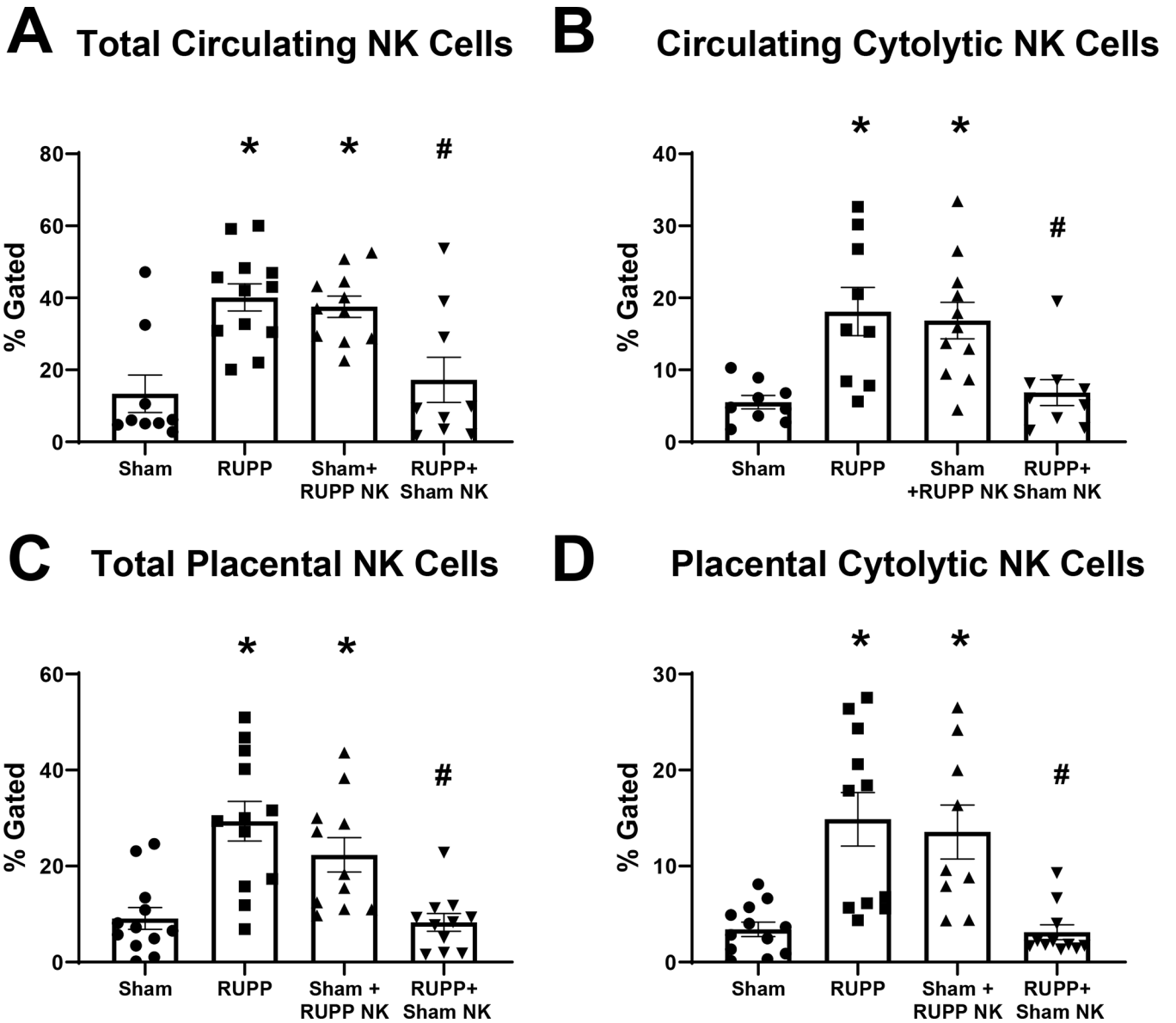
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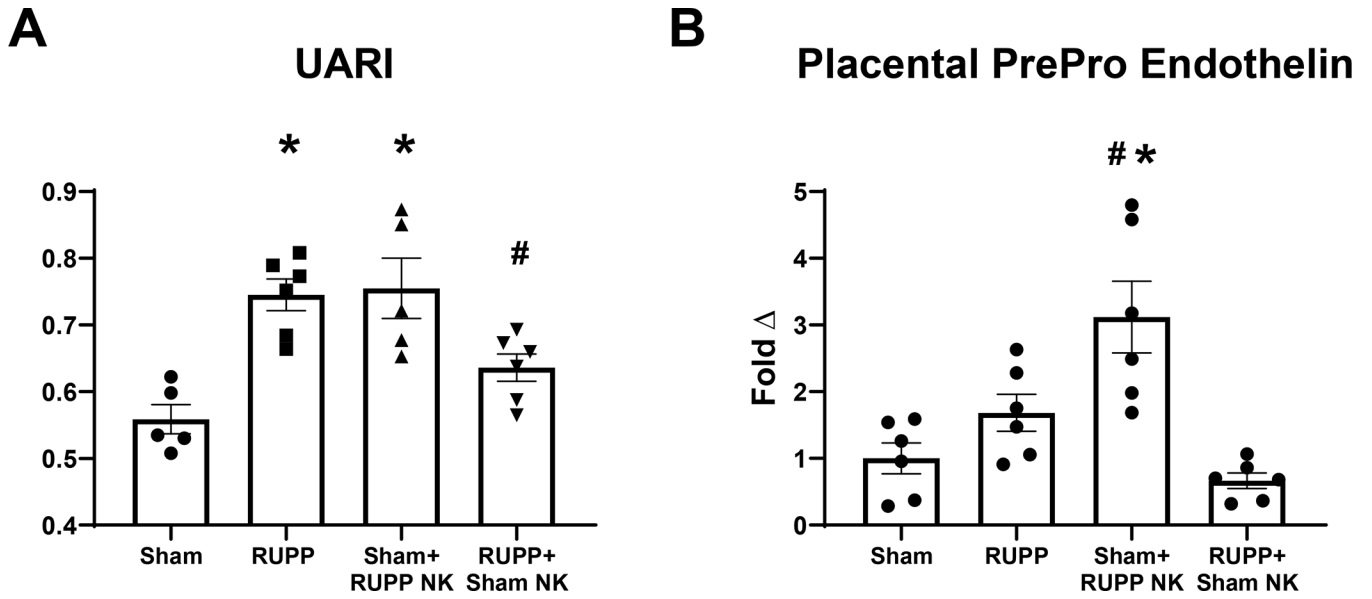


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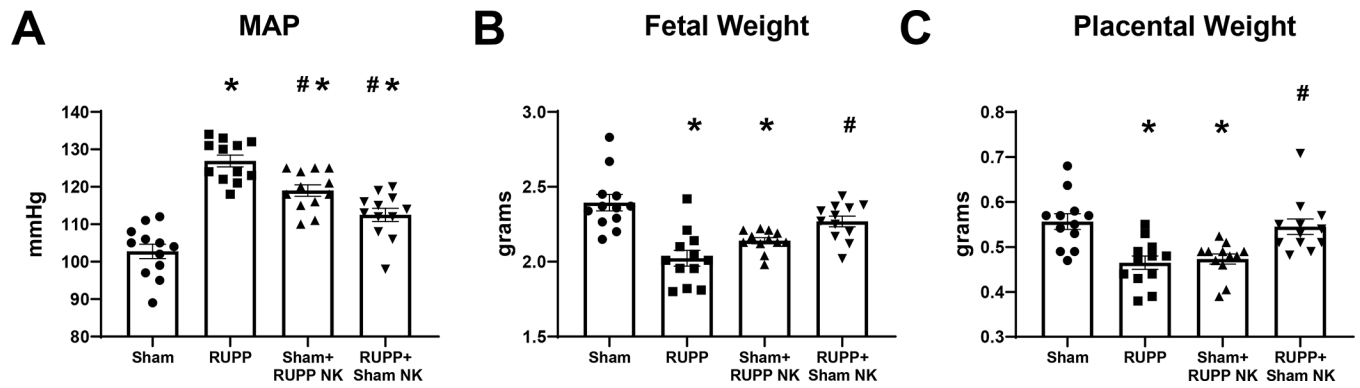


**Figure 1. Effects of Natural Killer (NK) Cell Adoptive Transfer on Circulating and Placental NK Populations in Pregnant Rats.**

On Gestation Day (GD) 14, vehicle or  $5 \times 10^6$  Reduced Uterine Perfusion Pressure (RUPP) NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD19, blood and placentas were collected, processed, and analyzed via flow cytometry to obtain levels of (A) circulating total NK cells and (B) circulating cytolytic NK cells along with (C) placental total NK cells, and (D) placental cytolytic NK cells. Sham n=9–12; RUPP n=9–12; Sham+RUPP NK n=9–11; RUPP+Sham NK n=9–11. All data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey’s post-hoc test. \*p<0.05 versus Sham; #p<0.05 vs RUPP.

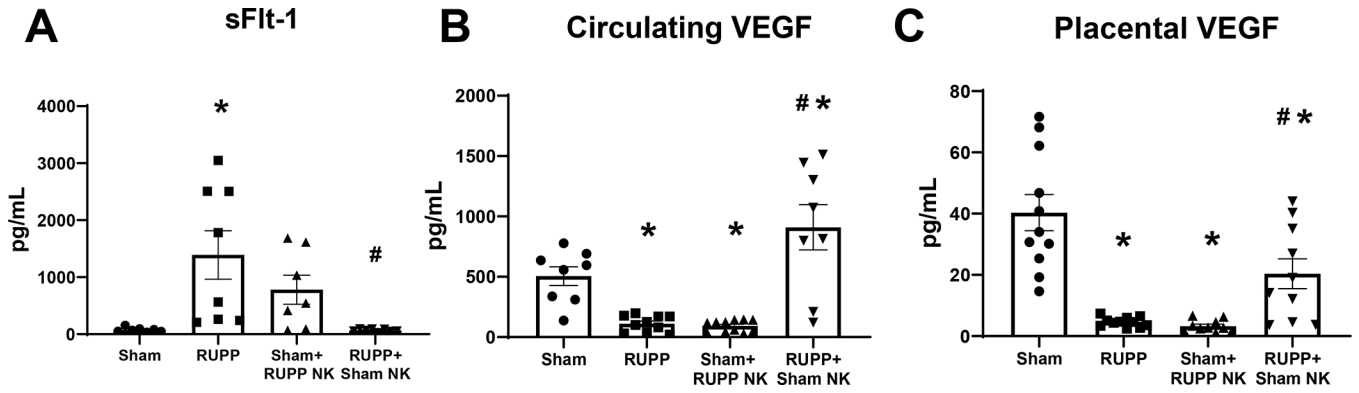


**Figure 2. Effects of Natural Killer (NK) Cell Adoptive Transfer on Maternal Uterine Artery Resistance Index (UARI) and placental PrePro Endothelin (PPET).** On Gestation Day (GD) 14, vehicle or  $5 \times 10^6$  Reduced Uterine Perfusion Pressure (RUPP) NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD18, (A) UARI was measured using Doppler ultrasound. On GD19 animals were sacrificed and placentas were collected and frozen for analysis of (B) PPET mRNA levels. Sham: n=5–6, RUPP: n=6, Sham+RUPP NK: n=6, RUPP+Sham NK: n=5–6. All data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey’s post-hoc test. \*p<0.05 versus Sham; #p<0.05 vs RUPP.



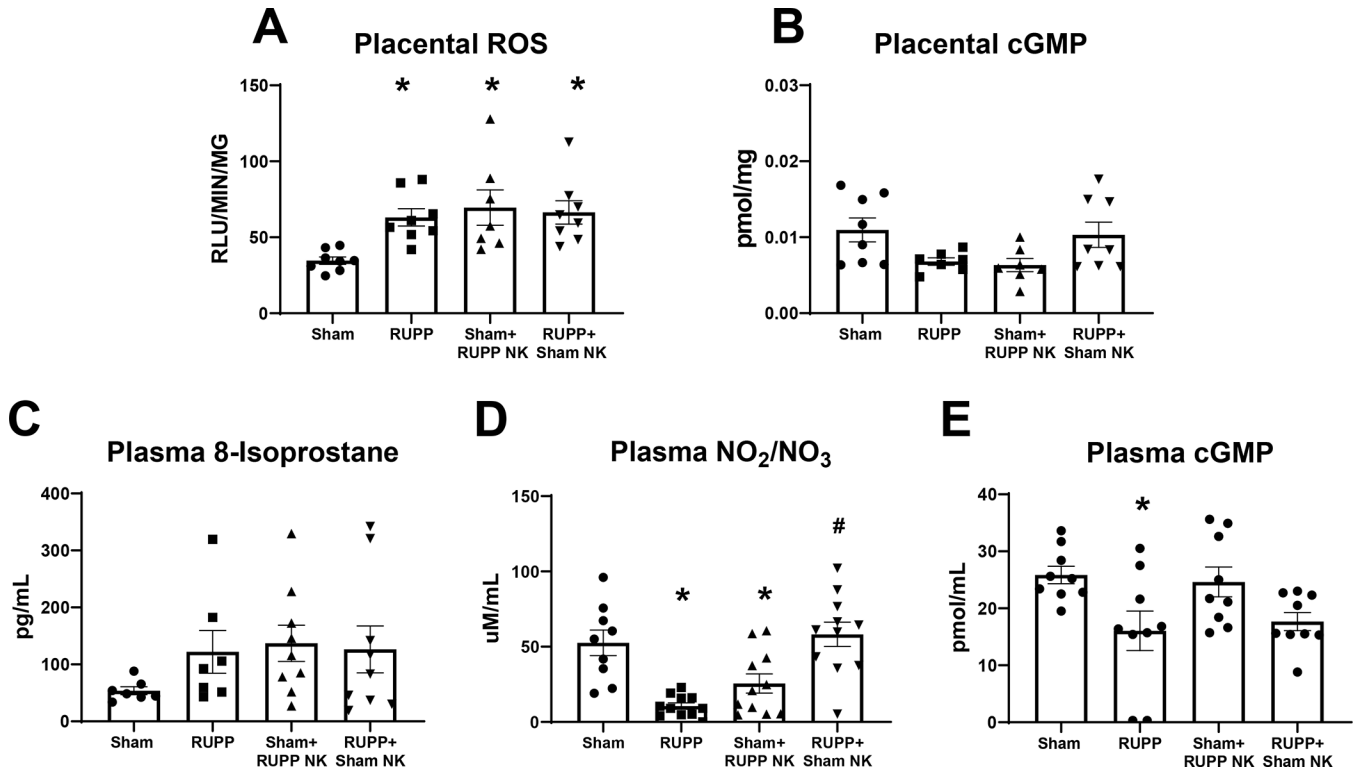
**Figure 3. Effects of Natural Killer (NK) Cell Adoptive Transfer on, Maternal Mean Arterial Pressure (MAP), and Fetal and Placental Weight.**

On Gestation Day (GD) 14, vehicle or  $5 \times 10^6$  Reduced Uterine Perfusion Pressure (RUPP) NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD19 (A) conscious mean arterial pressure was measured, and (B) fetal and (C) placental weights were recorded under isoflurane anesthesia. Sham: n=12, RUPP: n=12, Sham+RUPP NK: n=12, RUPP+Sham NK: n=12. All data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey's post-hoc test. \*p<0.05 versus Sham; #p<0.05 vs RUPP.



**Figure 4. Effects of Natural Killer (NK) Cell Adoptive Transfer on Circulating and Placental Angiogenic Factors.**

Gestation Day (GD) 14, vehicle or  $5 \times 10^6$  Reduced Uterine Perfusion Pressure (RUPP) NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD19, blood and placentas were collected and processed for further analysis. (A) Plasma levels of sFlt-1 was assessed using a commercial ELISA. Circulating (B) and Placental (C) levels of VEGF were measured using the Bio-Plex Pro Rat Cytokine Immunoassay Kit. All data are expressed as mean  $\pm$  SEM. Sham n=7–11, RUPP n=8–11, Sham+RUPP NK n=7–11, RUPP+Sham NK n=8–10. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey’s post-hoc test. \*p<0.05 versus Sham; #p<0.05 vs RUPP.



**Figure 5. Effects of Natural Killer (NK) Cell Adoptive Transfer on Placental and Circulating Oxidative Stress, Cyclic GMP, and Circulating Nitrate/Nitrite levels in Pregnant Rats.** On Gestation Day (GD) 14, vehicle or  $5 \times 10^6$  Reduced Uterine Perfusion Pressure (RUPP) NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD19, blood and placentas were collected following sacrifice and processed for further analysis. (A) Reactive oxygen species in placental homogenates were assessed using lucigenin and (B) placental cyclic GMP was measured via ELISA. (C) Circulating levels of 8-isoprostane were measured via ELISA. (D) Plasma total nitrate/nitrite was assessed using a colorimetric assay and (E) plasma cyclic GMP was measured via ELISA. All data are expressed as mean  $\pm$  SEM. Sham n=7–9, RUPP n=7–11, Sham+RUPP NK n=7–10, RUPP+Sham NK n=7–10. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey’s post-hoc test. \*p<0.05 versus Sham; #p<0.05 vs RUPP.

**Table 1.**

Effects of Natural Killer (NK) Cell Adoptive Transfer on Circulating and Placental Cytokines in Pregnant Rats.

Circulating Cytokines					
Cytokine (pg/mL)	Sham	RUPP	Sham+RUPP NK	RUPP+Sham NK	n/group
IL-6	138±21	426±56 <sup>*</sup>	338±40 <sup>*</sup>	245±56 <sup>#</sup>	8-9
IL-17	6.8±1.6	30.5±4.9 <sup>*</sup>	23.8±3.7	45.5±8.9 <sup>*</sup>	8-10
TNF- $\alpha$	30.3±5.0	152±16.6 <sup>*</sup>	108.7±13.6 <sup>*</sup>	115.4±17 <sup>*</sup>	10-11
IFN- $\gamma$	19.3±3.1	95.2±14.8 <sup>*</sup>	44.6±6.5 <sup>#</sup>	74.7±12.8 <sup>*</sup>	10-11
Placental Cytokines					
Cytokine (pg/mg)	Sham	RUPP	Sham+RUPP NK	RUPP+Sham NK	n/ group
IL-17	0.63±0.24	4.1±0.72 <sup>*</sup>	0.57±0.22 <sup>#</sup>	5.1±0.73 <sup>*</sup>	10-12
TNF- $\alpha$	36.3±4.0	64.3±7.2 <sup>*</sup>	46.3±4.5	71.7±5.4 <sup>*</sup>	9-11
IFN- $\gamma$	5.1±1.4	15.5±3.0 <sup>*</sup>	14.6±2.0 <sup>*</sup>	5.9±1.8 <sup>#</sup>	11-12

On GD14, vehicle or  $5 \times 10^6$  RUPP NKs were infused i.v. into a subset of Sham rats and vehicle or  $5 \times 10^6$  Sham NKs were infused i.v. into a subset of RUPP rats. On GD19, blood and placentas were collected following sacrifice and processed for further analysis. Levels of cytokines were measured in blood and placental homogenates of each group using the Bio-Plex Pro Rat Cytokine Immunoassay Kit. Placental data are normalized to total protein. All data are expressed as mean±SEM. Statistical analyses were performed using one-way ANOVA with multiple comparisons followed by Tukey's post-hoc correction.

\* p<0.05 vs. Sham

# p<0.05 vs RUPP.

### Major Resources Tables

<b>Animals (in vivo studies)</b>				
<b>Species</b>	<b>Vendor or Source</b>	<b>Background Strain</b>	<b>Sex</b>	
Rat	Envigo (Indianapolis, IN)	Sprague Dawley	Female	
<b>Antibodies</b>				
<b>Target antigen</b>	<b>Vendor or Source</b>	<b>Catalog #</b>	<b>Working concentration</b>	<b>Lot # (preferred but not required)</b>
Rat CD3 - Biotin	Miltenyi Biotec, (Auburn, CA)	130101973	1:10	
Rat CD3-APCVio770	Miltenyi Biotec	130102675	1:10	
Rat CD161-PE	Miltenyi Biotec	130102712	1:10	
Rat ANK 61	Abcam (Cambridge, MA)	ab36392	1:50	
Mouse IgG-Alexa Flour 405	Abcam	Ab175663	1:100	
Rat ANK 44	Abcam	Ab36388	1:100	
Mouse IgG-FITC	Abcam	Ab97230	1:50	
Rat Beta-actin	MilliporeSigma (Darmstadt, Germany)	A1978	1:2,000	
Human eNOS	BD Transduction Laboratories (San Jose, CA)	610296	1:250	
Mouse IgG IRDye® 800CW	Rockland (Gilbertsville, PA)	610731002	1:10,000	