IL-17 mediated oxidative stress is an important stimulator of AT1-AA and hypertension during pregnancy

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ABSTRACT

Preeclampsia is associated with autoimmune cells, T_{H}17, secreting interleukin-17, autoantibodies activating the angiotensin II type I receptor (AT1-AA) and placental oxidative stress (ROS). The objective of our study was to determine if chronic IL-17 increases blood pressure by stimulating ROS and AT1-As during pregnancy. To answer this question four groups of rats were examined: Normal Pregnant NP (n=20), NP+ IL-17 (n=12), NP+ tempol (n=7) (a superoxide dismutase mimetic that scavenges ROS) and NP+IL-17+Tempol (n=11). IL-17 (150 pg/day) was infused into NP rats while tempol was administered via the drinking water ad libitum. On day 19 blood pressure (MAP) was recorded, plasma, urine, and tissue were collected for isolation of ROS detected by chemilluminescent technique. Urinary isoprostane was measured by ELISA. AT1-As were determined via cardiomyocyte assay and expressed as beats per minute. MAP increased from 98+/-3 in NP to 123+/-3 mmHg in IL-17 infused NP rats. Urinary isoprostane increased from 1029 +/- 1 pg/mg/day in NP to 3526 +/- 2 pg/mg/day in IL-17 infused rats, p<0.05. Placental ROS was 436 +/- 4 RLU/ml/min (n=4) in NP and 702 +/- 5 (n=5) RLU/ml/min in IL-17 treated rats. Importantly AT1-AA increased from 0.41 +/- 0.05 bpm in NP rats (n=8) to 18.4 +/- 1 in IL-17 rats (n=12). Administration of Tempol attenuated the hypertension, (101 +/- 3 mmHg) ROS (459 +/- 5 RLU/ml/min) and blunted AT1-As (7.3 +/- 0.6 bpm) in NP+IL-17+Tempol treated rats. Additionally, AT1 receptor blockade inhibited IL-17 induced hypertension and placental oxidative stress. MAP was 105 +/- 5 mmHg and ROS was 418 +/- 5 RLU/ml/min in NP+IL17 treated with losartan. These data indicate that IL-17 causes placental oxidative stress which serves as stimulus modulating AT1-As which may play an important role in mediating IL-17 induced hypertension during pregnancy.
INTRODUCTION

Preeclampsia is one of the major causes of maternal and perinatal mortality and morbidity and yet, the pathophysiology of this disease remains incompletely understood\(^5,14\). The initiating event of the disease is associated with abnormal cytotrophoblast invasion resulting in inadequate remodeling of the uterine spiral arteries leading to significantly reduced uterine perfusion pressure (RUPP) of the utero-placental unit\(^5,6\). This cytotrophoblast invasion is tightly regulated between various immune cell types, such as monocytes, macrophages, uterine natural killer cells, T lymphocytes and endothelial cells\(^5,6,8,14\). As preeclampsia develops, a dysregulation among these immune cell types begins early in implantation, thus lending to the overactivation of a chronic inflammatory immune response\(^8\). As a result the placenta becomes ischemic, releasing factors thought to contribute to the development of hypertension and other agents of disease expression such as proinflammatory cytokines, TNF alpha and IL-6 being released into the maternal peripheral circulation\(^5,6,8,14\). Additionally we have learned recently of autoimmune activities during preeclampsia such as the production of autoantibodies to the angiotensin II type I receptor\(^6,8\) and elevated circulating IL-17 and T\(_{H}17\) cells \(^16\).

One important physiological function of IL-17 producing T\(_{H}17\) cells is to recruit leukocytes such as neutrophils to the site of infection as a major host defense mechanism against extracellular bacteria. At the site of recruitment, IL-17 stimulates a myriad of other cytokines such as TNF alpha and Interleukin 6 (IL-6) which are important for intercellular communication. Importantly, IL-17 also induces neutrophilic release of antimicrobial substances and/or phagocytosis of microbes and dead tissues. Macrophages and neutrophils convert molecular oxygen into reactive oxygen species by the phagocyte oxidase system catalyzed by the
enzyme NADPH oxidase. Once neutrophils are activated, they can cause injury to normal host tissues, such as the placental unit, by the release of lysosomal enzymes, reactive oxygen species or nitric oxide. Many studies have indicated that preeclamptic women have elevated oxidative stress within the placental unit measured by increased NADPH subunits as well as elevated urinary 8 isoprostanes as a measure of whole body oxidative stress.

In accord with exacerbated immune function during preeclampsia, recent evidence implicates an imbalance between regulatory (Treg) and effector T cells, subclasses of CD4+ T lymphocytes, in preeclamptic women. There is considerable data both in humans and in mice for the importance of Interleukin 17 in the development and progression of chronic inflammatory and autoimmune diseases. IL-17-producing CD4+ T cells (Th17 cells) are the dominant pathogenic cellular component in autoimmune inflammatory diseases, including autoimmune arthritis, psoriasis and multiple sclerosis. In contrast, Tregs (Foxp3+ T cells) have anti-inflammatory properties and can cause quiescence of immune activation. In agreement with recent clinical studies performed in preeclamptic vs normal pregnant patients, we have demonstrated abnormal ratios of circulating Tregs(Foxp3+CD4+ T cells)/Th17 (ROR γ+CD4+ T cells) occurring in response to RUPP in the pregnant rat. In addition we have shown that adoptive transfer of total CD4+ T cells from RUPP control rats into NP recipient rats causes hypertension associated with elevated IL-17 and AT1-AA. Therefore the objective of this study was to determine if chronic IL-17 during pregnancy leads to increased blood pressure, placental oxidative stress and autoantibody production (AT1-AA) as mediators of the pathogenicity of preeclampsia.

**MATERIALS AND METHODS**

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

**Protocol 1a: Effect of IL-17 infusion on MAP in Pregnant Rats**

Recombinant mouse IL-17 (150 pg/day) (RnD Systems, Minneapolis, MN) was infused intraperitoneally from day 14 to day 19 of gestation via mini-osmotic pumps (model 2002, Alzet Scientific Corporation) into normal pregnant (NP) rats. IL-17 (150pg/day) was also infused into virgin (V) rats via mini-osmotic pumps for 5 days.

**Measurement of mean arterial pressure in chronically instrumented conscious rats**

Under isoflurane anesthesia on day 18 of gestation or the 5th day of IL-17 infusion for virgin rats, carotid arterial catheters were inserted for blood pressure measurements. The catheters inserted are V3 tubing (SCI) which is tunneled to the back of the neck and exteriorized. On day 19 of gestation arterial blood pressure was analyzed after placing the rats in individual restraining cages. Arterial pressure was monitored with a pressure transducer (Cobe III Transducer CDX Sema) and recorded continuously after a one hour stabilization period. Subsequently, a blood and urine sample was collected, kidneys and placentas were harvested, and litter size and pup weights were recorded under anesthesia.9, 12

**Determination of circulating T lymphocytes**

Circulating CD4+ T cell populations were measured from peripheral blood leukocytes (PBL) collected at day 19 of gestation from normal pregnant (NP) rats and from pregnant IL-17 infused rats. We utilized flow cytometry analysis to detect specific CD4+ T cell populations;
CD4⁺RORγ⁺ (retinoic acid receptor-related organ receptor gamma) isolated from chronic IL-17 treated and NP rats PBLs. At the time of tissue harvest, plasma was collected and PBLs were isolated from plasma by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical Corp) according to the manufacturer’s directions. For flow cytometric analysis equal numbers of leukocytes (1 x 10⁶) were incubated for 30 minutes at 4°C with antibodies against mouse CD4 (BD Biosciences, San Jose, CA). After washing, cells were labeled with the secondary fluorescein isothiocyanate (FITC) antibody (Southern Biotech, Birmingham, AL) for 30 minutes at 4°C. Cells were washed and permeabilized and stained with anti-rat RORγ conjugated to PE (BD Pharmingen) 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 anti-FITC and anti-PE secondary antibodies alone. Subsequently, cells were washed and resuspended in 500µl of Roswell Park Memorial Institute medium (RPMI) and analyzed for single and double staining on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percent of positive staining cells above the negative control was collected for each individual rat and mean values for each experimental group (NP and NP+IL-17) was calculated.

**Determination of IL-6** An important function of IL-17 is induced cytokines such as IL-6, which would induce expansion of the T_i17 and B lymphocytes, therefore, we utilized the rat IL-6 Quantikine ELISA. The assay displayed a sensitivity of 21 pg/mL, intra-assay variability is 7.4% and interassay is 8.4%.

**Determination of urinary Isoprostone**

On day 19 of gestation, urine was collected and utilized for determination of excreted isoprostanes measured via ELISA from Oxford Biomedical Research (Oxford, MI). The assay
displayed a sensitivity of 0.05ng/mL, inter-assay variability of 4.2%, and intra-assay variability of 4.7%.

**Determination of tissue ROS.**

Superoxide production in the placenta was measured by using the lucigenin technique as we have recently described.\(^{10,13}\) Rat placentas were snap frozen in liquid nitrogen directly after collection and stored at -80°C until further processing. Placentas were removed and homogenized in RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Santa Cruz, Santa Cruz, CA) as described previously.\(^{10,13}\) The samples were centrifuged at 12,000g for 20 min, the supernatant aspirated and the remaining cellular debris discarded. The supernatant was incubated with lucigenin at a final concentration of 5μmol/l. The samples were allowed to equilibrate for 15 min in the dark, and luminescence was measured every second for 10 sec with a luminometer (Berthold, Oak Ridge, TN). Luminescence was recorded as relative light units (RLU) per min. An assay blank with no homogenate but containing lucigenin was subtracted from the reading before transformation of the data. Each sample was repeated 5 times and the average 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/min/mg protein.

**Determination of circulating AT1-AA**

On day 18 of gestation blood was collected and immunoglobulin was isolated from 1mL of serum by specific anti-rat IgG column purification. AT1-AA was purified from rat IgG by epitope binding to the amino acid sequence corresponding to the second extracellular loop of the AT1 receptor covalently linked to Sepharose 4B CNBr-activated gel. Unbound IgG was washed away and bound IgG was eluted with 3M potassium thiocyanate. AT1-AA activity was measured
utilizing a bioassay that evaluates the beats/minute (bpm) of neonatal cardiomyocytes in
culture. AT1-AAs were assessed in NP controls and NP+IL-17 infused rats. Since AT1-AA is not present in non-pregnant rats, it was not determined in this study.

**Protocol 2: Effect of Tempol on IL-17-induced hypertension**

This protocol was performed to determine whether the effect of placental and cortical oxidative stress generated in response to chronic IL-17 to increase blood pressure by administering a superoxide dismutase mimetic, to NP and IL-17-infused rats. NP rats treated with tempol added to the drinking water were used as controls whereas IL-17 infused rats treated with tempol served as the experimental group. On day 14 of gestation tempol (30mg/kg/day) was administered in the drinking water. Mini-osmotic pumps (Alzet 2002) infusing 150pg/day of IL-17 were surgically implanted intraperitoneally on day 14 into 12 pregnant rats. Arterial pressure was determined in all groups of pregnant rats at day 19 of gestation as previously described in protocol 1a.

**Protocol 3: Effect of AT1 Receptor blockade on IL-17-induced hypertension**

This protocol was performed to determine whether activation of the AT1 receptor response to chronic IL-17 caused an increase blood pressure and placental ROS. In this protocol Losartan (5mg/kg/day) was administered to NP and IL-17-infused rats via the drinking water beginning on day 14 of gestation. Mini-osmotic pumps (Alzet 2002) infusing 150pg/day of IL-17 were surgically implanted intraperitoneally on day 14 into 5 pregnant rats losartan treated NP served as controls (n=4). Arterial pressure was determined in all groups of pregnant rats at day 19 of gestation as previously described in protocol 1a.

**Protocol 4: B cell depletion attenuates IL-17 induced increases in blood pressure**
Normal pregnant rats were treated with Rutiximab (250mg/kg), from day 14 to day 19 of gestation (n=7) via osmotic minipump insertion. Rituximab is a chimeric monoclonal anti-CD20 antibody which is used to induce B cell depletion in vivo\textsuperscript{10,17}. The dose used was based on the efficacy demonstrated in previous studies from our lab illustrating that administration of Rituximab depleted circulating B cells and suppressed AT1-AA in RUPP rats and RUPPCD4+ Tcell induced hypertensive pregnant rats. Arterial pressure and circulating T\textsubscript{H}17 cells were determined in pregnant rats at day 19 of gestation as described in protocol 1.

**Statistical Analysis**

All data are expressed as mean standard error of the mean. Anova was used to determine differences among blood pressure and T\textsubscript{H}17 cells between the groups the multiple experimental groups. Difference between control and experimental tissues were analyzed using the t-test and was considered statistically significant at P values < 0.05. NP was compared to IL-17 infused, while Tempol treated NP was compared to Tempol treated IL-17 infused, Losartan treated NP were compared to Losartan treated IL-17 infused pregnant rats.

**RESULTS**

**Protocol 1: IL-17 infusion into normal pregnant rats is associated with hypertension and oxidative stress**

*Effect of IL-17infusion on T\textsubscript{H}17 cells and blood pressure during pregnancy*

Mean arterial pressure was increased significantly in IL-17 infused NP rats 123±3 mmHg (n=12) compared to NP rats 98±3 mmHg (n=20) (Figure 1; p=0.001). As with other cytokine infusion studies, infusion of IL-17 into virgin rats had no effect on blood pressure, MAP was 124±2 mmHg in virgins versus 117±3mmHg in virgins treated with IL-17. We hypothesized that infusion of IL-17 would stimulate T\textsubscript{H}17 cells which would be found elevated in the circulation of
IL-17 treated rats, therefore we utilized flow cytometry to determine levels of circulating T\textsubscript{H}17 cells in NP and IL-17 treated rats. Figure 2 demonstrates that circulating CD\textsuperscript{4+} T\textsubscript{H}17 cells, identified by expression of the ROR\textgamma transcription factor, are significantly elevated in response to chronic IL-17 infusion during pregnancy compared to NP rats (13±7% NP vs. 41±5% NP+IL-17, P<0.02).

Pup weight nor litter size nor placental weight were different between NP and NP+IL17 treated pregnant rats. NP litter size was 14, pup weight was 2.27±0.05 g and placental weight was 0.66±0.07g in NP rats. IL-17 treated litter size was 13, pup weight was 2.24±0.133g and placental weight was 0.62±0.11g in IL-17 treated pregnant rats.

**Effect of IL-17 on Reactive oxygen species**

Urinary levels of isoprostane were found to be significantly elevated with chronic infusion of IL-17 from 1029±1 pg/mg/mL in NP to 3526±2 pg/mg/mL in NP+IL-17 rats (Figure 3; p<0.05). We observed an increase in placental ROS production with the addition of NADPH oxidase in IL-17 infused rats (702±5 RLU/mL/mg) compared to NP controls (436±4 RLU/ml/mg) (Figure 4A, p<0.001).

**Effect of IL-17 on Renin angiotensin system**

Infusion of IL-17 into normal pregnant rats increased production of the AT1-AA to 18.4±1 bpm (n=12) compared to normal pregnant control rats 0.41±0.05 bpm (n=8) (Figure 5, p<0.001). Plasma ANG II, measured at Wake Forest, increased slightly but was not significantly different with IL-17 infusion in pregnant rats. In control pregnant rats, plasma ANGII was 54±16 versus 86±24 pg/ml in IL-17 treated pregnant rats.

**IL-6 is stimulated during IL-17 induced hypertension**
We hypothesized that as a result of elevated IL-17 during pregnancy, IL-6 would be induced and be an important player in intercellular communication to facilitate TH17 and potentially B cell activation. Therefore, we measured circulating IL-6 in IL-17 induced hypertensive pregnant rats. IL-6 increased significantly in response to IL-17 infusion and was 323 +/- 83 pg/ml in IL-17 infused rats (n=5) and was 89 +/- 13 pg/ml in NP rats (P<0.005) (n=8).

Protocol 2: Tempol attenuates placental oxidative stress, AT1-AA and hypertension during pregnancy

**Effect of Tempol on placental oxidative stress**

Administration of tempol decreased NADPH-stimulated placental production of ROS from IL-17 infused pregnant rats (459 +/- 5 RLU/min/mg) to levels no longer statistically different when compared to placental ROS from tempol-treated NP controls (474 +/- 5 RLU/min/mg; n=3) (Figure 4B; p=0.096). In addition, Tempol administration significantly decreased circulating AT1-AAs produce in response to IL-17 to 7.3 +/- 6 bpm (n=7) in NP+IL-17 rats (Figure 5, p<0.001). Importantly, the blood pressure increase to chronic IL-17 was completely attenuated with Tempol administration and was no different in Tempol treated IL-17 infused rats when compared to Tempol treated control rats. MAP was 102 +/- 5 mmHg (n=7) in Tempol control NP rats versus 101 +/- 2 mmHg (n=7) in IL-17 Tempol treated pregnant rats (Figure 1).

Protocol 3: AT1 receptor blockade attenuates IL-17 induced increases in blood pressure and placental oxidative stress

Importantly, Losartan administration attenuated the blood pressure increase to chronic IL-17; MAP was no different in Losartan treated IL-17 infused rats when compared to Losartan treated control rats. MAP was 98 +/- 5 mmHg (n=4) in Losartan control NP rats versus 105 +/-
2mmHg (n=5) in IL-17 Losartan treated pregnant rats (Figure 1). Furthermore, administration of losartan decreased both basal and NADPH-stimulated placental production of ROS from IL-17 infused pregnant rats (185±38 RLU/min/mg n=3, basal) to levels no longer statistically different when compared to basal placental ROS from losartan-treated NP controls (243±36 RLU/min/mg; n=4). NADPH-stimulated placental production of ROS from IL-17 infused pregnant rats (418±5 RLU/min/mg n=5) to levels no longer statistically different when compared to placental ROS from losartan-treated NP controls (619±5 RLU/min/mg; n=4).

**Protocol 4: B cell depleting agent, Rituximab, decreased blood pressure in response to chronic IL-17 during pregnancy.**

Importantly, administration of Rituximab significantly decreased blood during IL-17 infusion, however, this decrease was not as profound as the blockade with either Tempol or Losartan. MAP was reduced from 123±3 mmHg in IL-17 treated controls to 115±/-4mmHg in IL-17 Rituximab treated rats(P<0.05) compared to NP rats 98±3 mmHg (P<0.05). In addition, Rituximab decreased circulating T\(\text{H}17\) cells to 21%+/-4.7, which was significantly less compared to IL-17 induced hypertension pregnant (P<0.05). However, T\(\text{H}17\) cells in IL-17 + Rituximab was still significantly greater than that in the NP control group. (13+/-7%, P<0.05)

**DISCUSSION**

IL-17 producing T\(\text{H}17\) cells is a newly discovered subset of T helper cell which is believed to be a mediator for many autoimmune diseases such as Crohn’s disease, psoriasis, rheumatoid arthritis and multiple sclerosis. We are now learning of a potential role for IL17 and T\(\text{H}17\) cells as mediators for the pathophysiology associated with preeclampsia\(^1,7,16\). Although IL-17 and T\(\text{H}17\) cells are elevated in preeclampsia, it remains unknown if either IL-17 or T\(\text{H}17\) cells cause hypertension or any state of disease expression that is associated with preeclampsia.
Therefore, in this study we demonstrate, for the first time, that chronic IL-17 increases blood pressure and TH17 cells in pregnant rats but not in virgin rats. It is important to note that infusion of this dose of IL-17 had no hypertensive effects on the nonpregnant rats, just as we have seen with both TNF alpha and IL-6, indicating that moderate doses of cytokines cause hypertension during pregnancy most likely by stimulating some placental factor or factors to cause much of the disease characteristics.

IL-17 stimulates neutrophilic reactions and the production of antimicrobial substances such as defensins and reactive oxygen species. We demonstrate that IL-17 stimulates oxidative stress as illustrated by the increased in urinary isoprostanes and NADPH stimulated placental oxidative stress. With preeclampsia being associated with autoantibodies, and IL-17 being primarily an autoimmune cytokine, we wanted to determine if IL-17 induced hypertension was mediated through production of AT1-AA. Indeed, as with TNF alpha and IL-6 induced hypertension during pregnancy, IL-17 induced hypertension in pregnant rats is associated with production of the AT1-AA. Other investigators have demonstrated a clear link with IL-17 and angiotensin II induced hypertension,\(^\text{17}\) therefore we measured plasma ANG II and found only a small increase that was not significantly different between normal pregnant controls and IL-17 infused pregnant rats. Although, elevations in ANGII are not associated with preeclampsia, a link with increased ANGII sensitivity is speculated to play a role in this disease, we believe this may be due to production of the AT1-AA acting in concert with ANGII to activate the AT1 receptor. In order to determine a role for activation of AT1 receptors we treated a group of rats with losartan in the presence or absence of IL-17 infusion. We found that losartan attenuated the blood pressure response to IL-17 during pregnancy but also surprisingly inhibited placental
oxidative stress in IL-17 treated pregnant rats. This could be due to blockade of both AT1-AA and ANGII activation of the AT1 receptor.

In order to determine if the increase in oxidative stress caused the hypertension observed with IL-17 infusion, we repeated the infusion study in pregnant rats treated with the SOD mimetic in their drinking water (Tempol). We found that administration of Tempol not only attenuated the placental oxidative stress but also the increase in blood pressure in response to IL-17 infusion. Surprisingly, we found that administration of Tempol and decreased ROS in the placenta was associated with significantly lowered AT1-AA in response to IL-17. These data indicate the importance of reactive oxygen species as signaling molecules between immune cells and tissues to cause much of the pathology associated with preeclampsia and IL-17 induced hypertension during pregnancy.

In a previous study we infused rat AT1-AA into normal pregnant rats and found that placental oxidative stress was significantly elevated when compared to the nonhypertensive control normal pregnant rats. In that study we administered Tempol and found that we could attenuate the placental oxidative stress and the hypertension in that model. Therefore, in the current study, we examined the role of the AT1-AA to mediate the hypertension, stimulated in response to IL-17 as mediators of pathophysiology of preeclampsia. We have recently demonstrated that administration of Rituximab decreased circulating B cells and AT1-AA in two previous experiments. First we administered the B cell depleting agent to RUPP rats and found that AT1-AA, local ET-1 and blood pressure were decreased in response to placental ischemia in this rat model of preeclampsia. Most recently, we administered Rituximab to NP recipient rats of RUPP CD4+ T cells. In this T cell induced preeclampsia rat model blood pressure and circulating AT1-AA were significantly elevated compared to NP recipients of NP CD4+ T cells.
Administration of Losartan decreased the hypertension. Furthermore, administration of Rituximab was efficient at B cell depletion and AT1-AA suppression and attenuation of hypertension in response to CD4+ T cells from RUPP rats\textsuperscript{17}. We demonstrate, in this study, that administration of Rituximab significantly decreased the blood pressure in response to IL-17 infusion, suggesting an important role for the AT1-AA to cause hypertension in this model. However, it is important to note that as seen in RUPP rats, B cell depletion by administration of Rituximab did not attenuate but significantly blunted the blood pressure response to IL-17; MAP in IL-17+Rituximab treated rats was still significantly elevated compared to NP control rats. Furthermore, we demonstrate that administration of Rituximab significantly decreased circulating $T_h$17 cells stimulated in response to IL-17 infusion. However, this response was not completely attenuated and circulating $T_h$17 cells stimulated in response to IL-17 infusion was still significantly higher than that of NP control rats. Therefore we conclude that the remaining increased in blood pressure in this group could be due to the presence of excess circulating $T_h$17 cells or ANGII or other inflammatory cytokines stimulated in response to IL-17 infusion. However, since the blood pressure response nor the $T_h$17 stimulation was completely abolished we did not examine these additional factors. Nevertheless, these data highlight the importance of many factors caused by IL-17 excess during pregnancy, such as IL-17 stimulated oxidative stress, other inflammatory cytokines and $T_h$17 cells to mediate the elevation in blood pressure and pathophysiology associated with preeclampsia.

Although we recognize that ROS is a product of neutrophils and monocytes, we did not determine nor isolate and culture these cell types from our IL-17 infused pregnant rats. These phagocytic cells contain NADPH subunits and produce ROS, therefore they could be the producers of elevated whole body oxidative stress indicated by the sharp increase in urinary 8-
isoprostanes. We did, however, demonstrate that in response to chronic IL-17 infusion, T\textsubscript{H}17 cells are elevated in the circulation of pregnant rats. The stimulus for such could be a typical cellular response resulting from IL-6 and other cytokines such as IL-8, TNF alpha or IL-1, all of which synthesis is induced by elevated IL-17. Importantly, we found that indeed in response to chronic IL-17 administration, IL-6 is significantly elevated above that observed in NP rats. IL-17 is critical for function of T\textsubscript{H}17 cells which are associated with immune and autoimmune phenomena. It is accepted that elevated cytokines stimulate immune cells and intercellular/intracellular communication between leukocytes. Therefore, as demonstrated here, one result of chronic IL-17 infusion is elevated T\textsubscript{H}17 cells, oxidative stress and AT1-AA as mechanisms of hypertension during pregnancy. A secondary mediator could be elevated IL-6, which facilitates proliferation of both T\textsubscript{H}17 cells and B cells and as we have previously shown, is an important player in hypertension and AT1-AA production in response to placental ischemia\textsuperscript{19}. Future studies of interest would be to determine if what immune cell type is source of oxidative stress in the placenta and what importance do T\textsubscript{H}17 cells play in causing hypertension and AT1-AA production from B cells when IL-17 is elevated or in response to placental ischemia. Importantly, this study highlights the importance of IL-17 stimulated ROS as signaling molecules linking immune activation with the development of hypertension during pregnancy. Future studies blocking IL-17 and possibly T\textsubscript{H}17 cells in response to placental ischemia will be important to determine a causal role for IL-17 in placental oxidative stress and hypertension during preeclampsia.

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Figure legends

**Figure 1.** IL-17 induced hypertension is attenuated with a SOD mimetic or AT1 receptor blockade. Chronic infusion of IL-17 into normal pregnant rats causes hypertension during pregnancy. This blood pressure response is attenuated by administration of a superoxide dismutase (SOD) mimetic or Losartan, an AT1 receptor blocker. Data are expressed as Mean±S.E.M. * P<0.05 vs. normal pregnant controls

**Figure 2.** Infusion of IL-17 into NP rats increased circulating CD4+/ROR gamma + T cells when compared to NP control rats. Data are expressed as Mean±S.E.M. *P<0.05 vs normal pregnant controls

**Figure 3.** IL-17 infusion increases urinary isoprostane excretion. Urinary isoprostane excretion is increased in response to chronic IL-17 infusion, indicating that IL-17 increases oxidative stress. Data are expressed as Mean±S.E.M. * P<0.05 vs. normal pregnant controls

**Figure 4.** IL-17 infused placental oxidative stress is blunted by a superoxide dismutase mimetic or Losartan. A. Chronic infusion of IL-17 into normal pregnant (NP) rats significantly produces greater NADPH stimulated placental reactive oxygen species. B. Chronic administration of
tempol to IL-17 infused rats decreases NADPH stimulated placental oxidative stress to levels no longer significantly different than that of tempol-treated controls. C. Chronic AT1 receptor blockade with losartan significantly decreased NADPH stimulated placental oxidative stress in IL-17 treated rats. Data are expressed as Mean±S.E.M., RLU, relative light units. * P<0.05 vs. normal pregnant controls.

**Figure 5.** Tempol administration decreases AT1-AA in response to chronic infusion of IL-17. AT1-AA isolated from serum of rats chronically infused with IL-17 is significantly increased compared to normal pregnant rats, but is blunted in response to tempol administration. Data are expressed as Mean±S.E.M. *** P<0.001 vs. IL-17 infused rats
Figure 2

TH17 cells
CD4+/ROR+ (%gated)

NP NP+IL-17

**
Figure 5

AT1-AA (Basis per minute)

NP  NP+IL-17  NP+IL-17+Tempol

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