CD4\(^+\) T Cells Are Important Mediators of Oxidative Stress That Cause Hypertension in Response to Placental Ischemia

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Abstract—Preeclampsia is associated with oxidative stress, which is suspected to play a role in hypertension, placental ischemia, and fetal demise associated with the disease. Various cellular sources of oxidative stress, such as neutrophils, monocytes, and CD4\(^+\) T cells have been suggested as culprits in the pathophysiology of preeclampsia. The objective of this study was to examine a role of circulating and placental CD4\(^+\) T cells in oxidative stress in response to placental ischemia during pregnancy. CD4\(^+\) T cells and oxidative stress were measured in preeclamptic and normal pregnant women, placental ischemic and normal pregnant rats, and normal pregnant recipient rats of placental ischemic CD4\(^+\) T cells. Women with preeclampsia had significantly increased circulating (\(P=0.02\)) and placental CD4\(^+\) T cells (\(P=0.0001\)); lymphocyte secretion of myeloperoxidase (\(P=0.004\)); and placental reactive oxygen species (\(P=0.0004\)) when compared with normal pregnant women. CD4\(^+\) T cells from placental ischemic rats cause many facets of preeclampsia when injected into normal pregnant recipient rats on gestational day 13. On gestational day 19, blood pressure increased in normal pregnant recipients of placental ischemic CD4\(^+\) T cells (\(P=0.002\)) compared with that in normal pregnant rats. Similar to preeclamptic patients, CD4\(^+\) T cells from placental ischemic rats secreted significantly more myeloperoxidase (\(P=0.003\)) and induced oxidative stress in cultured vascular cells (\(P=0.003\)) than normal pregnant rat CD4\(^+\) T cells. Apocynin, a nicotinamide adenine dinucleotide phosphate inhibitor, attenuated hypertension and all oxidative stress markers in placental ischemic and normal pregnant recipient rats of placental ischemic CD4\(^+\) T cells (\(P=0.05\)). These data demonstrate an important role for CD4\(^+\) T cells in mediating another factor, oxidative stress, to cause hypertension during preeclampsia. (Hypertension. 2014;64:00-00.) • Online Data Supplement

Key Words: hypertension • oxidative stress • preeclampsia

Preeclampsia, defined as new onset hypertension and proteinuria during pregnancy, is one of the leading causes of maternal and perinatal morbidity.\(^1,2\) Abnormal trophoblast invasion into the maternal uterine spiral arteries is postulated to be the initiating event in preeclampsia, resulting in reduced uterine perfusion pressure (RUPP) to the placenta.\(^3\) As preeclampsia develops, dysregulation of various immune cells, such as monocytes, macrophages, T lymphocytes, and endothelial cells, occur thus leading to an alteration in various circulating factors that are thought to increase blood pressure and decrease renal excretory function.\(^4,5\) One such factor is oxidative stress. Macrophages and neutrophils and proinflammatory T cells are capable of converting molecular oxygen into reactive oxygen species (ROS) in pathological disease states, such as in preeclampsia.\(^6,7\) These immune cells, released on placental ischemia, can generate superoxide within the maternal vascular endothelium.\(^7\) Indeed increased levels of ROS and 8-isoPGF\(_2\alpha\), the free form of isoprostane, are found in placentas and in the circulation of women with preeclampsia,\(^8,9\) indicating that preeclampsia is accompanied by an increase in pro-oxidant factors. During hypertensive disorders, peroxynitrite concentrations\(^10,11\) and ROS are also released by the maternal vascular endothelium and smooth muscle cells stimulated by stress or angiotensin II. In the case of preeclampsia, smooth muscle cell and endothelial cells (human umbilical vein endothelial cells [HUVECs]) in culture have been shown to release increased ROS when stimulated by autoantibodies to the angiotensin II type I receptor or by antiangiogenic factors soluble fms-like tyrosine kinase-1.\(^12,13\) Therefore, during preeclampsia, there are multiple stimulators and pathways of oxidative stress, which are known to be important mediators of endothelial dysfunction and hypertension. However, not many studies have illustrated a direct role for T cells as producers or mediators of oxidative stress during preeclampsia. Many studies have demonstrated, however, an increase in circulating CD4\(^+\) T cells in women with preeclampsia when compared with those in normal pregnant (NP) women. In addition, this profile has been further characterized by an increase in Thelper 17 (Th17) and decrease in...
Tregulatory (Treg) cells. In addition, women with preeclampsia were found to have decreased mRNA expression of transcription factors directly associated with Treg cells and increased mRNA expression of transcription factors directly associated with Th17 cells in both the blood and the decidual when compared with NP women. It is thought that placental ischemia stimulates factors circulating in preeclampsia that causes much of the disease pathology. However, no studies have identified if this altered circulating T-cell ratio is reflective of that occurring within the placenta or if it is associated with elevated ROS.

In an animal model of RUPP, our laboratory has recently demonstrated that markers of oxidative stress and circulating CD4+ T cells are increased when compared with NP rats, similar to what is seen in women with preeclampsia. Previous work has shown that malondialdehyde, superoxide, and myeloperoxidase activity is increased in RUPP placentas similar to what is observed in placentas from women with preeclampsia. We have further shown that adoptive transfer of placental ischemia stimulated CD4+ T cells into NP recipient rats, resulted in hypertension, increases in inflammatory cytokines, angiotensin II type 1 receptor autoantibody, and endothelial activation in NP recipient rats. One mechanism whereby hypertension and oxidative stress may be increased during placental ischemia is via T cells.

Although antioxidant therapy has shown not to benefit mother and baby during preeclampsia, these studies only supplemented mothers and did not attempt to target cellular sources to reduce oxidative free radicals from being released. It is understandable that such studies may be difficult to perform in a clinical cohort; therefore, it is important to ascertain such knowledge from reliable and repeatable animal models of disease. Therefore, the objective of this study was 2-fold, first to determine whether placental CD4+ T cells have the same profile as circulating T cells from women with preeclampsia and whether they are important mediators of oxidative stress. Second, to determine whether placental ischemic stimulated CD4+ T cells are a source of oxidative stress and whether specific therapy targeting ROS could reduce blood pressure or T cells in animal models of preeclampsia.

**Materials and Methods**

**Protocol 1: Human Study Subjects**

Women diagnosed with preeclampsia or as having a normal pregnancy at the University of Mississippi Medical Center (UMMC) were consented and enrolled in this study approved by the Institutional Review Board. All participants were scheduled for cesarean section delivery, after which placentas were immediately collected. Plasma was collected for analysis of immune cells.

**Isolation of CD4+ Placental Lymphocytes**

Placental white blood cells were isolated from the placenta and used for the next set of studies (Methods in the online-only Data Supplement). CD4+ lymphocytes were isolated from placentas to determine their role in contributing to oxidative stress. Briefly, lymphocytes were resuspended in degassed MACS running buffer (Miltenyi Biotec) according to manufacturer’s directions (Methods in the online-only Data Supplement). The resulting CD4+ lymphocytes were cultured overnight at standard atmospheric conditions in lymphocyte media.

**Determination of CD4+ Treg and Th17 Lymphocytes**

Flow cytometry was used to measure CD4+ T cell subpopulations in lymphocytes isolated from placentas and whole-blood samples. To determine whether Treg and Th17 cells were increased and increased, respectively, we measured markers for Treg cells (CD4+CD25(high)-FoxP3+) and Th17 (CD4+CD25-ROr(+) cells (Methods in the online-only Data Supplement; Gating strategies are in Figures S1 and S2 in the online-only Data Supplement).

**Determination of Oxidative Stress**

Superoxide production in the placenta was measured using the lucigenin technique (Methods in the online-only Data Supplement). Each sample was repeated 5x and the average was used for data transformation. The protein concentration was measured using a protein assay. Data are expressed as relative light units per minute per milligram protein.

Myeloperoxidase was measured in plasma and cell culture supernatants according to manufacturer’s directions (RnD Systems).

To determine whether placentas and placental CD4+ T lymphocytes could cause an increase in vascular ROS production, previously collected tissue and cell culture media were placed over HUVECs, and oxidative stress was measured via dihydroethidium staining (Methods in the online-only Data Supplement).

**Protocol 2: Animal Study**

All animal 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 experimental procedures were in accordance with the National Institutes of Health guidelines for use and care of animals and approved by the Institutional Animal Care and Use Committee at University of Mississippi Medical Center.

**RUPP (Rat Model of Preeclampsia)**

Reduction in uterine perfusion pressure was performed on gestational day (GD) 14 as previously described (Methods in the online-only Data Supplement). Mean arterial pressure (MAP) was measured via the carotid artery (Methods in the online-only Data Supplement), blood, placentas, urine, and spleens were isolated and pups collected and weighed on GD19.

Placental explants were cultured as previously described for 24 hours, after which culture media were collected. To determine whether placental and splenic CD4+ T lymphocytes from RUPP rats could cause an increase in vascular ROS production, we repeated the HUVEC and dihydroethidium studies described above.

**Adoptive Transfer of RUPP CD4+ T Cells Into NP Recipient Rats**

The current study was designed to determine the effect of RUPP CD4+ T cells to increase oxidative stress as a mechanism of hypertension in NP recipient rats. As such CD4+ T cells were isolated from NP and RUPP spleens as previously described (Methods in the online-only Data Supplement). The 4 groups examined were NP rats (n=7), NP rats injected with NP CD4+ T cells (NP+NPT cells; n=3), RUPP rats (n=8), and NP rats injected with CD4+ RUPP T cells (NP+RPT cells; n=6). Because adoptive transfer of NP or RUPP CD4+ T cells into virgin rats has been shown to not increase blood pressure, these groups were not examined.

**Isolation and Measurement of Rat Lymphocytes From Whole Blood**

One milliliter of whole blood was mixed with 9 mL of Roswell Park Memorial Institute 1640 and placed over a Lymphoprep gradient and centrifuged at 1200 rpm for 25 min. Flow cytometry was used to measure CD4+ T cells after cells were stained with fluorescein isothiocyanate conjugated mouse antirat CD4 (BD Pharmingen). As we have previously reported that experimental placental ischemia does not significantly increase circulating CD8+ T cells, we did not measure them in the current study.

**Determination of Oxidative Stress**

Myeloperoxidase was measured in CD4+ T cell culture supernatants and 15-isoprostane F2, a marker of in vivo oxidative stress was measured in urine, according to manufacturer’s protocol (RnD Systems; Oxford Biomedical Research, Rochester Hills, MI). Superoxide
production in the placenta and renal cortex was measured using lucigenin techniques.\

**Effect of Antioxidant Therapy on CD4\(^+\) T-Cell–Induced Hypertension**

Because the generation of oxygen free radicals and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity are contributors to hypertension, we sought to determine whether suppression of either of these mechanisms affects hypertension in response to adoptive transfer of RUPP-stimulated CD4\(^+\) T cells. The superoxide dismutase mimetic tempol (TEM; Sigma) and the antioxidant, apocynin (APO; Sigma) were used. On GD13, TEM (5 mg/kg per day) or APO (100 mg/kg per day) was administered in the drinking water ad libitum of pregnant rats until GD19. The groups examined were as follows: NP+TEM (n=6); NP+NPTcells+TEM (n=3); RUPP+TEM (n=4); RPTcells+TEM (n=6) and NP+APO (n=6); NP+NPTcells+APO (n=2); RUPP+APO (n=4); NP+RPTcells+APO (n=6). MAP and tissues were measured/collected in all groups of pregnant rats on GD19.

**Statistical Analysis**

All data are expressed as mean±SEM. Differences between control and experimental groups were analyzed via 1-way ANOVA, and post hoc analyses were obtained through Bonferroni post hoc test. Student t test was used to compare groups treated with TEM or APO to their untreated groups. For confocal studies, 3 separate frames per experimental condition were taken per experiment; n=6 per experimental condition. All conditions of image collections, including gain, offset, pinhole, and laser power were identical among all samples. Values of P<0.05 were considered significant.

**Results**

**Protocol 1: Human Study**

Twenty women undergoing scheduled cesarean section were enrolled in the current study. There was not a significant difference between women with preeclampsia (n=10) and NP women (n=10) in maternal age at delivery (P=0.19; Table) or in body mass index at admission (P=0.583; Table). Women with preeclampsia delivered at a significantly earlier gestational age when compared with NP women (P=0.0001) had significantly smaller babies (P=0.0001; Table). MAP in women with preeclampsia was significantly higher when compared with NP women (15.91±2.9% versus 2.81±0.86%; P=0.003). Tregs were significantly decreased (0.67±0.23% versus 2.08±0.45%; P=0.02) and Th17s were increased (14.02±1.7% versus 6.4±0.34; P=0.0001) when compared with NP women (Figure 1B). There was a negative correlation between the number of Tregs and the gestational age at delivery in both circulating (r=0.663; n=10; P=0.037) and placental Tregs (r=0.653; n=10; P=0.041). CD8\(^+\) T cells were not significantly increased in the circulation (P=0.55) but were significantly increased in the placenta (P=0.02; online-only Data Supplement).

**Oxidative Stress Is Increased in Women With Preeclampsia**

As demonstrated in Figure 1C, placentas from women with preeclampsia (n=5) produce significantly more ROS when compared with NP women (n=5; 658.5±112.8 versus 267.8±72.8 RLU/min per milligram; P=0.019). The same is true when stimulated with NADPH oxidase (1333±53.7 versus 688.1±98.9 RLU/min per milligram; P=0.0004). Women with preeclampsia had significantly higher levels of circulating myeloperoxidase when compared with NP women (23.81±2.4 versus 13.78±1.8 ng/mL; P=0.004; Figure 1D). Placental white blood cells from women with preeclampsia secreted significantly more myeloperoxidase into cell culture media when compared with NP women (65.67±2 versus 31.53±2.7 ng/mL per milliliter; P=0.0004; Figure 1D).

Conditioned media from preeclamptic placentals explants tended to increase ROS production in HUVECs (59.68±4.4% signal intensity; n=4) when compared with conditioned media from NP explants (46.98±3.87%; n=4; P=0.07; Figure 2). The mean intensity of HUVECs exposed to media from preeclamptic CD4\(^+\) T cells was 78.87±1.86% versus 67.52±5.7% in HUVECs exposed to media from NP+NPTcells (P=0.10; Figure 2A–2E).

**Protocol 2: Animal Study**

**Placental Ischemia Stimulates Oxidative Stress**

Conditioned media from RUPP placentas (62.98±1.96%) significantly increased ROS in HUVECs when compared with placentas from NP rats (54.97±0.96%; n=4 per group; P=0.01; Table). Women with preeclampsia had significantly decreased Tregs when compared with NP women (0.51±0.29% versus 4.58±1.2%; P=0.01) and significantly increased Th17s (15.75±1.18% versus 6.84±1.25%; P=0.0008; Figure 1A). Women with preeclampsia had increased CD4\(^+\) T cells when compared with NP women (15.91±2.9% versus 2.81±0.86%; P=0.003). Tregs were significantly decreased (0.67±0.23% versus 2.08±0.45%; P=0.02) and Th17s were increased (14.02±1.7% versus 6.4±0.34; P=0.0001) when compared with NP women (Figure 1B). There was a negative correlation between the number of Tregs and the gestational age at delivery in both circulating (r=0.663; n=10; P=0.037) and placental Tregs (r=0.653; n=10; P=0.041). CD8\(^+\) T cells were not significantly increased in the circulation (P=0.55) but were significantly increased in the placenta (P=0.02; online-only Data Supplement).

**Table. Demographic Data for Women With Normal and Preeclamptic Pregnancies**

<table>
<thead>
<tr>
<th>Patient Parameters</th>
<th>Normal Pregnant (n=10)</th>
<th>Preeclamptic (n=10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>28.9±1.78 (17–37)</td>
<td>25.6±1.66 (19–35)</td>
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<tr>
<td>BMI at admission</td>
<td>34.3±3.03 (25.1–49.4)</td>
<td>37.1±4.10 (21–55.6)</td>
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<td>Gestational age, wk</td>
<td>38.9±0.15 (38.2–39.4)</td>
<td>33.9±0.91 (30–38.2)*</td>
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<tr>
<td>Fetal birth weight, g</td>
<td>3505±100.8 (3155–4005)</td>
<td>1697±244.9 (951–3645)*</td>
<td>0.0001</td>
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<tr>
<td>MAP</td>
<td>84.2±2.16 (71–93.67)</td>
<td>114.5±4.86 (83.33–133.3)</td>
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<tr>
<td>Systolic</td>
<td>119.4±2.01 (111–131)</td>
<td>155.1±6.65 (131–200)*</td>
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</tr>
<tr>
<td>Diastolic</td>
<td>66.5±2.54 (54–78)</td>
<td>94.2±4.65 (73–115)*</td>
<td>0.0001</td>
</tr>
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</table>

BMI indicates body mass index.

*P<0.05 compared with normal pregnant women.
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Figure 2F). Conditioned media from CD4+ T cells from RUPP rats (89.43±1.54%) significantly increased ROS in HUVECs when compared with media from NP+NPT cells (80.54±1.1%, n=4 per group; *P=0.003; Figure 2F–2J).

CD4+ T cells isolated from the spleens of RUPP rats secreted significantly more myeloperoxidase when compared with NP rats (n=6; 116.9±15.2 versus 48.5±8.3 ng/mg per milliliter; *P=0.003; Figure 3A).

Adoptive Transfer of RUPP CD4+ T Cells Increases MAP and CD4+ T Cells in Recipient NP Rats

In the current study, MAP increased from 100±2 mmHg in NP rats to 126±4 mmHg in RUPP rats (*P=0.0001) and to 118±4 mmHg in NP+RPTcell rats (P=0.002), which was significantly greater than NP+NPTcells (93±4 mmHg; P=0.006; Figure 3B). MAP in RUPP rats was significantly increased when compared with MAP in NP+NPTcell rats (P=0.0008; Figure 3B). Litter survival and pup weight for all groups treated with and without APO and TEM were unchanged among the groups but is reported in the online-only Data Supplement.

When compared with NP rats (22.6±1.2%), circulating levels of CD4+ T cells were significantly increased in RUPP (35.51±3.8%; *P=0.01; Figure 3C) and NP+RPTcell rats (29.55±2.5%; P=0.03). There were no significant differences in circulating levels of CD4+ T cells between NP+NPTcells (21.68±1.4%) and NP rats (P=0.64), which would indicate that RUPPCD4+ T cells stimulate endogenous T cells in the circulation in NP recipient rats.

Oxidative Stress Is Increased in Response to Adoptive Transfer of RUPP CD4+ T Cells

Urinary isoprostane excretion was significantly increased in RUPP and NP+RPTcell rats when compared with NP and NP+NPTcell rats (online-only Data Supplement; Figure 3D). Basal placental production of ROS between the groups was not statistically significant (P=0.403; data not shown). With the addition of NADPH oxidase, placental ROS production in RUPP rats (830.6±68 RLU/
min per milligram) and NP+RPTcell rats (726.2±43.73 RLU/min per milligram) was significantly increased when compared with that in NP rats (420.7±51.4 RLU/min per milligram; \(P=0.009\) and \(P=0.004\)) and NP+NPTcell rats (449±36.58 RLU/min per milligram; \(P=0.002\) and \(P=0.001\); Figure 3A).

Basal renal cortical ROS production between the groups was not statistically significant (\(P=0.808\); data not shown). With the addition of NADPH oxidase renal cortical ROS production in RUPP rats (966.9±53.15 RLU/min per milligram) was significantly increased when compared with NP rats (634.6±54.4 RLU/min per milligram; \(P=0.007\)) and NP+NPTcell rats (348.9±71.9 RLU/min per milligram; \(P=0.0007\); Figure 3B). NP+RPTcell rats (798.9±22 RLU/min per milligram) produced significantly more cortical ROS when compared with NP controls (\(P=0.025\)) and NP+NPTcell rats (\(P=0.004\); Figure 3B).

Apocynin Decreases MAP and CD4+ T Cells in NP Recipients of RUPP CD4+ T Cells

MAP in RUPP rats treated with APO (111±2 mm Hg) was significantly decreased when compared with MAP in untreated RUPP rats (\(P=0.03\); Figure 4C). Administration of TEM did not significantly decrease RUPPCD4+ T-cell–induced MAP (online-only Data Supplement); however, the administration of APO to NP+RPTcell rats significantly decreased MAP (102±3 mm Hg; \(P=0.01\) when compared with NP+RPTcell rats not receiving APO. There was not a significant effect of APO on NP rats (\(P=0.330\)) or on NP+NPTcell rats (\(P=0.877\); Figure 4C) when compared with untreated NP rats.

Importantly, CD4+ T cells were decreased on APO administration in both RUPP rats (decreased to 17.28±1.8%; \(P=0.002\)) and NP+RPTcell rats (decreased to 15.96±3.1%; \(P=0.008\); Figure 4A).

APO Decreases Tissue ROS Production in NP Recipients of RUPP CD4+ T Cells

APO administration decreased NADPH-induced placental ROS production in RUPP rats (622.5±40.3 RLU/min per milligram; \(P=0.05\)) and in NP+RPTcell rats (431.4±52.5 RLU/min per milligram; \(P=0.006\); Figure 5B). Cortical ROS production in RUPP rats treated with APO was significantly decreased (789.3±17 RLU/min per milligram; \(P=0.04\)) when

Figure 3. Adoptive transfer of reduced uterine perfusion pressure (RUPP) CD4+ T cells into normal pregnant (NP) rats increases mean arterial pressure (MAP) and circulating CD4+ T cells. Splenic CD4+ T cells isolated from placental ischemic (RUPP) rats and cultured for 24 hours produced significantly more myeloperoxidase (MPO) compared with CD4+ T cells isolated from NP rats (A). MAP was significantly increased in RUPP rats (B) when compared with NP rats and NP+NPCD4+ T cell rats (NP+NPTcells; B). NP recipients of RUPP CD4+ T cells (NP+RPTcells) had significantly increased circulating CD4+ T cells when compared with NP+NP Tcell rats and NP rats. CD4+ T cells are increased in placental ischemic RUPP rats and in NP+RPTcell rats (C). Excretion of urinary isoprostane was significantly increased in RUPP and NP+RPTcell rats when compared with NP and NP+NPTcell rats (D). Data are expressed as means±SEM. *\(P<0.05\), **\(P<0.005\), and ***\(P<0.0005\) between indicated groups.

Figure 4. Local reactive oxygen species production is increased in RUPP and NP recipients of RUPP CD4+ T cells. Placental (A) and renal cortical (B) production of reactive oxygen species was significantly increased on addition of nicotinamide adenine dinucleotide phosphate oxidase. Mean arterial pressure (MAP) was significantly decreased in reduced uterine perfusion pressure (RUPP) and normal pregnant (NP) recipients of RUPP CD4+ T cells (NP+RPTcells) on apocynin (APO) administration (C). Data are expressed as means±SEM. *\(P<0.05\), **\(P<0.005\), and ***\(P<0.0005\) between indicated groups.
compared with untreated RUPP rats. APO administration significantly decreased cortical ROS production in NP+RPTcell rats (693.9±15.7 RLU/min per milligram; \(P=0.02\); Figure 5C).

Administration of APO decreased urinary excretion of isoprostane from RUPP rats (54.83±4.4 ng/mg per milliliter; \(P=0.003\); Figure 5D). Urinary isoprostane excretion showed a trend to decrease in NP+RPTcell rats administered APO (78.57 ng/mg per milliliter; \(P=0.13\); Figure 5D) but did not reach statistical significance.

**Discussion**

Many characteristics of preeclampsia are associated with those of various immune diseases, such as elevated circulating inflammatory cytokines, oxidative stress, and an imbalance in T cells. In this study, we demonstrate for the first time that when measured by flow cytometry, placental T-cell ratio mirrors that of circulating T-cell alterations among patients with preeclampsia (Figure 1). In addition, we demonstrate that circulating and placental cells are a source of oxidative stress from patients with preeclampsia and not from those with normal pregnancies (Figure 1). Furthermore, exposure of endothelial cells from conditioned media from preeclamptic T cells or placental explants stimulated endothelial cell oxidative stress, indicating the importance of these cells to cause vascular oxidative stress systemically (Figure 2).

In addition, we demonstrate in our model of adoptive transfer of placental ischemic CD4+ T cells from RUPP rats into NP rats that placental and renal oxidative stress and MAP are all increased in recipient rats. Furthermore, like preeclamptic CD4+ T cell media, RUPPCD4+ T cell media directly stimulated oxidative stress in vascular endothelial cells (Figure 2). In addition, conditioned media from RUPP rat placental explants stimulated oxidative stress in vascular endothelial cells. Importantly, we demonstrate hypertension and circulating CD4+ T cells both decrease on treatment with apocynin, an inhibitor of NADPH oxidase, which correlated with lower placental and renal NADPH-stimulated oxidative stress (Figure 5). Although previous supplementation of women with preeclampsia with antioxidants vitamin E and C did not prove beneficial, we demonstrate here that oxidative stress stimulated via CD4+ T cells plays an important role in hypertension during pregnancy. This study highlights the importance of continued drug discovery for safe alternatives to suppress CD4+ T cells as a mechanism to decrease oxidative stress and many other important mediators in the pathology of preeclampsia, which would prove beneficial for the health of both mothers and babies affected by this disease.

There is increasing evidence demonstrating a role of T cells in the pathogenesis of preeclampsia. In the current study, we demonstrate that T cells are increased in the circulation and placentas of women with preeclampsia when compared with those with normal pregnancies. As Tregs peak during the second trimester and then begin to decrease as the pregnancy continues, we determined whether the changes in the number of Tregs between NP and women with preeclampsia was possibly a function of pregnancy. There was a negative correlation between gestational age at delivery and the number of Tregs, indicating that the decrease in Tregs in the preeclamptic group is not because of an earlier gestational delivery date. Furthermore, we show that white blood cells from preeclamptic placentas secrete greater myeloperoxidase, suggesting that these cells, once activated by placental ischemia, may play a role in contributing to placental oxidative stress. Although there are studies that trophoblasts and vascular smooth muscle cells release ROS during preeclampsia, to our knowledge, there is currently no data on the role of CD4+ T cells in contributing to the development of oxidative stress–mediated hypertension in preeclampsia.

In the current study, media collected from cultured placental CD4+ T cells from women with preeclampsia did not significantly stimulate oxidative stress in HUVECs. It is possible that because our isolated CD4+ cells from the placenta were >99% pure, there were potentially other cell types involved, which could contribute to the lack of statistical significance in the HUVEC studies. We have previously demonstrated that by adoptively transferring RUPP-stimulated CD4+ T cells into NP recipient rats, we can mimic some of the factors associated
with preeclampsia, thereby suggesting that CD4+ T cells play an important role in the hypertension and pathophysiological features associated with preeclampsia. The present study reveals that the hypertension associated with adoptive transfer of RUPP CD4+ T cells is accompanied by increases in the production of placental and renal ROS. Furthermore, we demonstrate that CD4+ T cells also secrete greater myeloperoxidase and stimulate myeloperoxidase in NP recipient rats, indicating their role to mediate multiple oxidative stress pathways as a pathophysiological mechanism triggered in response to placental ischemia. Interestingly, it has recently been reported that myeloperoxidase-specific CD4+ T cells contribute to renal injury in mice adoptively transferred with T cells, further supporting the idea that CD4+ T cells are indeed inducing oxidative stress.

Because oxidative stress is associated with hypertension in both clinical and experimental models, we sought to determine whether the increase in oxidative stress led to the development of the hypertension. Treatment with TEM has previously been shown to decrease oxidative stress and hypertension in experimental animal models of hypertension during pregnancy; however, the administration of TEM did not significantly decrease the blood pressure in NP recipients of RUPPCD4+ T cells. A study by De Miguel et al demonstrated that administration of TEM to Dahl salt-sensitive rats decreased hypertension but not T-cell infiltration. Therefore, it is possible that in this model of adoptive transfer of RUPPCD4+ T cells, the action of TEM is insufficient to decrease blood pressure. Therefore, we tried a more specific inhibitor of NADPH oxidase. We found that the administration of APO, an NADPH inhibitor, did significantly decrease the blood pressure in NP recipients of RUPPCD4+ T cells. In addition, administration of APO also decreased placental and cortical ROS in NP recipients of RUPP CD4+ T cells circulating CD4+ T cells in both RUPP control rats and NP recipient rats of RUPP CD4+ T cells (Figure 5). Importantly, urinary isoprostane excretion was not significantly decreased in NP+RPTcell rats receiving APO, which may indicate that although APO is effective at reducing NADPH-induced oxidative stress, other oxidative stress pathways may still be active in response to placental ischemic–stimulated CD4+ T cells. In addition, although T cells seemed to have been decreased in NP+RPTcell+APO, other immune cells that could be present in our cell prep used for adoptive transfer, such as neutrophils or monocytes, could be present releasing ROS molecules in these rats. These cell types, however, were not measured and may not have been affected by APO and could be contributing to the elevation in urinary isoprostanes. This study illustrates the importance of cellular communication between reactive oxygen molecules with immune cells and highlights how multifactorial placental ischemia can be in regard to immune activation. Although we acknowledge the importance of monocytes and neutrophils as producers of oxidative stress, we demonstrate a causal role for CD4+ T lymphocytes to mediate oxidative stress pathways as a mechanism of hypertension during pregnancy. This study further supports the importance of drug discovery to find novel immunosuppressive therapies applicable during pregnancy.

Perspectives
Preeclampsia has long been suggested to result from immunologic origins. Many studies worldwide have demonstrated that women with preeclampsia have exacerbated immune responses characterized by elevated tumor necrosis factor-α, interleukin-6, interleukin-17, autoantibodies, activated immune cells, and oxidative stress. Most recently, much focus has turned to characterizing the T-cell subsets activating and playing a role in this disease. We have focused our research attentions to deciphering a role for placental ischemia as a stimulus for dysregulation among the T-cell subsets Tregs/Th17s. In this study, we demonstrated that the placental T-cell profile mirrors that in the circulation (increased Th17/ decreased Tregs) and that these cells are secretors of oxidative stress molecules and stimulate ROS from vascular cells in vitro. Furthermore, we demonstrate for the first time that transferring CD4+ T cells mediates oxidative stress and we have previously shown that placental ischemic–stimulated CD4+ T cells mediate other pathologies associated with preeclampsia. The increase in placental and cortical oxidative stress, urinary isoprostane, and myeloperoxidase in recipients of RUPPCD4+ T cells indicates the importance of CD4+ T cells in stimulating oxidative stress during pregnancy. Importantly, NADPH oxidase inhibition attenuated oxidative stress, hypertension, and most importantly T cells from increasing in response to placental ischemia or in response to adoptive transfer of T cells during pregnancy. Collectively, the studies indicate the importance of continued research into drug discovery to improve oxidation and clinical symptoms and outcomes in women with preeclampsia.

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Disclosures
None.

References


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Supplemental Files

CD4+ T cells are important mediators of oxidative stress that cause hypertension in response to placental ischemia

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Supplemental Methods

Isolation of white blood cells (WBCs)

1mL of whole blood was mixed with 9mL’s of hanks balanced salt solution (HBSS; Sigma, St. Louis, MO), placed over a Histopaque gradient and centrifuged at 1200RPM for 25min. Placental tissues (~4-5g) were washed in phosphate buffered saline (PBS)+5% Penicillin/Streptomycin (Pen/Strep). Explants were digested in 4% collagenase I, 0.25U DNaSeI and 5% Pen/Strep in RPMI1640 buffer for an hr. The resulting digest was strained with 100µm filters and centrifuged at 1200RPM for 10min. The supernatant was discarded and the cell pellet reconstituted with HBSS and placed over a Histopaque gradient and centrifuged at 1200RPM for 25min. Cells were incubated overnight under standard conditions (5% CO2 at 37°C in a humidified atmosphere) in lymphocyte media (RPMI1640, 25mM HEPES, 2mM Glutamine, 1% Pen/Strep,10% fetal bovine serum (FBS), 1.022ng/mL IL-2 and 4ng/mL IL-12). Approximately 5mg of placental tissue per patient was cultured under standard atmospheric conditions for 24hrs in DMEM, 1% Pen/Strep, and 10% FBS. Media was collected and stored.

Staining procedures for CD4+ T regulatory (Treg) and THelper 17 (Th17) lymphocytes

Lymphocytes were separated into individual tubes at a concentration of 1x10^6 cells per tube. Cells were stained with fluorescein-isothiocyanate (FITC) conjugated anti-human CD4 (BD Pharmingen, Franklin Lakes, NJ) and phycoerythrin-cyanine 5.1 (PE-CY5) conjugated anti-human CD25 for 30min. at 4°C. After which 1mL of FACS buffer (HBSS+1% 0.5M ethylenediaminetetraacetic acid+5% FBS) was added to the cells and centrifuged for 10 min at 1200RPM as a wash step. Cells were next fixed with 1mL of 1% buffered formaldehyde for 10min. at 4°C, followed by another wash step. Cells were then permeabilized with flow cytometry FoxP3 staining buffer (RnD Systems) according to manufacturer’s directions, after which cells were labeled with either phycoerythrin (PE) conjugated anti-human forkhead box P3 (FOX3; Miltenyi Biotec, Cambridge, MA) or PE conjugated anti-human retinoic acid related orphan receptor gamma (RORγ; RnD Systems) for 30 min. at 4°C. Cells were then washed, reconstituted with FACS buffer and 50,000 events were analyzed using a Beckman Coulter Gallios Flow Cytometer equipped with a 488nm blue laser and a 638nm red laser, which is optimized for high sensitivity performance and has the capacity to acquire up to 25,000 events per second. Cells stained with FITC, PE and PECy5 were used as an isotype control. To examine Treg and Th17 cells, all cells were gated on CD4+ T cell populations which were based on the forward and side scatter (S1). Treg cells were FoxP3+ cells within the CD4+CD25^High gate and Th17 cells were RORγ+ cells within the CD4+CD25^- gate (S1). As CD4 is also present on monocytes, macrophages and dendritic cells, we also analyzed via flow cytometry the percentage of CD3+ T cells and CD20+ B cells within our selected gate, and then analyzed the percentage of T cells and B cells that were CD4+ (S2). Circulating and placental CD8+ cells were also analyzed via flow cytometry using APC conjugated anti-human CD8 (RnD Systems). Each patient served as their own control to account for patient to patient variability. Fluorescence minus one (FMO) controls were run with each Gallios usage to determine what compensation if any should be used.

Isolation of CD4+ placental lymphocytes

To isolate CD4+ lymphocytes from placentas, isolated lymphocytes were resuspended in degassed MACS running buffer (Miltenyi Biotec) according to manufacturer’s directions. Human CD4 microbeads were added to the lymphocytes and mixed at 4°C for 30 min. 2mL of MACS running buffer was added to lymphocytes and they were centrifuged at 3200RPM for 10min. The cell pellet was resuspended in MACS running buffer, filtered with a 70 micron filter
and passed through MS columns (Miltenyi Biotec). CD4⁺ lymphocytes were released from the columns via mechanical separation. The resulting CD4⁺ lymphocytes were cultured overnight at 5% CO₂ at 37°C in a humidified atmosphere in lymphocyte media at a concentration of 2x10⁵/mL, followed by media collection. This isolation resulted in on average 77.67% CD4⁺ T cell purity.

Determination of oxidative stress via lucigenin

Briefly, 5mg of placental explants were collected, rinsed in PBS and homogenized in radioimmunoprecipitation assay (RIPA) buffer + protease inhibitor cocktail (Roche, Madison, WI). The samples were centrifuged at 12,000g for 10min, the supernatant aspirated and the remaining cellular debris discarded. 50µL of the supernatant was incubated with lucigenin at a final concentration of 5µmol/l in Krebs-HEPES buffer with or without NADPH (Sigma). The samples were allowed to equilibrate for 15min in the dark, and luminescence was measured every second for 10sec. with a luminometer (Berthold, Oak Ridge, TN)¹, ². Luminescence was recorded as relative light units (RLU) per min.

Dihydroethidium staining of Human Umbilical Vein Endothelial Cells (HUVECs)

To determine if placentas and placental CD4⁺ T lymphocytes could cause an increase in vascular ROS production HUVECs were grown to 70% confluency on gelatin coated BD Falcon culture slides under standard atmospheric conditions in HUVEC media (Medium 199, 0.1mg/mL heparin, 5%fBS, 0.05mg/mL endothelial cell growth attachment factor, 1%Pen/Strep). After which cells were serum starved for 48hrs and then exposed to experimental media (50% conditioned media from human placentas or from placental CD4⁺ T lymphocytes and 50% HUVEC media) for 24hrs under standard atmospheric conditions. After 24hrs, media was removed and cells washed with HBSS. Dihydroethidium (DHE; Sigma) was added at a concentration of 5µM in HBSS to cells and incubated for 30min. at 37°C in the dark. The DHE stain was removed, mounting media added and slides were coverslipped. All experiments were performed in duplicate. Immunofluorescence intensity was quantified using Leica Confocal software and normalized by cell area (µm²).

Reduced Uterine Perfusion Pressure (RUPP rat model of preeclampsia)

To reduce uterine perfusion pressure on gestational day (GD)14 under isoflurane anesthesia normal pregnant (NP) rats underwent RUPP surgery with the application of a constrictive silver clip (0.203 mm) to the aorta superior to the iliac bifurcation performed while ovarian collateral circulation to the uterus was reduced with restrictive clips (0.100 mm) to the bilateral uterine arcades at the ovarian end ³⁻⁵.

Determination of mean arterial pressure in chronically instrumented conscious rats

On gestational day (GD) 18, a catheter of V-3 tubing was inserted into the carotid artery, tunneled to the back of the neck and exteriorized after implantation for blood pressure monitoring. On GD19, pregnant rats were placed in individual restraining cages for arterial pressure measurements. Arterial pressure was monitored with a pressure transducer and was recorded continuously for a 1 hr. period following a 1hr stabilization period ³⁻⁶.

Isolation of RUPP CD4⁺ T cells for adoptive transfer into normal pregnant (NP) recipient rats

Briefly, spleens from NP and RUPP rats were isolated at the time of sacrifice on GD19 and immediately placed in ice-cold PBS. Spleens were homogenized with RPMI 1640 medium containing 10% FBS and filtered through a 100µm cell strainer to obtain single cell suspensions. CD4⁺ T cells were isolated from the splenocytes via magnetic separation using CD4⁺ Dynabeads according to the manufacturer’s recommended protocol (Invitrogen, Grand Island, NY). Once released from the Dynabeads, CD4⁺ T cells were washed in PBS and cultured in lymphocyte
media for 24 hours at 5% CO2 at 37º C in a humidified atmosphere at a concentration of 2x10^5/mL. Following centrifugation, media was collected and cell pellets were washed with saline and adjusted to 1 x 10^6 cells/100 µl saline for intraperitoneal injection into recipient NP rats on their 13th day of gestation.

Supplemental Data

Placental CD8^+ T cells are significantly increased in women with preeclampsia.

There was no significant difference in circulating CD8^+ T cells in (3.14 ± 1.04% vs. 2.3 ± 0.71%; p=0.55) between NP and preeclamptic women. Placental CD8^+ T cells were increased in preeclamptic women compared to NP women (6.73 ± 2.19% vs. 0.41 ± 0.26%, p=0.021).

Reduction in uterine perfusion pressure decreases litter survival

There was no significant difference between NP (2.1 ± 0.06g), RUPP (2.04 ± 0.06g), NP+NPTCell (2.31 ± 0.11g), and NP+RPTCell (2.1 ± 0.08g) rats in pup weight (p=0.144). Litter survival in RUPP rats (46.8 ± 13.8%) was significantly decreased compared to all groups of rats (p=0.0011) who had between 98.25-92.93% litter survival. Treatment with Tempol (TEM) did not significantly increase pup weight in RUPP rats (1.81 ± 0.14g) compared to NP rats (2.2 ± 0.07g; p=0.02) administered TEM. Litter survival in TEM treated RUPP rats (42.64 ± 11.6%) was significantly decreased compared to the other groups treated with TEM (p<0.0001). There was no significant difference between NP, RUPP and NP CD4 recipient rats treated with apocynin (APO) in pup weight (p=0.208). Litter survival in apocynin treated RUPP rats (40.89 ± 14.21%) was significantly decreased compared to the other groups treated with APO (p=0.0009). Litter survival was determined by ((#live pups/(sum of live and reabsorbed pups))*100).

Oxidative stress is increased in response to adoptive transfer of RUPP CD4^+ T cells

RUPP rats excreted significantly more urinary isoprostane compared to NP rats (94.15 ± 6.5 vs. 64.49 ± 11.7 pg/mg/mL; p=0.046) and NP+NPTcell rats (36.75 ± 5.9 pg/mg/mL; p=0.0001; Figure 3D). NP+RPTcells also excreted significantly more isoprostane compared to NP rats (100.5 ± 10.7; p=0.04) and NP+NPTCell rats (p=0.0008; Figure 3D).

Tempol (TEM) does not prevent RUPPCD4^+ T cell induced increase in MAP

As we have previously reported, TEM significantly decreased MAP in placental ischemic RUPP rats compared to untreated RUPP rats (114.1 ± 8. vs. 128.1 ± 4mmHg in untreated RUPP; p=0.03). However, administration of TEM to NP recipients of RUPP CD4^+ T cells did not significantly decrease MAP when compared to NP+RPTcell rats not receiving TEM (114.8 ± 2.1 vs. 118.3 ± 4.2; p=0.477). Administration of TEM did not cause a statistically significant change in MAP in NP+NPTcell rats compared to untreated NP+NPTcell rats (102.3 ± 1.3 vs. 92.7 ± 3.7; p=0.07).

References.


S1. Gating strategy for triple stained lymphocytes (tissue and whole blood). Freshly isolated peripheral blood or tissue lymphocytes were isolated, stained and analyzed. Representative scatter plots and staining profiles are shown; gating strategy was the same for peripheral blood leukocytes, and tissue. Lymphocytes were gated in the forward and side scatter plot and the percentage of CD4 and CD25 were measured within the lymphocyte gate. FOXP3 was gated in the CD4$^{+}$CD25$^{High}$ subset of cells and RORgamma was gated in the CD4$^{+}$CD25$^{-}$ subset of cells.
S2. Distribution of CD4⁺ T and B cells. The percentage of CD4⁺ T and B cells was determined. Representative histograms and scatter plots are shown demonstrating that cells stained with CD4 are T cells.