CD4+ T Cells Play a Critical Role in Mediating Hypertension in Response to Placental Ischemia

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Abstract

Similar to preeclamptic women, hypertension in the chronic Reduced Uterine Perfusion Pressure Rat Model Of Preeclampsia (RUPP) is associated with increased CD4+ T cells, cytokines, sFlt-1 and agonistic autoantibodies to the AngII receptor (AT1-AA). We examined the effect inhibition of T cell co-stimulation in RUPP rats treated with (A) abatacept, 250 mg/kg, infused i.v. at gestation day 13), on hypertension and sFlt-1, TNF-α and AT1-AA. RUPP surgical procedure was performed on day 14. On day 19 MAP increased from 94±2 mmHg in Normal Pregnant (NP) to 123 ± 3 mmHg in RUPP control rats. This response was attenuated by Abatacept, MAP was 104 ± 2 mmHg in RUPP + A, and 96 ± 2 mmHg NP ± A. Percent circulating CD4+ T cells were 66 ± 3% in RUPPs compared to 55 ± 3% NP rats (p<0.04) but were normalized in RUPP ± A rats (54 ± 3%). The twofold increase in TNF alpha seen in RUPPs (277 ± 47 pg/ml) was decreased to 80 ± 16 pg/ml in RUPP + A. Placental sFlt-1 was reduced 70 % to 151 ± 28 in RUPP ± A compared 488 ± 61 pg/ml in RUPP (p<0.001). AT1-AA decreased from 20 ± 0.8 bpm in control RUPP to 6 ± 0.7 bpm in RUPP ± A. We next determined the effect of RUPP in causing hypertension in pregnant T cell deficient rats by examining MAP in NP (123 ± 5 mmHg) and RUPP athymic nude rats (123 ± 7 mmHg). In the absence of T cells, hypertension in response to placental ischemia was completely abolished. Collectively these data indicate that CD4+ T cells in response to placental ischemia play an important role in the pathophysiology of hypertension associated with preeclampsia.

Keywords: Hypertension; Preeclampsia; Placental ischemic insult

Introduction

Preeclampsia is a devastating disease of pregnancy affecting 5-7% of all pregnancies in the US [1,2]. While preeclampsia remains a significant contributor to maternal deaths, it is also a great cause of perinatal morbidity. For many patients, this disease is associated with devastating effects underscored by severe maternal morbidity. While the manifestations of preeclampsia do not appear until 20 weeks gestation, the disease process is hypothesized to begin at implantation [3-7]. During a normal pregnancy the invading trophoblasts remodel the uterine spiral arterioles creating a low resistance system allowing for increased blood volume delivery. When trophoblastic invasion is shallow, the arteriolar remodeling is blunted and the vascular resistance remains high, oxygenated blood flow is decreased and placental ischemia results. In addition to the fetal effects of impaired growth, placental ischemia has been shown to result in the production of vasoactive peptides and inflammatory cytokines [8-10]. These findings have been verified in the Reduced Uterine Perfusion Pressure (RUPP) model of preeclampsia, where pregnant rats that have surgically induced placental ischemia have elevated levels of circulating TNF-α, IL-6, IL-17, and sFlt-1 [8-13].

Newer research has begun to elucidate the role of the immune system in the pathogenesis of preeclampsia [14-21]. Many believe that partial failure of the maternal immune tolerance mechanisms precedes the development of placental oxidative stress and ischemia, both of which are major players in the pathophysiology of preeclampsia. This maternal immune tolerance involves crucial interactions between regulatory CD4+ T cells and uterine natural killer cells recognizing and accepting the fetal antigens and facilitating placental growth. Partial failure of this crucial step leads to poor placentation and dysfunctional placentation and chronic immune activation originating in the placenta. Importantly, analysis of circulating blood collected from preeclamptic women has demonstrated a decrease in the proportion of circulating T regulatory cells [4,18]. Again, these findings are replicated in an animal model of preeclampsia which utilizes the RUPP procedure which revealed that pregnant RUPP rats had a 47% decrease in FoxP3+ T regulatory cells in the peripheral circulation when compared to NP rats. This represents a shift from the normal anti-inflammatory Th2 state of pregnancy to the pro-inflammatory Th1 state [4,15,20].

While preeclampsia has not historically been classified as an autoimmune disorder, this shift in humoral immunity and production of autoantibodies certainly indicates that perhaps it should. In fact, T helper 17 cells, which have been known to upregulate in autoimmune disorders including lupus, psoriasis, and multiple sclerosis, are increased in preeclamptic women [4,15,18-20]. With a loss of T regulatory cells, the body loses the normal protection it has against the creation of autoantibodies. One of the most promising findings indicating the importance of T cells has been the discovery of a class 3 IgG molecule that stimulates the angiotensin type I receptor [17]. This agonistic autoantibody to the AT1receptor (AT1-AA) has been isolated from the sera of preeclamptic, but not normal pregnant women. These activating autoantibodies to the angiotensin type I receptor have been shown in an animal model to result in enhanced sensitivity to angiotensin [17]. Infusion of AT1-AA resulted in an exaggerated response to angiotensin II, a phenomenon which is known to occur in women with preeclampsia, the mechanism by which was previously unexplained. Further, chronic infusion of the AT1-AA has also been shown to increase production of sFlt-1 and soluble endoglin, activation of the ET-1 pathway and placental oxidative stress, all of
which are important players in the pathophysiology of preeclampsia [10-13,16,17,21,22].

We have recently demonstrated that magnetically separated and cultured CD4+ T cells from RUPP rats secrete greater amounts of TNF alpha, IL-6, IL-17 and sFlt1 into their cell culture media compared to NP CD4+ T cells [23]. Further, adoptive transfer of CD4+ T cells from RUPP dams into NP dams results in increased blood pressure and elevations in many factors shown to be stimulated by placental ischemia in the RUPP rat, such as sFlt-1, ET-1, TNF alpha, IL-6 and AT1-AA [11,23]. However a role for endogenous T cells to mediate secretion of such factors and hypertension in response to placental ischemia remained unidentified. Therefore the objective of the current study was to determine the effects of inhibiting endogenous T cell co-stimulation prior to placental injury on blood pressure and placental ischemia stimulated soluble factors. Two approaches were taken to answer this question. First, abatacept (Orencia) which is a fusion molecule of CTLA 4 designed to inhibit co-stimulation of T cells in response to antigens, was administered to pregnant rats on gestational day 13, RUPP was induced on day 14 and blood pressure and soluble factors were collected on day 19. Inhibition of T cell co-stimulation has proven efficient in other animal models of autoimmune disease and is now being clinically used to treat patients in various chronic inflammatory states such as hepatitis B and rheumatoid arthritis [5,6].

A second approach was to examine the hypertensive effect of RUPP on athymic nude T cell deficient pregnant rats.

Materials and Methods

All animal studies were performed in timed pregnant Sprague Dawley rats or Athymic Nude virgin or pregnant rats purchased from Harlan Inc (Indianapolis, IN). Animals were housed in a temperature controlled room (23°C) with a 12:12 light:dark cycle. Athymic nude rats were housed in an isolated barrier temperature controlled room (23°C) with a 12:12 light:dark cycle. All experimental procedures executed in this study were in accordance with the NIH guidelines for the use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and the University of Mississippi Medical Center.

Inhibition of T cell co-stimulation in the Reduced Uterine Perfusion Pressure (RUPP) rat model of preeclampsia

To determine the role of endogenous T cells in mediating hypertension in RUPP rats, four groups of pregnant rats were examined: normal pregnant (NP, n=20), Reduced uterine perfusion pressure (RUPP, n=20), NP + Orencia (Abatacept) (NP+A; n=12) and RUPP + Orencia (Abatacept) (RUPP ± A; n=19). Orencia, Abatacept, (250mg/kg) was infused IV via jugular catheter over a 20 minute period prior to placental ischemic insult on day 19 of gestation. 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 suspended in 500 µl of Roswell 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 each individual rat and mean values for each experimental group (RUPP and RUPP + A) was calculated.

Determination of AT1-AA production in response to blockade of CD4+ T cells prior to placental ischemic insult

Antibodies were detected by the chronotropic responses to AT1 receptor–mediated stimulation of cultured neonatal rat cardiomyocytes coupled with receptor-specific antagonists as previously described [10-13,16,17]. Briefly, on day 18 of gestation blood was collected and immunoglobulin was isolated from one ml of serum by specific anti-rat IgG column purification. Subsequently, AT1-AA was purified by the 7 amino acid epitope binding from the column purified rat IgG. AT1-AA activity was measured, as previously described, utilizing a bioassay that we have previously published in which the chronotropic response of rat neonatal cardiomyocytes in culture is detected and counted. Increased chronotropic response is counted and expressed as the change in beats/minute (Δbpm) of the rat neonatal cardiomyocytes in culture, therefore units of AT1-AA are expressed as Δbpm over basal levels.

TNF-α in response to inhibition of T cell co-stimulation prior to placental ischemic insult

A rat TNF-α colorimetric sandwich ELISA (R&D Systems) was used for quantification of serum TNF-α levels between 12.5 and 800 pg/ml. This assay displayed a sensitivity level of 5 pg/ml and interassay variability of 10% and intra-assay variability of 5.1%.

Placental sFlt-1 in response to inhibition of T cell co-stimulation prior to placental ischemic insult

We have shown that placental explants from RUPP rats or AT1-AA infused rats displayed increased placental production of sFlt-1. Further we demonstrated that RUPP CD4+ Tcells secrete sFlt-1 in vitro and stimulate sFlt-1 elevation in NP recipient rats of RUPP
CD4+ T cells. Therefore, we hypothesized that production of sFlt-1 would be attenuated by suppressing T cells in RUPP rats treated with Abatacept prior to the placental ischemic insult (described above). Freshly harvested placentas collected from four control RUPPs and six RUPP+A rats were rinsed in cold phosphate buffered saline and the myometrium layer was isolated from the decidua and the vascular bundle was excised. Villous explants were plated on matrigel 6-well cell culture filter inserts and cultured in Dulbecco’s modified Eagle medium/F12 supplemented with 0.25 μg/ml ascorbate and 10U Pen/Strep at 37°C, 6% O₂, 89% N₂, 5% CO₂ conditions [13]. Culture media was analyzed for sFlt-1 secretion via ELISA. Murine vascular endothelial growth factor R1 enzyme-linked immunosorbent assay from R&D Systems. The variability for intra-assay was 7.2% and interassay was 8.4%.

Uterine artery resistive index in response to inhibition of T cell co-stimulation prior to placental ischemic insult

In a method described by Tam Tam [24] Power Doppler velocimetry measurements were performed on anesthetized pregnant dams at an imaging station with a Vevo 770 unit (Visual sonics) using a 30 Hz transducer and an insonating angle <30°. The Peak Systolic flow Velocity (PSV) and End Diastolic flow Velocity (EDV) were recorded using the uterine artery Doppler waveform. The uterine artery resistive index was calculated using the following formula: UARI=(PSV-EDV)/PSV. Uterine artery resistive index was determined for the uterine artery bilaterally at three levels and the mean was calculated for control RUPPs and compared to RUPPs + A.

Statistical analysis

All data are expressed as mean ± standard error. Differences between control and experimental groups were analyzed using the Student’s t-test for renal function. Blood pressure analysis and AT1-AA levels were analyzed using 1 way ANOVA for comparison among multiple groups. Values were of p<0.05 were considered significant.

Results

Effect of inhibition of T cell stimulation prior to placental ischemic insult on blood pressure, AT1-AA, TNF alpha, placental sFlt-1 and uterine artery resistive index

Percent circulating CD4+ T cells increased in RUPPs compared to NP rats, 66 ± 3% and 55.5±2.7% respectively (p<0.04). Administration of Abatecept attenuated the rise in circulating T cells in RUPP rats compared to RUPP controls. Circulating CD4+ Tcells were normalized to 54.8 ± 2.5% in RUPP+A rats. Abatecept had no effect of circulating CD4+ Tcells in NP rats, CD4+ T cells were 59 ± 4% in NP+A (Figure 1). CD8+ T cells are not significantly different from NP to RUPP control rats (24 ± 4% to 29 ± 5%). Administration of abatacept had no effect to decrease CD8+ T cells in either NP (25 ± 5%) or RUPP rats (25 ± 5%) illustrating the specificity and efficiency of abatecept on CD4+ T cells (Figure 1).

MAP increased from 94 ± 2 mmHg in NP rats to 123 ± 3 mmHg in RUPP rats. This hypertensive response was attenuated with inhibition of CD4+ T cell stimulation in RUPP rats. MAP was 96 ± 2 mmHg in NP+A, and only increased 8 mmHg to 104 ± 2 mmHg in RUPP+A (Figure 2). Pup weights were significantly decreased in RUPP rats compared to NP rats (1.97 ± 0.05 vs 2.24 ± 0.05 grams) and decreased further to 1.8 ± 0.04 grams in RUPP+A but were unchanged in NP + A (2.13 ± 0.44grams). No other morphological changes were noted in pups of RUPP rats treated with Abatacept.

Inhibition of T cell stimulation blunts the blood pressure response to placental ischemia during pregnancy

Placental ischemia significantly increased AT1-AA production in RUPP compared to NP controls, as measured by an increase in cardiomyocyte chronotropic events expressed as beats per minute (bpm). NP rats display a 1.3 ± 0.6 bpm in cardiomyocyte chronotropic events while RUPP rats have significantly higher levels of 20 ± 0.41 bpm in cardiomyocyte chronotropic events. No change in cardiomyocyte chronotropic events were seen in NP rats treated with Abatecept (NP+A 0.67 ± 0.47 bpm). However, in stark contrast, inhibition of CD4+ T cell co-stimulation had a significant effect to decrease AT1-AA production in RUPP rats treated with Orencia, RUPP+A displayed 6.4 ± 0.755 bpm (Figure 3).
The two fold increase in circulating TNF alpha characteristically seen in RUPP rats (277 ± 47 pg/ml) was significantly decreased to 80 ± 18 pg/ml in RUPP+A. Placental explant secretion of sFlt-1 at 2 hrs of culture was 186 ± 60 in RUPP, but was decreased to 53 ± 7 pg/ml in RUPP rats treated with Abatacept. Placental explant secretion of sFlt-1 at 24 hrs culture was 488 ± 61 pg/ml in RUPP placentas but was significantly decreased to 151 ± 28 pg/ml in placentas from RUPP+A rats (p<0.001) (Figure 4). Uterine artery ressitive index increased from 0.6 ± 0.02 in NP dams to 0.68 ± 0.02 in RUPP dams. Blockade of CD4+ T cells normalized uterine artery ressitive index to 0.61 ± 0.03 in RUPP+A pregnant rats.

**Effect of RUPP on athymic nude pregnant rats**

Virgin and normal pregnant athymic nude rats were obtained from Harlan. Four underwent the RUPP procedure on day 14 of gestation and four were utilized as controls. Inducing placental ischemia in CD4+ T cell deficient pregnant rats had no effect to increase blood pressure during pregnancy. MAP in control athymic nude rats was 114 ± 5 mmHg. MAP in control athymic nude pregnant rats was 123 ± 5 mmHg. MAP in RUPP athymic nude rats was 123 ± 7 mmHg (Figure 5).

**Inhibition of T cell stimulation attenuates production of AT1-AA in response to placental ischemia during pregnancy**

It was also demonstrated that adoptive transfer of placental ischemic stimulated CD4+ T cells were responsible for alterations in renal hemodynamics [11]. As previously described by Alexander et al. in 2001 [25], reduction in uterine perfusion pressure during pregnancy is associated with decreased glomerular filtration rate, increased renal vascular resistance, and decreased renal perfusion flow. This profile of altered renal hemodynamics was similar to the profile attained with exposure to placental ischemic stimulated CD4+ T cells without any mechanical reduction in uterine perfusion [11]. This suggests that it is not the actual mechanical decrease in blood flow that leads to the altered renal hemodynamics, but rather, the immunologic response to that stimulus. It is likely that these findings also suggest that while immune activation plays a large role in the alteration of renal hemodynamics, it is just one of a myriad of stimuli working together in vivo to create the preeclamptic phenotype.

The hypertensive response to RUPP CD4+ T Lymphocytes was attenuated by AT1 receptor blockade with Losartan [11]. It is not clear whether the reduction in MAP was due to blockade of the AT1-AA or blockade of endogenous AngII activation of AT1 receptor. This question was answered with B cell depletion with Rituximab. Once the CD21 costimulating molecule is blocked, endogenous B cells are

**Figure 3:** Inhibition of T cell co-stimulation attenuates production of AT1-AA in response to placental ischemia during pregnancy. We have consistently demonstrated AT1-AA produced in response to placental ischemia. With T cell suppression, AT1-AA is significantly lowered in RUPP+A compared to control RUPPs (P<0.05).

**Figure 4:** Inhibition of T cell co-stimulation attenuates production of TNF-α and placental sFlt-1 in RUPP pregnant rats. Placental ischemia stimulates TNF alpha and placental sFlt-1. Administration of Abatecept and suppression of T cells decreased circulating TNF alpha and placental secreted sFlt-1 in RUPP+A compared to RUPP control rats.

**Figure 5:** T cells play a critical role in mediating hypertension in response to placental ischemia. Inducing placental ischemia in pregnant rats deficient in T cells had no effect to increase blood pressure during pregnancy. Both NP and RUPP in athymic/nude rats had blood pressures of 123 mmHg.

**Discussion**

While the immunologic profiles of preeclamptic women have been well described, the stimulus for the immunologic shift that occurs between a normal and preeclampsic pregnancy is not understood [3,7,15,18-20,23]. Previous work has demonstrated that both TNF-α and the AT1-AA can serve to stimulate the production of sFlt-1 from AT1-AA can serve to stimulate the production of sFlt-1 from the placenta during pregnancy [12]. Our previous adoptive transfer data shows that CD4+ T cells stimulated in response to placental ischemia can induce a preeclampsic like state in a normal pregnant rat without any additional stimulus [11,23].

The hypertensive response to RUPP CD4+ T Lymphocytes was attenuated by AT1 receptor blockade with Losartan [11]. It is not clear whether the reduction in MAP was due to blockade of the AT1-AA or blockade of endogenous AngII activation of AT1 receptor. This question was answered with B cell depletion with Rituximab. Once the CD21 costimulating molecule is blocked, endogenous B cells are
not stimulated to produce antibodies. By treating NP+RUPP CD4+ T cell injected rats with Rituximab, AT1-AA levels were significantly suppress and MAP was decreased to NP levels [11].

Because B cell depletion with an anti-CD20 compound has been demonstrated to decrease production of the AT1-AA during pregnancy in response to surgical placental ischemia [13], and it was demonstrated in this data that MAP in response to adoptive transfer of ischemic stimulated CD4+ T cells was reduced with the same compound, these findings posed the question whether blockade of the interaction between the antigen presenting cell and the T cell could have the same results. CTLA-4, homologous to CD28, is expressed on activated T cells and binds to its ligand B7 as an important co-stimulatory signal to T cells [26]. Orencia (Abatacept) is a CTLA-4 immunoglobulin used experimentally to inhibit the T cell immune response by blocking this interaction. In this study, treatment of RUPP rats with Orencia (abatacept) prior to the placental insult was shown to significantly decrease MAP when compared to untreated RUPP rats. The reduction in MAP occurs in parallel with a significant reduction in AT1-AA from treated RUPP rats (Figure 2 and 3). This reduction in MAP, and suppression of AT1-AAs production, was further explained by examining the absolute percentage of T cells in each group. The RUPP+A rats had circulating CD4+ T cell levels more similar to NP than RUPP rats (Figure 1). Further examination of the effect of CD4+ T cell blockade demonstrated that increased secretion of TNF-a and placental sFlt-1 in response to RUPP in pregnant rats were also significantly reduced. We have previously published that the RUPP rat model of PE has increased circulating TNF-a and placental secretion of the anti-angiogenic factor sFlt-1 (Figure 4) [6-13,23,24]. We have previously shown that RUPP CD4+ T cells are important sources of these factors. In this study we demonstrate the importance of T cells to be associated with increased secretion of these factors in response to placental ischemia by demonstrating that inhibition of CD4+ T cell stimulation prevents elevations in TNF alpha and placental sFlt-1 in association with hypertension in response to placental ischemia.

Additionally, we have shown that RUPP rats display a significant rise in uterine artery resistive index [24]. In 2011, Tam Tam, demonstrated similar findings in reductions in uterine artery resistive index in RUPP rats treated with an endothelin antagonist. The significant increase in uterine artery resistive seen in rats having undergone the RUPP procedure was also attenuated in the group of RUPP rats treated with Abatacept. These findings are similar to the renal hemodynamic findings, in that although it seems as though alterations in blood flow with RUPP are largely due to the mechanical obstruction caused by the RUPP procedure, there is an additional component stimulated by the response to placental ischemia that acts in concert to mediate the other pathophysiological components in RUPP rats that are seen in preeclamptic women. Interestingly, pup weights of RUPPs+A were less than the control RUPPs (Figure 2). However, no other fetal abnormalities were noted nor reductions in litter sizes were noted.

To further illustrate the importance of T cells in mediating hypertension in response to placental ischemia, we performed the RUPP procedure in athymic/nude pregnant rats, rats deficient in T cells. We demonstrate a lack of blood pressure response in RUPP athymic/nude pregnant rats, thereby confirming our hypothesis that T cells are important to cause hypertension in response to placental ischemia (Figure 5). These findings lend hope that one day there will be an acceptable treatment for preeclampsia. While no current treatments are able to modify the failed trophoblastic invasion of the uterine spiral arterioles, these findings in the RUPP model show that although surgically reduced uterine perfusion is present, the effects of preeclampsia with respect to T helper cells, MAP, AT1-AA production, and cytokine profiles can be pharmacologically improved to mimic normal pregnant rats and thus lead to a more normal pregnancy.

**Perspectives and Significance**

It has been demonstrated that CD4+ T cells in response to placental ischemia are responsible for hypertension, alterations in renal hemodynamics, production of inflammatory cytokines and anti-angiogenic molecules, as well as stimulating the production of agonistic autoantibodies to the angiotensin type I receptor. The effects of placental ischemia on blood pressure and many facets seen in preeclampsia can be attenuated with inhibition of T cells either by blockade of antigen presenting cells interacting with T cells or by overall T cell deficiency. Importantly, these data suggests the potential use of anti-inflammatory targets to target cell suppression as a therapeutic for preeclampsia. These studies highlight the importance of targeting CD4+ T cell suppression as an avenue to decrease blood pressure, cytokine production, anti-angiogenic factors, autoantibodies and uterine artery resistive index to improve preeclampsia symptoms late in pregnancy in PE women. Targeting the overactive immune system late in pregnancy in PE women could be a safe and potential therapeutic to improve pregnancy outcomes in women with this disease.

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