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Blockade of CD40 ligand for intercellular communication reduces hypertension, placental oxidative stress, and AT1-AA in response to adoptive transfer of CD4+ T lymphocytes from RUPP rats

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ABSTRACT

Preeclampsia (PE) is associated with altered immune activation during pregnancy. We have previously shown that adoptive transfer of CD4⁺ T cells from the reduced uterine perfusion pressure (RUPP) rat model of PE increases blood pressure, oxidative stress (ROS), and inflammation in normal pregnant recipient rats. The objective of this study was to determine if blockade of communication via the CD40-CD40 ligand interaction between placental ischemia-induced CD4⁺ T cells with endogenous normal pregnant (NP) cells would improve pathophysiology that was previously observed in NP recipient rats of RUPP CD4⁺ T cells. Splenic CD4⁺ T lymphocytes were magnetically separated, incubated with 2.5 µg/mL anti-CD40 ligand (αCD40L) overnight and transferred into NP rats on day 12 of gestation (NP+RUPP CD4⁺T+anti-CD40L). On day 19 of gestation, blood pressure (MAP), blood, and tissues were collected. MAP was 99±2 in NP (n=13), 116±4 in NP+RUPP CD4⁺ T cells (n=7; p<0.01); MAP only increased to 104 ±2 in NP+RUPP CD4⁺Tcells+CD40L (n=24) (p<0.05 vs NP+RUPP CD4⁺T cells). Mechanisms of hypertension in response to RUPP CD4⁺ T cells include ET-1, ROS, and AT1-AA were analyzed. Inhibition of CD40 ligand binding reduced placental ET-1 to 2.3 fold above NP rats, and normalized placental ROS from 318.6 ±89 in NP+RUPP CD4⁺T cells (p<0.05) to 118.7 ±24 in NP+RUPP CD4⁺T+anti-CD40L (p<0.05). AT1-AA was also normalized with inhibition of CD40 ligand. This data suggests that placental

ischemia-induced T cell communication via the CD40 ligand is one important mechanism leading to much of the pathophysiology of preeclampsia.

KEYWORDS: Hypertension, Preeclampsia, T-cells, B-cells

INTRODUCTION

Preeclampsia (PE) is a major cause of maternal and perinatal morbidity and mortality worldwide (10, 33, 43). Chronic inflammation and immune activation are major factors mediating pathophysiology associated with PE (40, 41, 46). Women with PE have an increase in circulating and placental levels of inflammatory cytokines, such as Interleukin (IL) -6, IL-17 and tumor necrosis factor alpha (TNF- α), as well as an increase in activation of immune cells, including CD4⁺ T helper cells and B cells secreting agonistic autoantibody to the Angiotensin II (ANGII) type I receptor (AT1-AA) (5, 18, 32, 44, 48). The reduced uterine perfusion pressure (RUPP) model of placental ischemia recapitulates the characteristics of PE, including hypertension, intrauterine growth restriction (IUGR), chronic inflammation, and immune activation. We have previously shown that adoptive transfer of CD4⁺ T cells from RUPP rats into NP rats results in the development of hypertension, mediated by AT1-AA, endothelin-1 system, and oxidative stress (ROS) (34, 35, 52, 53). Furthermore, this hypertension is associated with elevated soluble fms-like tyrosine kinase-1 (sFlt-1), and inflammatory cytokines (35, 51-53). In addition, we have shown that infusion of purified rat AT1-AA into normal pregnant rats causes hypertension mediated by ROS, sFlt-1 and the ET-1 system (20, 21, 38, 39). Therefore, we believe that T cell mediated AT1-AA is an important mechanism causing hypertension through stimulating ET-1, ROS and sFlt-1 in response to placental ischemia or during preeclampsia. T helper dependent activation of B cells occurs via interaction with multiple receptors. One interaction that is essential for long-term B cell activation is between CD40 on B cells and CD40 ligand (CD40L) on T cells (9). Hubel, et al. found that AT1-AA remained present in the circulation up to 1 year

postpartum in women who had PE demonstrating that the autoantibody is produced by long-lived, antibody-secreting plasma cells and memory B cells, thereby suggesting the AT1-AA is produced by a T cell dependent response (16).

CD40 is expressed on a variety of cell types including vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) (13). CD40L has been found on the surface of other immune cell types, under special conditions, but is mainly expressed on activated T lymphocytes (50). CD40-CD40L interactions have been implicated in ROS and inflammation in immune and non-immune cells, and, more recently, in vascular disease (13, 42). CD40 not only plays an important role in inflammation but is also a membrane antigen present on *activated platelets*. Exaggerated platelet activation and inflammation resulting from endothelial damage in preeclampsia and HELLP syndrome is associated with increased shedding of the CD40 ligand (sCD40L)(2). One of the consequences of increased sCD40L is increased activation of CD40 on the surface of multiple immune cells that are implicated in the pathophysiology of this disease. Therefore, we hypothesized that communication of activated T cells with endogenous immune and/or vascular cells via CD40-CD40L interactions during preeclampsia mediate pathophysiology in response to placental ischemia stimulated CD4+ T cells and is crucial to stimulating production of the AT1-AA.

The AT1-AA activates the AT1 receptor similar to ANGII and contributes to endothelial dysfunction during PE (14, 24). Previous studies have shown that AT1-AA mediated AT1 signaling activates the MAPK/ERK pathway and NADPH oxidase leading to NFκB activation, tissue factor expression, decreased trophoblast invasion and increased ROS production, all of which have been implicated in the pathophysiology of

PE (7, 29, 54). Long-term B cell antibody responses and differentiation into memory cells are T cell dependent mechanisms, therefore suggesting an important role for T-helper cells in B cell production of AT1-AA. Therefore, the objective of this study was to determine if blockade of communication between placental ischemia-stimulated CD4⁺ T cells with endogenous cells in Normal Pregnant (NP) recipient rats at the CD40-CD40 Ligand interaction would attenuate AT1-AA production and therefore reduce the hypertension, inflammation and oxidative stress previously observed with adoptive transfer of RUPP CD4⁺ T cells into NP recipient rats.

MATERIALS AND METHODS

Pregnant Sprague-Dawley rats purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) were used in this 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 Institutes 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.

Measurement of mean arterial pressure into chronically instrumented conscious rats

Carotid arterial catheters were inserted on day 18 of gestation for blood pressure measurements that were assessed on day 19 of gestation. 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 for one hour after a 30 minute stabilization period. Subsequently, blood and urine samples were collected, kidneys, placentas, and spleens were harvested, and litter size and pup weights were recorded under anesthesia.

Reduction of uterine perfusion pressure (RUPP)

The RUPP model is a well-established model of placental ischemia in pregnant rats and has been described in detail (11, 12, 17). On gestational day 14, under isoflurane anesthesia, normal pregnant (NP) rats underwent a reduction in uterine perfusion pressure (RUPP) with the application of a constrictive silver clip (0.203 mm) to the aorta superior to the iliac bifurcation while ovarian collateral circulation to the uterus was reduced with restrictive clips (0.100 mm) to the bilateral uterine arcades at the ovarian end. Rats were excluded from the study when the clipping procedure resulted in total reabsorption of all fetuses.

Separating splenic CD4⁺ T cell lymphocytes

At the time of harvest (gestational day 19), spleens were collected from RUPP rats and lymphocytes were isolated from spleens by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical & Scientific Corp., Westbury, NY) according to the manufacturer's instructions. Anti-CD4 antibodies (BD Biosciences, San Jose, CA) were biotinylated using the DSB-X™ Biotin Protein Labeling Kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Isolated lymphocytes were incubated with biotinylated anti-CD4 antibody. The CD4-positive (CD4⁺) population was isolated using FlowComp Dynabeads (Invitrogen, Oslo, Norway) according to the manufacturer's protocol. The isolated CD4⁺ T lymphocytes were incubated overnight in

RPMI containing HEPES (25Mm), Glutamine (2mM), Pen/Strep (100U/mL), 1.022 ng/mL interleukin-2, and 4ng/mL interleukin-12 with or without 2.5 µg/mL anti-CD40 ligand (αCD40L) at 5% CO₂, 37 °C in a humidified atmosphere. RUPP CD4⁺ T cells or RUPP CD4⁺ T cells +CD40L were diluted in sterile saline at a concentration of 2x10⁶ cells/ml and 1x10⁶ cells were injected intraperitoneally into gestation day 12 normal pregnant (NP) rats. Recipients of RUPP CD4⁺ T cells were designated NP+RUPP CD4⁺ T cells, and recipients of RUPP CD4⁺ T cells incubated with αCD40L were designated NP+ RUPP CD4⁺ T+anti-CD40L. The groups of rats examined in this study were NP (n=13), NP+ RUPP CD4⁺ T cells (n=7), and NP+ RUPP CD4⁺ T+anti-CD40L (n=24).

Determination of CD40 Ligand binding efficiency

RUPP CD4⁺ T lymphocytes incubated with or without αCD40L were analyzed for binding efficiency using flow cytometry. After incubation, 1 X 10⁶ cells were labeled with secondary Fluorescein isothiocyanate (FITC; Southern Biotech, Birmingham, AL) antibody for 30 minutes at 4 °C. As a negative control for each individual rat, cells incubated without αCD40L were also labeled with FITC secondary antibodies alone. Subsequently, cells were washed and suspended in 500µL of Rosswell Park Memorial Institute medium (RPMI) and analyzed for single staining on a Gallios flow cytometer (Beckman Coulter, Brea, CA). The percent of positive staining cells above the negative control was collected for 3 separate cultures.

Determination of placental ROS

Superoxide production in the placenta was measured by using the lucigenin technique as we have previously described (25, 39). Rat placentas from NP, NP+ RUPP CD4⁺ T

cells, and NP+ RUPP CD4⁺ T+anti-CD40L rats 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 (25, 39). The samples were centrifuged at 16,000g for 30 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 15min 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 placental preproendothelin mRNA levels.

The placenta of the rats pups were separated, weighed, and immediately snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted using the RNeasy[®] Protect Mini kit supplied by Qiagen Kit after the tissue was crushed in liquid nitrogen with a mortar and pestle. The isolation procedure was then performed as outlined in the instructions provided by the manufacturer. Real-time PCR was utilized, as previously described, to determine tissue preproendothelin-1 levels(26, 28). cDNA was synthesized from 1 µg of RNA with Bio-Rad iScript cDNA reverse transcription and real-

time PCR was performed using the Bio-Rad iQ SYBR Green Supermix and iCycler. The following primer sequences provided by Life technologies were PPET-1 as previously described: forward 1, CTAGGTCTAAGCGATCCTTG, and reverse 1, TCTTTGTCTGCTTGGC (27). Invitrogen's reverse-transcription PCR primer control kit was used to amplify B-Actin transcripts as control. Levels of mRNA were calculated using the mathematical formula for $2^{-\Delta\Delta Ct}$ ($2^{\text{avg. Ct gene of interest} - \text{avg Ct beta actin}}$) recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997).

Determination of cytokine production

Plasma collected from all pregnant rats were measured for IL-6, TNF- α , and sFlt-1 concentrations using commercial ELISA kits (Quantikine) available from R&D Systems according to the manufacturer's protocol.

Determination of circulating AT1-AA

On day 19 of gestation blood was collected and immunoglobulin was isolated from 200 μ l of serum by protein G sepharose protein purification system (Knauer, Germany). This IgG fraction was used in a bioassay. The AT1-AA activity was measured using spontaneously beating neonatal rat cardiomyocytes and antagonized specifically using AT1 receptor antagonists. The results express the difference between the basal beating rate of the cardiomyocytes and the beating rate measured after the addition of the AT1-AA (increase in number of beats/min or Δ beats / min) (16, 24, 38). AT1-AAs were assessed in NP, NP+ RUPP CD4⁺ T cells, and NP+ RUPP CD4⁺ T+anti-CD40L rats.

Statistical Analysis

All of the data are expressed as mean \pm SEM. Comparisons of control with experimental groups were analyzed by ANOVA with Tukey's multiple comparisons test as post hoc analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Blockade of CD40 ligand attenuates hypertension in NP rat recipients of placental

ischemia-induced CD4⁺ T lymphocytes CD4⁺ T lymphocytes were isolated from RUPP spleens and cultured as described above. Lymphocytes cultured with or without antibody to CD40 ligand were injected intraperitoneally into gestation day 12 NP rats at a concentration of 2×10^6 cells/mL. Flow cytometry was performed to determine binding efficiency of anti-CD40L antibody to RUPP CD4⁺ T cells. Eighty-eight percent of the cells cultured with antibody to CD40L stained positive to for anti-CD40L-FITC (**Figure 1**), thereby inhibiting this receptor from intercellular communication with endogenous cells in recipient rats. Mean arterial pressure was measured on day 19 of gestation in NP, NP+ RUPP CD4⁺ T cells, and NP+ RUPP CD4⁺ T+anti-CD40L rats. (**Figure 2**). The MAP increased significantly from 99 ± 2 in NP rats ($n=13$) to 116 ± 4.3 in NP+ RUPP CD4⁺ T cells rats ($n=7$; $p < 0.01$). Antibody mediated neutralization of CD40L on RUPP CD4⁺ T lymphocytes prior to adoptive transfer caused a significant decrease in blood pressure to 104 ± 1.9 in NP+ RUPP CD4⁺ T+anti-CD40L rats ($n=24$; $p < 0.05$ vs NP+RUPP T cells). These results indicate the increase in blood pressure in response to placental ischemia induced CD4⁺ T lymphocytes is in part mediated by T cell communication with endogenous cells through CD40-CD40L interactions.

Inhibition of CD40-CD40L between RUPP CD4⁺ T Cells and endogenous B cells

attenuates the increased AT1-AA in response to adoptive transfer AT1-AA was 1.9 ± 0.3 in NP (n=9), 7 ± 1.3 in NP+ RUPP CD4⁺ T cells (n=7; $p < 0.001$ vs NP) and was only 1.5 ± 0.6 pg/ml in NP+ RUPP CD4⁺ T+anti-CD40L (n=17; $p < 0.0001$ vs NP+RUPP CD4⁺ T cells) (**Figure 3**). This therefore suggests that B cell production of AT1-AA occurs in a T cell dependent manner that requires CD40 and CD40L interactions between B cells and CD4⁺ T cells.

Inhibition of CD40-CD40L interactions blunts activation of the endothelin-1 system in

response to adoptive transfer of RUPP CD4⁺ T cells We have previously shown that the placental endothelin-1 system is activated in response to placental ischemia-stimulated CD4⁺ T cells. After adoptive transfer of RUPP CD4⁺ T cells into NP rats, placental PPET-1 was increased 3.5 fold in NP+ RUPP CD4⁺ T cells (n=6; $p < 0.0001$); and only increased 2.28 fold in NP+ RUPP CD4⁺ T+anti-CD40L (n=6; $p < 0.001$ vs NP+RUPP CD4⁺ T cell) (**Figure 4**).

Inhibition of CD40-CD40L interactions blunts Placental ROS in response to adoptive

transfer of RUPP CD4⁺ T cells to NP rats One mechanism by which CD40-CD40L causes endothelial dysfunction is through its mediation of ROS production (1, 4, 42, 49). Placental ROS increased from 143.4 ± 22 in NP (n=6) to 318.6 ± 89 in NP+ RUPP CD4⁺ T cells (n=3; $p < 0.05$). CD40L binding reduced placental ROS to 118.7 ± 24 in NP+ RUPP CD4⁺ T+anti-CD40L (n=6; $p < 0.05$ vs NP+RUPP CD4⁺ T Cell) (**Figure 5**).

Inhibition of CD40-CD40L interactions blunts TNF- α and IL-6 in response to adoptive transfer of RUPP CD4⁺ T cells to NP rats Plasma IL-6 increased from 35.52 \pm 6 in NP (n=7) to 84.8 \pm 12 in NP+ RUPP CD4⁺ T cells (n=8; p<0.01 vs NP) and was significantly decreased to 46.9 \pm 2.9 pg/ml in NP+ RUPP CD4⁺ T+anti-CD40L (n=5, p<0.05 vs NP+ RUPP CD4⁺ T cells), (**Figure 6A**). Additionally, TNF- α was 7.8 \pm 2 in NP, 18.8 \pm 8 in NP+ RUPP CD4⁺ T cells and was only 13.9 \pm 4 pg/ml in NP+ RUPP CD4⁺ T+anti-CD40L (**Figure 6B**). These data demonstrates that while blockade of CD40-CD40L interactions does not inhibit increases in circulating and TNF- α , it does inhibit increases in circulating IL-6 in response to RUPP CD4⁺ T cells. CD40 expressing B cells are known to secrete IL-6(6), therefore, neutralization of CD40L may have decreased CD40 activated B cell production of IL-6. The lack of inhibition of TNF- α expression-and the presence of some residual IL-6 is not surprising as activated T cells express these cytokines and blockade of CD40L with antibody is not expected to reverse the activation of the adoptively transferred CD4⁺ T cells.

Inhibition of CD40-CD40L interactions blunts sFlt-1 in response to adoptive transfer of RUPP CD4⁺ T cells to NP rats Plasma sFLT-1 increased from 79 \pm 14 in NP (n=4) to 107 \pm 9 in NP+ RUPP CD4⁺ T cells (n=4, n.s.) and was only 74 \pm 21 pg/ml in NP+ RUPP CD4⁺ T+anti-CD40L (n=4) (**Figure 6C**). However, these differences did not reach statistical significance

DISCUSSION

Altered immune activation and vascular dysfunction are key mediators of pathophysiology in PE. Clinical studies have shown that T lymphocyte numbers and activation are increased in women with PE compared to women with normal pregnancies (5, 8, 32). Preclinical studies in our RUPP model mirror these findings. We have previously determined a role for the activated CD4⁺ T helper cell population in mediating hypertension, ET-1 activation, inflammation, oxidative stress, and AT1-AA production in response to placental ischemia; all of which are mediators of PE pathophysiology (34, 51-53). In addition we have shown an important role for AT1-AA to stimulate ET-1, ROS, and sFlt-1 as mechanisms of increasing blood pressure during pregnancy. Inhibition of AT1-AA secretion with Rituximab lowered AT1-AA and blood pressure in the RUPP rat model of placental ischemia (25). Furthermore, we have previously demonstrated that inhibition of T cell activation attenuates such mediators and lowers blood pressure in response to placental ischemia (34). However, inhibition of T or B cells and temporarily leaving the mother immunocompromised is not a plausible treatment option for PE because a controlled inflammatory response and immune protection from outside pathogens is absolutely required for a healthy, successful pregnancy.

Much research concerning the stimulus for and the route of production of the AT1-AA has been performed in recent years. Based on the autoantibodies' ability to bind and activate the AT1- receptor, one may assume that AT1-AAs are derived from a very specific epitope region on the AT1-receptor. However, it is important to note that autoantibodies can also be generated by molecular mimicry (36, 47). The seven amino acid epitope region on the second extracellular loop of the AT1 receptor, which binds to

the AT1-AA, shares a six amino acid homology with capsid proteins of the human parvovirus B19 (PVB19) which is associated with several auto-immune diseases as well as PE (15, 47). Herse *et al*, showed that antibodies generated against the VP2 capsid protein of the PVB19 increases the beating rate of rat neonatal cardiomyocytes to the same level as observed after incubation with AT1-AAs isolated from preeclamptic patients (15). Furthermore, the increase in beats per minute stimulated by autoantibodies against PVB19 were suppressed by losartan and the specific 7 amino acid sequence, which neutralizes AT1-AA activation of the AT1- receptor. These experiments suggest the VP2 capsid protein of PVB19 could serve as the antigen for AT1-AA generation. An earlier study by Stephan *et al*, examined women with abnormal uterine perfusion and with normal uterine perfusion during the 2nd and 3rd trimester pregnancy and found no correlation between PVB19 positive patients and the presence of AT1-AAs in the maternal circulation of normal and abnormal pregnant women. They also showed no differences in AT1-AA activity in PVB19 positive or negative patients. Thus Stephan and colleagues concluded that AT1-AA generation is independent of epitope mimicry of the AT1 receptor by PVB19 (47). Whether or not PVB19 is an epitope that mimics the AT1-receptor for AT1-AA production is still unclear. Future studies administering the PVB19 capsid protein antibodies into pregnant rodents are warranted to determine whether or not these antibodies generate PE symptoms in rodents similar to AT1-AA infusion.

Another recent theory of AT1-AA generation arises from posttranslational modification of the AT1 receptor by tissue transglutaminase (TG2) (30, 31). TG2 can modify the AT1 receptor to create an antigen for AT1-AA production, and placentas

from women with PE have an increase in TG2 activity. Cystamine, an endogenous inhibitor of TG2, is decreased in cultured explants of PE placentas. Furthermore the administration of cystamine to pregnant mice inhibits AT1-AA induced hypertension and proteinuria (30). Administration of LIGHT, a new TNF- α superfamily member also known as tumor necrosis factor superfamily member 14, was injected into pregnant mice and elicited all the hallmarks of PE, including increased circulating AT1-AAs during pregnancy. In these mice TG2 activity was increased in the placenta and cystamine administration (via the drinking water) decreased TG2 activity, circulating AT1-AAs, hypertension, and proteinuria (31). Data from these studies suggest that posttranslational modifications to the AT1 receptor may also have a role in AT1-AA production, but more studies are needed to verify these conclusions.

With the route of production still unknown, we hypothesized that CD4⁺ T cells are necessary for the production of the AT1-AA. To answer this question, we inhibited communication through CD40 ligand, one of the mechanisms for T cell communication to B cells, necessary for B cell proliferation. Therefore the objective of this study was to determine if inhibition of T cell to B cell communication via CD40-CD40L interaction would attenuate AT1-AA and other pathophysiology observed in response to adoptive transfer of CD4⁺ T cells from the RUPP rat model of placental ischemia.

In the current study, we demonstrate that antibody-mediated neutralization of CD40L on placental ischemia-stimulated CD4⁺ T cells, prior to adoptive transfer, attenuates the hypertension, placental oxidative stress and AT1-AA production in NP recipient rats. This suggests that communication of activated CD4⁺ T cell with endogenous cells including B cells via CD40-CD40L signal plays a role to mediate these

pathophysiologies in PE. CD40 is expressed on immune cells and vascular cells, including endothelial and smooth muscle cells. Activation of CD40 on B cells by CD40L on T cells not only activates B cells to produce antibodies, but also mediates differentiation into memory B cells and facilitates class switching to IgG. It is important to note that AT1-AA is detected as IgG antibody in preeclamptic women and in the RUPP rat. Therefore, it could be that this interaction plays a central role in the production of AT1-AA. AT1-AA and mediates its pathophysiological effects, including endothelial dysfunction and oxidative stress, through activation of the AT1 receptor (7, 14, 24).

We have previously shown that placental ischemia, RUPP CD4⁺ T cells and AT1-AA lead to increased placental PPET-1 mRNA expression and secretion of ET-1 by cultured human umbilical endothelial vascular cells in culture (20, 22), suggesting that placental activation of the ET-1 system may be important in the pathology of the disease. We believe this shows the importance of T cells and AT1-AA to impact the placenta thereby leading to increased ET-1 which could ultimately play a role in the hypertension. We previously showed that NP recipients of RUPP CD4⁺ T cells had lower blood pressure response when treated with an ETA receptor antagonist, thus emphasizing the importance of ET-1 in response to RUPP CD4⁺ T cells. In this study, RUPP CD4⁺ T cell mediated increased placental PPET-1 mRNA levels which could be a contributing factor to hypertension in this study. Importantly, blockade of CD40L resulted in decreased AT1-AA production and therefore, contributed to the decreased oxidative stress and ET-1 production leading to lower blood pressures in this study.

However, because CD40 is also expressed on endothelial and vascular smooth muscle cells, the vascular dysfunction and increase in ROS production could be a result of direct interactions between CD40L on the activated CD4⁺ T cells and CD40 on vascular cells. The CD40-CD40L signaling axis has a role to induce endothelin-1 expression in endothelial cells directly and indirectly by inducing expression of Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (45). Furthermore, Chen, et al. demonstrated that treatment of endothelial cells with recombinant CD40L resulted in increased superoxide production (3). It has been shown that *activated platelets* express CD40L and their interaction with vascular cells can lead to endothelial dysfunction in cardiovascular disease. However, Isler, et al. previously provided evidence that activated platelets are not present in the RUPP rat model of placental ischemia (17). Therefore we conclude that in this preclinical PE model, CD40L on activated T cells, not activated platelets, has a role to directly contribute to endothelial dysfunction and oxidative stress through CD40-CD40L interactions on vascular cells.

Although there was a downward trend, blockade of CD40L on CD4⁺ T cells did not have profound effects to decrease plasma sFlt-1 or TNF- α in response to RUPP CD4⁺ T cells. This is not necessarily surprising, as T cells only exhibit CD40L surface expression after they are activated. Binding CD40L with neutralizing antibody would not be expected to reverse activation of the T cell, and would therefore not have an effect to decrease secretion of sFlt-1 or inflammatory cytokines, which we have shown to be secreted by RUPP CD4⁺ T cells(53). Importantly, blockade of CD40L on CD4⁺ T cells significantly decreased circulating IL-6. This may be due to decreased CD40 mediated activation of B cells, as CD40-activated B cells have been shown to secrete IL-6 (6).

Therefore, the decrease in IL-6 may also be due to decreased B cell activation by the RUPP CD4⁺T cells which would account for less AT1-AA and support our hypothesis that AT1-AA is a CD4⁺ T cell mediated event.

The impact of CD40L antibody binding on proper maternal immune and vascular function are important factors to consider in determining the feasibility of this approach as a therapy for women with PE. Furthermore assessment of the effects of this treatment on fetal outcomes are important to weigh the benefits against any risks associated with use of this biologic. TNF- α inhibitors have been well studied during pregnancy; and were found to have no teratogenic effects on the fetus. However an increased risk of infection after birth was associated with administration during late pregnancy (37). Limited clinical studies have shown that inhibition of T cell activation with abatecept during pregnancy had no pattern of negative effects on the mother or fetus (19). Even fewer studies are available on the use of other biologics, such as rituximab, anakinra, tocilizumab, and belimumab during pregnancy (37). Therefore, further studies are required to assess the viability of neutralization of CD40L as a therapy in the treatment of PE.

PERSPECTIVES AND SIGNIFICANCE

In this study, we have demonstrated that interaction between endogenous cells and placental ischemia-stimulated CD4⁺ T cells via CD40-CD40L binding is one important mechanism that leads to much of the pathophysiology of PE. Inhibition of this intercellular communication through blockade of CD40L on CD4⁺ T cells attenuated hypertension, activation of the ET-1 system, placental oxidative stress, and AT1-AA production. Data presented in this study highlight the consequences of improper

immune activation during pregnancy and identify activated CD4⁺ T cells as a potential therapeutic target for the management/treatment of PE.

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REFERENCES

1. **Aggarwal A, Blum A, Schneider DJ, Sobel BE, and Dauerman HL.** Soluble CD40 ligand is an early initiator of inflammation after coronary intervention. *Coronary artery disease* 15: 471-475, 2004.
2. **Azzam HA, Abousamra NK, Goda H, El-Shouky R, and El-Gilany AH.** The expression and concentration of CD40 ligand in normal pregnancy, preeclampsia, and hemolytic anemia, elevated liver enzymes and low platelet count (HELLP) syndrome. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis* 24: 71-75, 2013.
3. **Chen C, Chai H, Wang X, Jiang J, Jamaluddin MS, Liao D, Zhang Y, Wang H, Bharadwaj U, Zhang S, Li M, Lin P, and Yao Q.** Soluble CD40 ligand induces endothelial dysfunction in human and porcine coronary artery endothelial cells. *Blood* 112: 3205-3216, 2008.
4. **Cipollone F, Chiarelli F, Davi G, Ferri C, Desideri G, Fazia M, Iezzi A, Santilli F, Pini B, Cuccurullo C, Tumini S, Del Ponte A, Santucci A, Cuccurullo F, and Mezzetti A.** Enhanced soluble CD40 ligand contributes to endothelial cell dysfunction in vitro and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control. *Diabetologia* 48: 1216-1224, 2005.
5. **Darmochwal-Kolarz D, Saito S, Rolinski J, Tabarkiewicz J, Kolarz B, Leszczynska-Gorzela B, and Oleszczuk J.** Activated T lymphocytes in pre-eclampsia. *American journal of reproductive immunology* 58: 39-45, 2007.
6. **Daudelin JF, Mathieu M, Boulet S, and Labrecque N.** IL-6 production by dendritic cells is dispensable for CD8+ memory T-cell generation. *BioMed research international* 2013: 126189, 2013.
7. **Dechend R, Viedt C, Muller DN, Ugele B, Brandes RP, Wallukat G, Park JK, Janke J, Barta P, Theuer J, Fiebeler A, Homuth V, Dietz R, Haller H, Kreuzer J, and Luft FC.** AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase. *Circulation* 107: 1632-1639, 2003.
8. **Dimitrakova-Dzhambazova E, Milchev N, Pavlov P, and Dimitrakov D.** [Lymphocyte populations and subpopulations in peripheral blood of pregnant women with pre-eclampsia]. *Akusherstvo i ginekologija* 44: 40-44, 2005.
9. **Dorner T, Radbruch A, and Burmester GR.** B-cell-directed therapies for autoimmune disease. *Nature reviews Rheumatology* 5: 433-441, 2009.
10. **George EM, and Granger JP.** Recent insights into the pathophysiology of preeclampsia. *Expert review of obstetrics & gynecology* 5: 557-566, 2010.
11. **Gilbert J, Dukes M, LaMarca B, Cockrell K, Babcock S, and Granger J.** Effects of reduced uterine perfusion pressure on blood pressure and metabolic factors in pregnant rats. *American journal of hypertension* 20: 686-691, 2007.
12. **Granger JP, LaMarca BB, Cockrell K, Sedeek M, Balzi C, Chandler D, and Bennett W.** Reduced uterine perfusion pressure (RUPP) model for studying cardiovascular-renal dysfunction in response to placental ischemia. *Methods in molecular medicine* 122: 383-392, 2006.
13. **Hassan GS, Merhi Y, and Mourad W.** CD40 ligand: a neo-inflammatory molecule in vascular diseases. *Immunobiology* 217: 521-532, 2012.

14. **Herse F, and LaMarca B.** Angiotensin II type 1 receptor autoantibody (AT1-AA)-mediated pregnancy hypertension. *American journal of reproductive immunology* 69: 413-418, 2013.
15. **Herse F, Verlohren S, Wenzel K, Pape J, Muller DN, Modrow S, Wallukat G, Luft FC, Redman CW, and Dechend R.** Prevalence of agonistic autoantibodies against the angiotensin II type 1 receptor and soluble fms-like tyrosine kinase 1 in a gestational age-matched case study. *Hypertension* 53: 393-398, 2009.
16. **Hubel CA, Wallukat G, Wolf M, Herse F, Rajakumar A, Roberts JM, Markovic N, Thadhani R, Luft FC, and Dechend R.** Agonistic angiotensin II type 1 receptor autoantibodies in postpartum women with a history of preeclampsia. *Hypertension* 49: 612-617, 2007.
17. **Isler CM, Bennett WA, Rinewalt AN, Cockrell KL, Martin JN, Jr., Morrison JC, and Granger JP.** Evaluation of a rat model of preeclampsia for HELLP syndrome characteristics. *Journal of the Society for Gynecologic Investigation* 10: 151-153, 2003.
18. **Jonsson Y, Ruber M, Matthiesen L, Berg G, Nieminen K, Sharma S, Ernerudh J, and Ekerfelt C.** Cytokine mapping of sera from women with preeclampsia and normal pregnancies. *Journal of reproductive immunology* 70: 83-91, 2006.
19. **Kumar M, Ray L, Vemuri S, and Simon TA.** Pregnancy outcomes following exposure to abatacept during pregnancy. *Seminars in arthritis and rheumatism* 2015.
20. **LaMarca B, Parrish M, Ray LF, Murphy SR, Roberts L, Glover P, Wallukat G, Wenzel K, Cockrell K, Martin JN, Jr., Ryan MJ, and Dechend R.** Hypertension in response to autoantibodies to the angiotensin II type I receptor (AT1-AA) in pregnant rats: role of endothelin-1. *Hypertension* 54: 905-909, 2009.
21. **LaMarca B, Parrish MR, and Wallace K.** Agonistic autoantibodies to the angiotensin II type I receptor cause pathophysiologic characteristics of preeclampsia. *Gender medicine* 9: 139-146, 2012.
22. **LaMarca B, Speed J, Fournier L, Babcock SA, Berry H, Cockrell K, and Granger JP.** Hypertension in response to chronic reductions in uterine perfusion in pregnant rats: effect of tumor necrosis factor-alpha blockade. *Hypertension* 52: 1161-1167, 2008.
23. **Lamarca B, Speed J, Ray LF, Cockrell K, Wallukat G, Dechend R, and Granger J.** Hypertension in response to IL-6 during pregnancy: role of AT1-receptor activation. *International journal of interferon, cytokine and mediator research : IJIM* 2011: 65-70, 2011.
24. **LaMarca B, Wallace K, and Granger J.** Role of angiotensin II type I receptor agonistic autoantibodies (AT1-AA) in preeclampsia. *Current opinion in pharmacology* 11: 175-179, 2011.
25. **LaMarca B, Wallace K, Herse F, Wallukat G, Martin JN, Jr., Weimer A, and Dechend R.** Hypertension in response to placental ischemia during pregnancy: role of B lymphocytes. *Hypertension* 57: 865-871, 2011.
26. **LaMarca BB, Bennett WA, Alexander BT, Cockrell K, and Granger JP.** Hypertension produced by reductions in uterine perfusion in the pregnant rat: role of tumor necrosis factor-alpha. *Hypertension* 46: 1022-1025, 2005.
27. **LaMarca BB, Cockrell K, Sullivan E, Bennett W, and Granger JP.** Role of endothelin in mediating tumor necrosis factor-induced hypertension in pregnant rats. *Hypertension* 46: 82-86, 2005.

28. **LaMarca BD, Gilbert J, and Granger JP.** Recent progress toward the understanding of the pathophysiology of hypertension during preeclampsia. *Hypertension* 51: 982-988, 2008.
29. **Lindheimer MD, and Umans JG.** Explaining and predicting preeclampsia. *The New England journal of medicine* 355: 1056-1058, 2006.
30. **Liu C, Wang W, Parchim N, Irani RA, Blackwell SC, Sibai B, Jin J, Kellems RE, and Xia Y.** Tissue transglutaminase contributes to the pathogenesis of preeclampsia and stabilizes placental angiotensin receptor type 1 by ubiquitination-preventing isopeptide modification. *Hypertension* 63: 353-361, 2014.
31. **Luo R, Liu C, Elliott S, Wang W, Daugherty P, Kellems RE, and Xia Y.** [259-POS]: Elevated transglutaminase promotes the production of AT1-AA in preeclampsia. *Pregnancy hypertension* 5: 130, 2015.
32. **Mahmoud F, Omu A, Abul H, El-Rayes S, and Haines D.** Lymphocyte subpopulations in pregnancy complicated by hypertension. *Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology* 23: 20-26, 2003.
33. **Noris M, Perico N, and Remuzzi G.** Mechanisms of disease: Pre-eclampsia. *Nature clinical practice Nephrology* 1: 98-114; quiz 120, 2005.
34. **Novotny S, Wallace K, Herse F, Moseley J, Darby M, Heath J, Gill J, Wallukat G, Martin JN, Dechend R, and LaMarca B.** CD4+ T Cells Play a Critical Role in Mediating Hypertension in Response to Placental Ischemia. *Journal of Hypertension* 2: 116, 2013.
35. **Novotny SR, Wallace K, Heath J, Moseley J, Dhillon P, Weimer A, Wallukat G, Herse F, Wenzel K, Martin JN, Jr., Dechend R, and LaMarca B.** Activating autoantibodies to the angiotensin II type I receptor play an important role in mediating hypertension in response to adoptive transfer of CD4+ T lymphocytes from placental ischemic rats. *American journal of physiology Regulatory, integrative and comparative physiology* 302: R1197-1201, 2012.
36. **Oldstone MB.** Molecular mimicry and immune-mediated diseases. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 12: 1255-1265, 1998.
37. **Ostensen M.** Safety issues of biologics in pregnant patients with rheumatic diseases. *Annals of the New York Academy of Sciences* 1317: 32-38, 2014.
38. **Parrish MR, Ryan MJ, Glover P, Brewer J, Ray L, Dechend R, Martin JN, Jr., and LaMarca BB.** Angiotensin II type 1 autoantibody induced hypertension during pregnancy is associated with renal endothelial dysfunction. *Gender medicine* 8: 184-188, 2011.
39. **Parrish MR, Wallace K, Tam Tam KB, Herse F, Weimer A, Wenzel K, Wallukat G, Ray LF, Arany M, Cockrell K, Martin JN, Dechend R, and LaMarca B.** Hypertension in response to AT1-AA: role of reactive oxygen species in pregnancy-induced hypertension. *American journal of hypertension* 24: 835-840, 2011.
40. **Redman CW, Sacks GP, and Sargent IL.** Preeclampsia: an excessive maternal inflammatory response to pregnancy. *American journal of obstetrics and gynecology* 180: 499-506, 1999.
41. **Redman CW, and Sargent IL.** Latest advances in understanding preeclampsia. *Science* 308: 1592-1594, 2005.

42. **Rizvi M, Pathak D, Freedman JE, and Chakrabarti S.** CD40-CD40 ligand interactions in oxidative stress, inflammation and vascular disease. *Trends in molecular medicine* 14: 530-538, 2008.
43. **Saito S.** Th17 cells and regulatory T cells: new light on pathophysiology of preeclampsia. *Immunology and cell biology* 88: 615-617, 2010.
44. **Saito S, Umekage H, Sakamoto Y, Sakai M, Tanebe K, Sasaki Y, and Morikawa H.** Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with preeclampsia. *American journal of reproductive immunology* 41: 297-306, 1999.
45. **Sakurai K, Cominacini L, Garbin U, Fratta Pasini A, Sasaki N, Takuwa Y, Masaki T, and Sawamura T.** Induction of endothelin-1 production in endothelial cells via co-operative action between CD40 and lectin-like oxidized LDL receptor (LOX-1). *Journal of cardiovascular pharmacology* 44 Suppl 1: S173-180, 2004.
46. **Steegers EA, von Dadelszen P, Duvkot JJ, and Pijnenborg R.** Pre-eclampsia. *Lancet* 376: 631-644, 2010.
47. **Stepan H, Wallukat G, Schultheiss HP, Faber R, and Walther T.** Is parvovirus B19 the cause for autoimmunity against the angiotensin II type receptor? *Journal of reproductive immunology* 73: 130-134, 2007.
48. **Toldi G, Rigo J, Jr., Stenczer B, Vasarhelyi B, and Molvarec A.** Increased prevalence of IL-17-producing peripheral blood lymphocytes in pre-eclampsia. *American journal of reproductive immunology* 66: 223-229, 2011.
49. **Ueland T, Aukrust P, Yndestad A, Otterdal K, Froland SS, Dickstein K, Kjekshus J, Gullestad L, and Damas JK.** Soluble CD40 ligand in acute and chronic heart failure. *European heart journal* 26: 1101-1107, 2005.
50. **van Kooten C, and Banchereau J.** CD40-CD40 ligand. *Journal of leukocyte biology* 67: 2-17, 2000.
51. **Wallace K, Cornelius DC, Scott J, Heath J, Moseley J, Chatman K, and LaMarca B.** CD4+ T cells are important mediators of oxidative stress that cause hypertension in response to placental ischemia. *Hypertension* 64: 1151-1158, 2014.
52. **Wallace K, Novotny S, Heath J, Moseley J, Martin JN, Jr., Owens MY, and LaMarca B.** Hypertension in response to CD4(+) T cells from reduced uterine perfusion pregnant rats is associated with activation of the endothelin-1 system. *American journal of physiology Regulatory, integrative and comparative physiology* 303: R144-149, 2012.
53. **Wallace K, Richards S, Dhillon P, Weimer A, Edholm ES, Bengten E, Wilson M, Martin JN, Jr., and LaMarca B.** CD4+ T-helper cells stimulated in response to placental ischemia mediate hypertension during pregnancy. *Hypertension* 57: 949-955, 2011.
54. **Zhou CC, Ahmad S, Mi T, Xia L, Abbasi S, Hewett PW, Sun C, Ahmed A, Kellems RE, and Xia Y.** Angiotensin II induces soluble fms-Like tyrosine kinase-1 release via calcineurin signaling pathway in pregnancy. *Circulation research* 100: 88-95, 2007.

Figure Legends

Figure 1. Binding efficiency of CD40L antibody to RUPP CD4[±] T cells. To examine binding of α CD40L to placental ischemia-stimulated CD4⁺ T cells, a subset of cells cultured with or without CD40L antibody were stained with a secondary rat anti-mouse IgG2-FITC antibody. Cells were gated in the forward and side scatter plot. The percentage of CD40L-FITC positive cells were measured within the gate. Representative flow cytometry plot showing % binding of CD40L antibody in culture.

Figure 2. Hypertension in response to placental ischemia-stimulated CD4[±] T cells is attenuated with CD40L neutralization. MAP was measured on day 19 in NP (n=13), NP+ RUPP CD4⁺ T cells (n=7), and NP+ RUPP CD4⁺ T+anti-CD40L (n=24) rats. Hypertension in response to adoptive transfer of placental ischemia-stimulated CD4⁺ T cells is attenuated after CD40L binding with neutralizing antibody (*p<0.05, **p<0.01).

Figure 3. AT1-AA production is attenuated after α CD40L binding. AT1-AA levels was measured via chronotropic events in cardiomyocytes in culture. AT1-AAs are significantly increased in NP+ RUPP CD4⁺ T cells (n=7) rats compared to NP rats (n=9). Blockade of CD40L significantly decreased circulating AT1-AA in NP+ RUPP CD4⁺ T+anti-CD40L rats (n=17) (**p<0.001, ****p<0.0001).

Figure 4. α CD40L binding blunts activation of the placental ET-1 system. mRNA of pre-proendothelin was measured in the placentas of NP (n=6), NP+ RUPP CD4⁺ T cells (n=6), and NP+ RUPP CD4⁺ T+anti-CD40L (n=6) rats. Pre-proendothelin is increased in placenta of NP+ RUPP CD4⁺ T cells compared to NP rats. CD40L binding blunted the increased transcription of preproendothelin in NP+ RUPP CD4⁺ T+anti-CD40L rat placentas (**p<0.001, ****p<0.0001)..

Figure 5. Placental ROS in response to RUPP-stimulated CD4⁺ T cells is mediated by CD40-CD40L interactions. Placental oxidative stress was measured using chemiluminescence. In response to adoptive transfer of placental ischemia-CD4⁺ T cells, placental ROS was elevated compared to NP animals. Increased ROS production was attenuated after CD40L binding to RUPP CD4⁺ T cells (*p<0.05).

Figure 6. Inflammatory and angiogenic factors secreted from placental ischemia-stimulated CD4⁺ T cells are not attenuated with CD40L neutralization. Circulating levels of inflammatory cytokines, IL-6 and TNF- α , and the angiogenic factor sFLT-1, were measured in all groups. Increased circulating IL-6 in response to adoptive transfer of RUPP CD4⁺ T cells into NP rats was attenuated with antibody mediated neutralization of CD40L (A). Plasma TNF- α sFLT-1 expression in response to placental ischemia-stimulated CD4⁺ T cells was not inhibited with CD40L neutralization (*p<0.05, **p<0.01).