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Inhibition of T cell activation attenuates hypertension, $\text{TNF}\alpha$, IL-17 and blood brain barrier permeability in pregnant rats with angiogenic imbalance

Cynthia Bean, MD¹, Shauna-Kay Spencer, MS¹, Teylor Bowles¹, Patrick B. Kyle, PhD², Jan Michael Williams, PhD³, Jacob Gibbens, MD¹, and Kedra Wallace, PhD¹

¹Department of Obstetrics & Gynecology, University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216

²Department of Pathology, University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216

³Department of Pharmacology & Toxicology, University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216

Abstract

Problem—Angiogenic imbalance during pregnancy is associated with immune activation, hypertension, increased T cell infiltration, and neurological insults.

Method of Study—On gestational day (GD) 12 timed-pregnant rats were infused with anti-angiogenic factors sFlt-1 and sEndoglin (4.7 and 7 $\mu\text{g/kg}$) to create HELLP syndrome via mini-osmotic pumps for 8 days, with a subset of these rats having Orencia (2mg/kg) infused on GD13. On GD19, blood brain barrier (BBB) permeability was evaluated via Evan's Blue infusion, blood was collected for T cell measurements, inflammatory cytokine secretion. Brain tissues were also collected to examine inflammatory cytokine infiltration.

Results—T cell attenuation with Orencia decreased circulating CD4^+ and CD8^+ T cells, circulating $\text{TNF}\alpha$ and IL-17, BBB permeability and significantly decreased biochemical evidence of HELLP compared to untreated HELLP rats.

Conclusions—These data support the hypothesis that T cells have a critical role in contributing to the pathophysiology that is seen in angiogenic imbalance during pregnancy.

Keywords

blood brain barrier; CD4^+ T cells; HELLP syndrome; hypertension pregnancy brain; $\text{TNF}\alpha$

INTRODUCTION

Hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome affects 1 to 2 out of 1000 pregnant women in the United States every year and occurs in 10–20% of women with preeclampsia (PE)^{1, 2}. Similar to PE, HELLP syndrome is believed to develop downstream of placental ischemia and is characterized after the 20th week of gestation with hypertension, neurological complications, and abnormal laboratory values indicating hepatic damage, microangiopathic hemolytic anemia, and dysfunction of the maternal vascular endothelium^{1, 3}. Women with HELLP syndrome can also develop a variety of neurologically related maternal morbidities which include cerebral hemorrhage, blurred vision and cognitive impairments during gestation and in the immediate post-partum period^{4–6}. We recently reported that plasma from women with HELLP syndrome significantly increased blood brain barrier (BBB) permeability, which suggests that factors in the circulation are capable of impairing the BBB⁷.

sFlt-1 (soluble fms-like tyrosine kinase-1) and sEng (soluble endoglin) are two soluble anti-angiogenic factors that are significantly increased in women with HELLP syndrome compared to normal pregnant and PE women⁸. Infusion or overexpression of sFlt-1 and sEng into normal pregnant rats leads to a HELLP like phenotype in the recipient rats. This phenotype is expressed not only by the increase in hemolysis, elevated liver enzymes and decreased platelets, but also by a significant increase in mean arterial pressure, inflammatory cytokines, endothelin-1, CD4⁺ and CD8⁺ T lymphocytes^{8–10}. A substantial number of studies have demonstrated that systemic inflammation mediated by inflammatory cytokines, anti-angiogenic growth factors, CD4⁺ T cells, or the damaged vascular endothelium can impair the BBB^{11–13}.

We have previously published data demonstrating that adoptive transfer of placental ischemic CD4⁺ T cells into normal pregnant rats leads to hypertension and an increase in systemic inflammatory cytokines, such as tumor necrosis factor alpha (TNF α)¹⁴. To date the majority of T cell studies focus on CD4⁺ T cell infiltration into the brain and their role in BBB permeability with very few studies focusing on CD8⁺ T cells. As CD8⁺ T cells are commonly involved in viral responses and tumors, they are frequently overlooked in pregnancy and HELLP syndrome. However, CD8⁺ T cells are associated with neuroinflammatory conditions such as neurological paraneoplastic syndromes and multiple sclerosis^{15, 16}. As CD4⁺ and CD8⁺ T cells are increased in response to infusion of sFlt-1 and sEng^{9, 10} we sought to determine if 1) rats with HELLP syndrome had an increase in BBB permeability and 2) if T cells contributed to the inflammation and any potential increase in BBB permeability in an animal model of HELLP syndrome.

METHODS

All studies were performed in timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN). Animals were housed in a temperature controlled room with a 12:12 light:dark cycle. All experimental procedures used in this study were in accordance with the National Institutes of Health guidelines for use and care of animals. All experimental procedures in

this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center.

Protocol 1: HELLP Rat Model

On gestational day (GD) 12 we infused sFlt-1 and sEng (4.7 and 7ug/kg respectively; R&D systems; Minneapolis, MN) via mini-osmotic pumps (Alzet Scientific, Cupertino, CA) into normal pregnant rats (n=18) to create HELLP syndrome for a period of 8 days^{9, 10}. Normal pregnant rats that were not infused served as normal pregnant (NP) controls (n=14).

Measurement of Mean Arterial Pressure in Conscious Rats and Determination of Hemolysis, Liver Enzymes and Platelet Counts—

To determine mean arterial pressure (MAP) on GD18 a catheter of V3 tubing (SCI, Lake Havasu City, AZ) was inserted into the carotid artery, the catheter was tunneled to the back of the neck and exteriorized after implantation while animals were under isoflurane anesthesia. On GD19, pregnant rats were placed in individual restraining cages for MAP measurements. After a 30 minute stabilization period, arterial pressure was monitored and recorded continuously for 30 minutes with a pressure transducer (Cobe III Transducer CDX Sema, Birmingham, AL) and connected to the indwelling carotid artery catheter as previously described^{9, 14, 17}.

Immediately following MAP measurement, whole blood was collected to assess lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and platelet counts^{9, 10}. Whole blood was also collected for flow cytometry and enzyme linked immunosorbent immunoassay (ELISA) experiments. Unless animals were being assessed for BBB permeability or flow cytometry, brains were dissected into frontal cortex, posterior cortex, cerebellum and brainstem sections, snap frozen and stored at -80°C for future studies.

Assessment of BBB permeability—To evaluate BBB permeability rats were infused with 4% Evan's Blue (EB; MP Biomedical, Santa Ana, CA) in 0.9% saline via a catheter inserted into the jugular vein and allowed to circulate for 25 minutes followed by infusion of 0.9% sterile saline via the jugular vein while the rat was under anesthesia. Once the circulation was free of any remaining EB, the animal was euthanized, brains collected and divided into the frontal cortex, posterior cortex and the cerebellum/brainstem. As HELLP syndrome is a multi-organ disease, we also collected a single placenta, kidney, spleen and a portion of the liver to examine EB leakage into these organs. All tissues were weighed and immediately homogenized in 50% TCA (trichloroacetic acid) buffer diluted in 0.9% saline at a 1:3 – 1:5 dilution per mg of tissue as previously described¹⁸. Plasma collected after EB infusion was diluted 1:9 in 0.9% saline. Briefly, after tissue homogenization, samples and plasma were centrifuged at 10,000g for 20 minutes at 4°C. 30μL of plasma (final dilution of 1:10,000) and samples along with EB standards were loaded in triplicate onto a clear 96-well plate followed by 90uL of 95% ethanol. Fluorescence was measured with a microplate reader at an excitation of 620nm and emission of 680nm. Data is represented as the concentration of EB dye (pg/mL)/tissue weight (g)/plasma concentration (ng/mL).

Fluorescent activated cell staining (FACS) in the brain—Cells positive for CD4⁺ and CD8⁺ were stained the frontal and posterior cortex, cerebellum and brainstem of NP and HELLP rats (Methods Supplement).

Determination of cytokine production in the brain—TNF α , interleukins-17 (IL-17), IL-10 and IL-6 and IL-1 β levels were measured in homogenates from the frontal cortex, posterior cortex, cerebellum and brainstem of rats using commercially available ELISA kits (R&D Systems; EMD Millipore, Billerica, MA; eBioscience, San Diego, CA) and assayed according to the manufacturer's protocol. All samples were normalized to their total protein concentration (via the bicinchoninic acid method; ThermoFisher, Rockford, IL).

Protocol 2: Administration of Orencia to HELLP rats

To determine if T cells contributed to the inflammation and any potential increase in BBB permeability in an experimental animal model of HELLP syndrome, 2mg/kg of Orencia (Abatacept) was infused via the jugular vein on GD13 into a subset of HELLP rats (n=17) or control NP rats (n=13). Orencia is a fusion protein fused to the extracellular domain of CTLA-4 which when administered inhibits the co-stimulation of T cells.

Determination of T Lymphocytes—To determine if Orencia decreased circulating CD4⁺ and CD8⁺ T cells, lymphocytes were isolated from plasma and stained for CD4 and CD8 as previously described^{9, 10, 14, 19} (Methods Supplement).

Determination of circulating cytokine production—Anti-angiogenic factors sFlt-1, sEng, and inflammatory cytokines TNF α , IL-6, IL-1 β , IL-17 and the anti-inflammatory cytokine IL-10 were measured in the circulation of NP, NP+Orncia, HELLP and HELLP +Orncia rats via commercially available ELISAs according to the manufacturer's protocol (R&D Systems, EMD Millipore, eBiosciences).

Statistical Analysis

All of the data are reported as mean $\pm$ standard error mean. Differences between groups were analyzed using one way analysis of variance with Bonferroni for post-hoc analysis or Student's t-test when only two groups were analyzed. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Protocol 1: Rats with HELLP syndrome have significantly increased BBB permeability

HELLP rats had a significant increase in BBB permeability, as expressed by EB dye extravasation in the posterior cortex ($p=0.009$, Figure 1A) and in the cerebellum/brainstem ($p=0.0015$; Figure 1A) compared to NP rats. While there were no statistically significant differences in BBB permeability between NP and HELLP rats in the frontal cortex ($p=0.537$).

Livers of HELLP rats had significantly more EB extravasation compared to NP livers (0.035 ± 0.006 vs. 0.101 ± 0.018 pg·g tissue-plasma concentration; $p=0.009$). There were not any significant differences in permeability between the two groups in the spleen ($p=0.259$), the placenta ($p=0.161$) or in the kidney ($p=0.745$).

T cells are increased in brains of HELLP rats—CD4⁺ T cells were significantly increased in the frontal cortex (p=0.031), posterior cortex (p=0.0002) and brainstem (p=0.044) of HELLP rats compared to NP rats (Supplemental Figure 1). There were no statistically significant differences in CD4⁺ T cell infiltration in the cerebellum between the two groups (p=0.408). CD8⁺ T cells were found to be significantly increased in the posterior cortex (p=0.031) but not in the frontal cortex (p=0.151), cerebellum (p=0.421) or brainstem (p=0.082).

IL-17 and TNF α are increased in the Brainstems of HELLP rats—There were no statistically significant differences in IL-17 (p=0.445), IL-6 (p=0.542), TNF α (p=0.258) or IL-1 β (p=0.930) levels in the frontal cortex between NP and HELLP rats. Similar results were seen in the posterior cortex and the cerebellum between NP and HELLP rats in and IL-17 (p=0.302, p=0.169), IL-6 (p=0.721, p=0.619), TNF α (p=0.422, p=0.146) and IL-1 β (p=0.689, p=0.284) levels. However, both TNF α (p=0.0001) and IL-17 (p=0.0001) were significantly increased in the brainstems of HELLP rats compared to NP rats. IL-6 levels were not statistically significant between brainstems from HELLP rats compared to NP rats (p=0.279) nor was IL-1 β (p=0.291).

There were no statistically significant differences in levels of the anti-inflammatory cytokine IL-10 in the frontal cortex (p=0.242) or the posterior cortex between NP and HELLP rats (p=0.848). However, IL-10 was significantly decreased in the cerebellum of HELLP rats compared to NP rats (p=0.045), whereas in the brainstem IL-10 levels were significantly increased in HELLP rats compared to NP rats (p=0.037).

Protocol 2: Angiogenic imbalance in pregnancy creates symptoms of HELLP syndrome

As we have previously reported^{9, 10}, infusion of sFlt-1 and sEng into NP rats led to the development of HELLP syndrome as evidenced by a significant increase in hemolysis (p=0.014), liver enzymes (p=0.009) and a significant decrease in circulating platelets (p=0.008, Figure 2). Additionally, there was a significant increase in MAP in rats with HELLP syndrome compared to NP rats (p=0.0001).

Orencia is currently used clinically to treat patients with moderate to severe rheumatoid arthritis to help reduce the T-cell induced inflammation associated with rheumatoid arthritis. As we have previously demonstrated that Orencia also decreases T cells and inflammation in placental ischemic rats¹⁹, we sought to determine if T cell attenuation in HELLP rats improved the symptomology of HELLP syndrome. Hemolysis (p=0.033), liver enzymes (p=0.043), platelets (p=0.01) and MAP (p<0.0001) were all significantly improved in HELLP+Orencia rats compared to untreated HELLP rats (Figure 2). NP+Orencia rats had no significant changes in these parameters compared to NP rats, but were still statistically lower in comparison to untreated HELLP rats (Figure 2).

There were no significant differences between NP and HELLP rats in organ weights (Table I). Pups born to HELLP rats who received Orencia weighed significantly less compared to pups born to untreated HELLP rats (p=0.020, Table I). Orencia treatment to HELLP rats did not significantly change the fetus reabsorption rate or maternal organ weight (Table I).

Orencia inhibits circulating T cells in HELLP rats—HELLP rats had significantly increased circulating CD4⁺ T cells compared to NP rats ($p=0.017$). Administration of Orencia to HELLP rats significantly decreased CD4⁺ T cells compared to HELLP rats ($p=0.02$) and was not statistically significant when compared to circulating CD4⁺ T cells in NP rats ($p=0.583$; Figure 1A). NP+Orencia rats had significantly less circulating CD4⁺ T cells compared to untreated HELLP rats ($p=0.002$) but not when compared with NP ($p=0.110$) or HELLP rats treated with Orencia ($p=0.055$; Figure 1A).

In comparison to NP rats, circulating CD8⁺ T cells were significantly increased in HELLP rats ($p=0.0002$) and significantly decreased in HELLP+Orencia rats compared to untreated HELLP rats ($p=0.0005$); however there was no statistical difference between NP and HELLP+Orencia rats ($p=0.273$; Figure 3A). NP+Orencia rats had significantly less circulating CD8⁺ T cells compared to untreated HELLP rats ($p<0.0001$) and NP rats ($p=0.003$; Figure 3A). There was not a statistically significant difference between NP and HELLP rats treated with Orencia ($p=0.231$).

Circulating sFlt-1, sEng, IL-17 and TNF α are decreased in response to T cell inhibition—Circulating sFlt-1 and sEng were significantly increased in HELLP rats compared to NP rats ($p<0.0001$). Administration of Orencia to HELLP and NP rats led to a significant decrease in both circulating levels of sFlt-1 and sEng ($p=0.0017$; Supplemental Figure 2).

Circulating IL-17 ($p=0.0006$) and TNF α ($p=0.004$) were significantly increased in HELLP rats compared to NP rats (Figure 3). In HELLP+Orencia rats levels of IL-17 ($p=0.0009$) and TNF α ($p=0.02$) were all significantly decreased compared to HELLP rats (Figure 3), with similar results being seen when HELLP rats were compared to NP+Orencia rats ($p<0.0001$; $p=0.002$ respectively; Figure 3). Circulating IL-6 was also significantly increased in HELLP rats compared to NP rats ($p=0.008$), however administration of Orencia to HELLP rats did not significantly decrease circulating IL-6 compared to untreated HELLP rats ($p=0.192$). There were no statistically significant differences in circulating levels of IL-1 β between the 4 groups of rats ($p=0.979$). The anti-inflammatory cytokine IL-10 was significantly higher in HELLP rats compared to NP rats (13.26 ± 2.74 vs. 62.24 ± 11.43 pg/mL; $p=0.003$) and to NP+Orencia rats (11.53 ± 7.08 ; $p=0.02$). Administration of Orencia to HELLP rats did not significantly affect IL-10 levels when compared to untreated HELLP ($p=0.595$) rats, nor were there any statistically significant differences when NP+Orencia or HELLP+Orencia rats were compared to NP ($p=0.794$; $p=0.06$ respectively) rats.

To determine if there was a correlation between CD4⁺ and CD8⁺ T cells all cytokines and growth factors were correlated with each subset of T cells per treatment group. There was a positive correlation between CD8⁺ T cells and circulating TNF α ($r=0.900$, $p=0.014$) in HELLP rats but not in other groups regardless of the T cell subset.

Orencia attenuates the increased BBB permeability in rats with HELLP syndrome—There were no statistically significant differences due to Orencia administration in BBB permeability in the frontal cortex ($p=0.192$; Figure 1A). Orencia administration to HELLP rats decreased BBB permeability in the posterior cortex ($p=0.002$)

and in the cerebellum/brainstem ($p<0.0001$) compared to untreated HELLP rats (Figure 1A). There was not a statistically significant difference in the posterior cortex or in the cerebellum/brainstem BBB permeability between NP rats and HELLP+Orencia rats ($p=0.300$; $p=0.0804$ respectively). There were no statistically significant differences in circulating levels of EB dye between the groups of rats ($p=0.987$). Administration of Orencia to HELLP rats significantly decreased liver permeability (0.038 ± 0.021 pg-g tissue-plasma concentration; $p=0.048$) compared to HELLP rats.

TNF α protein levels were significantly increased in response to Orencia in the brainstem ($p=0.0006$), the posterior cortex ($p=0.009$) and the cerebellum ($p=0.046$). IL-1 β was increased in the frontal ($p=0.0008$) and the posterior cortex ($p<0.0001$). Whereas IL-17 was significantly increased ($p<0.0001$) in the brainstem, frontal and posterior cortex and in the cerebellum ($p=0.006$). Orencia administration led to a significant increase in IL-10 production in the brainstem ($p=0.0009$) and posterior cortex ($p<0.0001$).

DISCUSSION

Similar to PE, women with HELLP syndrome have evidence of systemic inflammation^{20, 21} and most recently we have reported that plasma collected from women with HELLP syndrome is capable of increasing BBB permeability compared to plasma from women with a normal pregnancy⁷. These findings suggest that plasma from women with HELLP syndrome contains certain factors that potentially contribute to BBB disruption. Cerebral complications seen in high-risk pregnancies such as HELLP syndrome and PE are an important pathological feature and may lead to the development of seizure and/or stroke^{22, 23}. The mechanisms for these complications have yet to be elucidated; however, factors released by placental debris into the maternal circulation or released by the damaged vascular endothelium have been proposed as potential contributors^{24, 25}. Systemic inflammation has been reported in a variety of disease states to contribute to BBB disruption. The vascular endothelium of the BBB is subject to inflammation and infiltration of inflammatory and cytotoxic mediators such as T cells, inflammatory cytokines and chemokines^{26–29}.

In the current study, we infused the anti-angiogenic factors sFlt-1 and sEng into timed-pregnant rats to create an animal model of HELLP syndrome that has pathological features similar to that seen in women with HELLP syndrome^{9, 10}. We sought to determine if T cells, particularly CD4⁺ T cells, which are capable of secreting inflammatory cytokines, inducing hypertension and vascular endothelial activation/dysfunction¹⁴ contributed to the angiogenic imbalance, inflammation and any BBB disruption in this animal model. In the present study, we found that there was a regional difference in BBB permeability in rats with HELLP syndrome compared to NP and NP+Orencia rats, which was accompanied by an increase in inflammatory cytokines TNF α and IL-17 in the brainstem. The anti-inflammatory cytokine IL-10 was found to be decreased in the cerebellum but increased in the brainstem. Clinical studies have reported that women with HELLP syndrome have increased risk of stroke (infarction and/or hemorrhage) in the brainstem, cerebellar and posterior regions of the brains relative to other regions^{30, 31}. These areas were also the target of inflammation and had increased permeability in the current study. One possible explanation for the differences

in cytokine localization could be the migration of cytokine secreting microglia and astrocytes³² in brain regions of HELLP rats compared to NP rats. A study by Johnson et al reported that in an animal model of severe PE, there were significantly more activated microglia in the posterior regions of the brain compared to NP control rats³³. Given the similarity between PE and HELLP syndrome, the same could be true in this case.

Additionally, as the brainstem has a distinct role in the development of neurogenic hypertension³⁴, inflammation and increased T cell infiltration in this area of the brain could possibly contribute to the hypertension experienced during HELLP syndrome³⁵. T cells, primarily CD4⁺ T cells, were found to be significantly increased in the brain in response to HELLP syndrome with the highest population of infiltrating cells residing in the brainstem (Supplemental Figure 1). As T cells are involved in neurogenic hypertension it is possible that the combination of the systemic inflammation and compromised BBB enhance infiltration of T cells into the brain which can contribute in part to neuroinflammation, neuropathological diseases and hypertension. However, future studies will need to identify adaptive immune cells (macrophages) as well as microglia in the brains of HELLP and NP rats to get further insight into the immune cells and their inflammatory properties.

In the current study, a single dose of Orencia, administered 24hours after the initial infusion of sFlt-1 and sEng, was associated with a significant improvement in the biochemical parameters that define HELLP syndrome along with a significant decrease in the MAP when compared to untreated HELLP rats. The decrease in MAP was accompanied by a decrease in circulating CD4⁺ and CD8⁺ T cells, sFlt-1, sEng and the inflammatory cytokines TNF α and IL-17. As it has previously been published that all of these factors contribute to an increase in BBB permeability^{13, 27, 29, 36} we next examined the BBB permeability in Orencia treated rats. We found that BBB permeability was significantly reduced in response to administration of Orencia. There was a decrease in fetal pup weight in response to Orencia administration, but there were no other differences between the litters. This is similar to what we have previously reported in which Orencia administration reduced fetal weight in our animal model of PE¹⁹. There is limited clinical data on the safety of Orencia during pregnancy, however what has been reported is that while there are some fetal abnormalities associated with exposure to Orencia they low risk to the fetus is believe to be outweighed by the benefit to the mother³⁷. In our studies we did not see a decrease in fetal weight in the NP +Orencia group which suggests that the angiogenic imbalance in addition to Orencia might be causing the decrease in fetal weight as opposed to treatment of Orencia.

Orencia decreased both CD4 and CD8 T cells in the current study, however the role of CD8⁺ T cells in contributing to hypertension is still not clear³⁸. CD4⁺ T cells have been implicated in a number of studies as contributing to not only the inflammation that occurs during pregnancy but also to the hypertension that is present in these animal models^{19, 39, 40}. We have previously reported that rats with HELLP syndrome have a significant decrease in the ratio of circulating T regulatory (CD4⁺CD25⁺FOXP3⁺) to T helper 17 (TH17; CD4⁺CD25⁻ROR γ ⁺) cells which was due to the increase in TH17 cells in HELLP rats⁹. As Orencia decreases the population of active T cells we did not examine the proportion of T regulatory or TH17 cells in the current study. CD8 cells were also decreased in response to Orencia administration. As Orencia targets CTLA-4 which is not only located on CD8 but is

expressed at a higher affinity on CD8 cells compared to CD4 T cells⁴¹. However future studies will examine not only the role of CD4 and CD8 in specifically causing BBB damage in this model, but will also examine the functional role of T regulatory and TH17 cells.

There was also increased permeability of EB into the liver of rats with HELLP syndrome. The liver is a primary site of damage in HELLP syndrome³ and the increased permeability to this organ could contribute to some of its vulnerability. We have previously reported that livers from rats with HELLP syndrome have increased T cells⁹, which could contribute to some of the damage that occurs in the liver.

In the current study, T cell inhibition selectively decreased circulating TNF α and IL-17, but did not have a statistically significant effect on circulating IL-6 and IL-1 β levels. Interestingly, all of these inflammatory cytokines have been reported to increase BBB permeability and contribute to neuroinflammation^{42, 43}. Recent reports by Warrington et al suggest that direct infusion of TNF α into pregnant rats alone is not sufficient to lead to increases in BBB permeability³⁶. Data in the current study suggests that animals with angiogenic imbalance, increased IL-17, IL-6 and TNF α did have increases in BBB which when combined with the study from Warrington et al leads us to believe that other factors are needed in addition to TNF α to affect BBB permeability. In the current study, we only measured BBB disruption via EB leakage; however, tight junctions located between vascular endothelial cells in the BBB help regulate up to 98% of the small substances that can pass into the brain and serve as a target for inflammatory mediators^{44, 45}. The endothelium of the brain also has efflux and influx transporters which actively control entry of cytokines and chemokines into the brain. The function of the primary efflux transporter, P-glycoprotein, has been found to be compromised in response to increased levels of both CD4⁺ T cells and TNF α in the microvasculature^{46, 47}, which might serve as another mechanism whereby neuroinflammation may occur. A study by Maharaj et al reported that overexpression of sFlt-1 and sEng increased vascular permeability, brain edema and hypertension in non-pregnant mice, which supports the current study implying that angiogenic imbalance does lead to changes in the brain¹³.

Surprisingly HELLP syndrome increased IL-10 in the brainstem compared to NP rats. As the brainstem was also the area of the brain which had the largest population of infiltrating CD4⁺ T cells and BBB permeability we can tell the source of IL-10. It is possible that there is also an influx of microglia and astrocytes into that area which could possibly be working towards repairing the areas of damage which could explain the increase in IL-10 in this area. Studies investigating immune cells in the brain will help address this finding.

In summary, our data demonstrates that administration of sFlt-1 and sEng during pregnancy has regional effects on BBB permeability, leading to EB leakage in the posterior cortex and cerebellum/brainstem. Inhibition of circulating T cells by administration of Orencia decreases circulating CD4⁺ and CD8⁺ T cells, which was associated with decreases in circulating sFlt-1, IL-17, sEng and TNF α . Along with an improvement in the hemolysis, elevated liver enzymes and low platelets and hypertension associated with HELLP syndrome. In addition, a single dose of Orencia to rats with HELLP syndrome attenuated EB leakage in the posterior cortex and cerebellum/brainstem regions of brains. While data from

this study indicate that T cells may have a potential role in contributing to the biochemistry and inflammation associated with HELLP syndrome, further studies will need to be conducted to truly understand the specific role of T cells in contributing to systemic inflammation, cerebrovascular and neuro-immune factors that plague women with severe PE and HELLP syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Abildgaard U, Heimdal K. Pathogenesis of the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP): a review. *European Journal of Obstetrics and Gynecology and Reproductive Biology*. 2013; 166:117–123. [PubMed: 23107053]
2. Haram K, Svendsen E, Abildgaard U. The HELLP syndrome: Clinical issues and management. A Review. *BMC Pregnancy and Childbirth*. 2009;9. [PubMed: 19250517]
3. Geary M. The HELLP syndrome. *British Journal of Obstetrics and Gynaecology*. 1997;104.
4. Sibai B, Ramadan M, Chari R, Friedman S. Pregnancies complicated by HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets): subsequent pregnancy outcome and long-term prognosis. *Am J Obstet Gynecol*. 1995; 172:125–129. [PubMed: 7847520]
5. Martin J Jr, Brewer J, Wallace K, Sunesara I, Canizaro A, Blake P, Lamarca B, Owens M. HELLP syndrome and composite major maternal morbidity: Importance of Mississippi classification system. *J Maternal Fetal and Neonatal Medicine*. 2013; 26:1201–1206.
6. Van Pampus M, Wolf H, Mayruhu G, Treffers P, Bleker O. Long-term follow-up in patients with a history of (H)ELLP syndrome. *Hypertensi Pregnancy*. 2001; 20:15–23.
7. Wallace K, Tremble S, Owens M, Morris R, Cipolla M. Plasma from patients with HELLP Syndrome Increases Blood-brain barrier permeability. *Reproductive Science*. 2015; 22:278–284.
8. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim Y, Bdolah Y, Lim K, Yuan K, Libermann T, Stillman I, Roberts D, D'Amore P, Epstein F, Sellke F, Romero R, Sukhatme V, Letarte M, Karumanchi S. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature Medicine*. 2006; 12:642–649.
9. Wallace K, Morris R, Kyle P, Cornelius D, Darby M, Scott J, Moseley J, Chatman K, Lamarca B. Hypertension, inflammation and T lymphocytes are increased in a rat model of HELLP syndrome. *Hypertension in Pregnancy*. 2014; 33:41–54. [PubMed: 24380504]
10. Morris R, Spencer S, Kyle P, Williams J, Harris A, Owens M, Wallace K. Hypertension in an animal model of HELLP syndrome is associated with activation of endothelin-1. *Reproductive Science*. 2016; 130:409–419.
11. Warrington J, Fan F, Murphy S, Roman R, Drummond H, Granger J, Ryan M. Placental ischemia in pregnant rats impairs cerebral blood flow autoregulation and increases blood-brain barrier permeability. *Physiol Rep*. 2014;2.
12. Li-kun W, Zhen H, Guo-feng W, Chang L. Perihematoma endothelin-1 level is associated with an increase in blood-brain barrier permeability in a rabbit model of intracerebral hematoma. *Chinese Medical Journal*. 2013; 126:3433–3438. [PubMed: 24034085]
13. Maharaj A, Walshe T, Saint-Geniez M, Venkatesha S, Maldonado A, Himes N. VEGF and TGF- β are required for the maintenance of the choroid plexus and ependyma. *JEM*. 2008; 205:491–501.

14. Wallace K, Richards S, Dhillon P, Weimer A, Edholm E, Bengten E, Wilson M, Martin JJ, LaMarca B. CD4⁺ T Helper cells stimulated in response to placental ischemia mediate hypertension during pregnancy. *Hypertension*. 2011; 57:949–955. [PubMed: 21464392]
15. Neumann H, Medana I, Bauer J, Lassmann H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci*. 2002; 25:313–319. [PubMed: 12086750]
16. Huseby E, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8⁺ T cells in a model for multiple sclerosis. *J Exp Med*. 2001; 194:669–676. [PubMed: 11535634]
17. Wallace K, Cornelius D, Scott J, Heath J, Moseley J, Chatman K, LaMarca B. CD4⁺ T cells are important mediators of oxidative stress that cause hypertension in response to placental ischemia. *Hypertension*. 2014; 64:1151–1158. [PubMed: 25259742]
18. Wang H, Lai T. Optimization of Evans blue quantitation in limited rat tissue samples. *Scientific Reports*. 2014; 4:6588. [PubMed: 25300427]
19. Novotny S, Wallace K, Herse F, Moseley J, Darby M, Heath J, Gill J, Wallukat G, Martin J Jr, Dechend R, LaMarca B. CD4⁺ T cells play a critical role in mediating hypertension in response to placental ischemia. *Journal of Hypertension*. 2013; 2:1–6.
20. Visser W, Beckmann I, Bremer H, Lim H, Wallenburg H. Bioactive tumour necrosis factor alpha in pre-eclamptic patients with and without the HELLP syndrome. *Br J Obstet Gynaecol*. 1994; 101:1752–1757.
21. van Runnard Heimel P, Kavelaars A, Heijnen C, Peters W, Huisjes A, Franx A, Bruinse H. HELLP Syndrome is Associated with an Increased Inflammatory Response, which may be Inhibited by Administration of Prednisolone. *Hypertension in Pregnancy*. 2008; 27:253–265. [PubMed: 18696354]
22. Cipolla M, Sweet J, Chan S. Cerebral vascular adaptation to pregnancy and its role in the neurological complications of eclampsia. *Journal of Applied Physiology*. 2011; 110:329–339. [PubMed: 21071591]
23. Cuero M, Varelas P. Neurologic complications in pregnancy. *Crit Care Clin*. 2016; 32:43–59. [PubMed: 26600443]
24. Shen F, Wei J, Snowise S, DeSousa J, Stone P, Viall C, Chen Q, Chamley L. Trophoblast debris extruded from preeclamptic placentae activates endothelial cells: A mechanism by which the placenta communicates with the maternal endothelium. *Placenta*. 2014; 35:839–847. [PubMed: 25096950]
25. Young B, Levine R, Karumanchi S. Pathogenesis of preeclampsia. *Annu Rev Pathol*. 2010; 5:173–192. [PubMed: 20078220]
26. Wheway J, Obeid S, Couraud P, Combes V, Grau G. The brain microvascular endothelium supports T cell proliferation and has potential for alloantigen presentation. *PLoS One*. 2013; 8:e52586. [PubMed: 23320074]
27. Quandt J, Dorovini-Zis K. The beta chemokines CCL4 and CCL5 enhance adhesion of specific CD4⁺ T cell subsets to human brain endothelial cells. *J Neuropathol Exp Neurol*. 2004; 63:350–362. [PubMed: 15099025]
28. Wong D, Dorovini-Zis K, Vincent S. Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood brain barrier. *Exp Neurol*. 2004; 190:446–455. [PubMed: 15530883]
29. Liu K, Dorovini-Zis K. Differential regulation of CD4⁺ T cell Adhesion to cerebral microvascular endothelium by the B-Chemokines CCL2 and CCL3. *Int J Mol Sci*. 2012; 13:16119–16140. [PubMed: 23203188]
30. Imaizumi H, Nara S, Kaneko M, Chiba S, Tamakawa M. Magnetic resonance evaluation of brainstem dysfunction in eclampsia and the HELLP syndrome. *J Emerg Med*. 1995; 13:191–194. [PubMed: 7775789]
31. Paul B, Juneja S, Paul G, Gupta S. Spectrum of neurological complications in HELLP syndrome. *Neurol India*. 2013; 61:467–471. [PubMed: 24262446]
32. Carvalho da Fonseca A, Matias D, Garcia C, Amaral R, Geraldo L, Freitas C, Lima F. The impact of microglial activation on blood-brain barrier in brain diseases. *Frontiers in Cellular Neuroscience*. 2014

33. Johnson A, Tremble S, Chan S, Moseley J, Lamarca B, Nagle K, Cipolla M. Magnesium sulfate treatment reverses seizure susceptibility and decreases neuroinflammation in a rat model of severe preeclampsia. *PLoS One*. 2014; 9:e113670. [PubMed: 25409522]
34. Sved A, Ito S, Sved J. Brainstem mechanisms of hypertension: role of the rostral ventrolateral medulla. *Curr Hypertens Rep*. 2003; 5:262–268. [PubMed: 12724060]
35. Marvar P, Lob H, Vinh A, Zarreen F, Harrison D. The central nervous system and inflammation in hypertension. *Current Opinions in Pharmacology*. 2011; 11:156–161.
36. Warrington J, Drummond H, Granger J, Ryan M. Placental ischemia-induced increases in brain water content and cerebrovascular permeability: role of TNF- α . *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*. 2015; 309:R1425–R1431.
37. Kumar M, Ray L, Vemuri S, Simon T. Pregnancy outcomes following exposure to abatacept during pregnancy. *Semin Arthritis Rheum*. 2015; 45:351–356. [PubMed: 26210783]
38. Zhang J, Crowley S. Role of T lymphocytes in hypertension. *Current Opinion in Pharmacology*. 2015; 21:14–19. [PubMed: 25523165]
39. Zenclussen A, Fest S, Joachim R, Klapp B, Arck P. Introducing a mouse model for preeclampsia: adoptive transfer of activated Th1 cells leads to pre-eclampsia-like symptoms exclusively in pregnant mice. *European Journal of Immunology*. 2004; 34:377–387. [PubMed: 14768042]
40. Wallace K, Novotny S, Heath J, Moseley J, Martin J, Owens M, Lamarca B. Hypertension in response to CD4⁺ T cells from reduced uterine perfusion pregnant rats is associated with activation of the endothelin-1 system. *Am J Physiol Regul Integr Comp Physiol*. 2012; 303:R144–149. [PubMed: 22647295]
41. Pandiyan P, Hegel J, Frueger M, Quandt D, Brummer-Weinzierl M. High IFN- γ production of individual CD8 T lymphocytes is controlled by CD152 (CTLA-4). *J Immunol*. 2007; 178:2132–2140. [PubMed: 17277117]
42. de Vries H, Kuiper J, de Boer A, Van Berkel T, Breimer D. The blood-brain barrier in neuroinflammatory diseases. *Pharmacol Rev*. 1997; 49:143–155. [PubMed: 9228664]
43. Waisman A, Hauptmann J, Regen T. The role of IL-17 in CNS diseases. *Acta Neuropathol*. 2015; 129:625–637. [PubMed: 25716179]
44. Wallez Y, Huber P. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochimica et Biophysica Acta - Biomembranes*. 2008; 1778:794–809.
45. Harhaj N, Antonetti D. Regulation of tight junctions and loss of barrier function in pathophysiology. *Int J Biochem Cell Biology*. 2004; 36:1206–1237.
46. Bauer B, Hartz A, Miller D. Tumor necrosis factor- α and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier. *Mol Pharm*. 2006; 71:667–675.
47. Kooij G, van Horssen J, de Lange E, Reijerkerk A, Van der Pol S, Van het Hof B, Drexhage J, Vennegoor A, Killestein J, Scheffer G, Oerlemans R, Schepers R, van der Valk P, Dijkstra C, De Vries H. T lymphocytes impair P-glycoprotein function during neuroinflammation. *J Autoimmun*. 2010; 34:416–425. [PubMed: 19959334]

Figure 1. Blood brain barrier disruption and neuroinflammation in HELLP rats

Rats with HELLP syndrome had significantly more EB leakage into the posterior cortex and cerebellum/brainstem regions of the brain compared to normal pregnant rats (**A**). Administration of Orencia to HELLP rats significantly decreased EB leakage in the posterior cortex and cerebellum/brainstem compared to HELLP rats (**A**; $n=6/\text{group}$). Circulating CD4⁺ and CD8⁺ T cells were significantly increased in HELLP rats ($n=5$) compared to NP rats ($n=6$), but was decreased in response to Orencia ($n=5$; **B**). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ between the indicated groups. # denotes statistical significance compared to NP+Orencia rats.

Figure 2. Orenia decreases the parameters of HELLP syndrome

HELLP rats (n=13) have increased lactate dehydrogenase (A), increased alanine aminotransferase (B), decreased platelet count (C), and increased mean arterial pressure (D) compared to NP rats (n=10). HELLP + Orenia rats (n=10) had significantly decreased hemolysis and liver enzymes compared to HELLP rats. Platelets were also significantly increased and mean arterial pressure was decreased in response to Orenia administration. *p < 0.05, **p < 0.005, ***p < 0.0005 between indicated groups. # denotes statistical significance compared to NP+Orenia rats.

Figure 3. Orencia decreases circulating IL-17 and TNFα.

Circulating IL-17, IL-6 and TNFα were increased in HELLP rats compared to NP rats.

Orencia administration to either NP or HELLP rats decreased IL-17 and TNFα. **p<0.005,

***p<0.0005 between indicated groups. # indicates p<0.05 compared to NP+Orencia rats.

Maternal organs and rat pups weights were analyzed via analysis of variance with Student's T test for post-hoc analysis (when applicable). Fetal reabsorption was calculated as the number percent dead pups/live pups. Data is reported as mean $\pm$ standard error mean.

Table I

	Normal Pregnant	NP +Orencia	HELLP	HELLP+Orencia	P value
Liver	12.07 $\pm$ 0.36	11.85 $\pm$ 0.34	11.64 $\pm$ 0.30	11.57 $\pm$ 0.38	0.715
Spleen	0.888 $\pm$ 0.09	0.919 $\pm$ 0.04	0.739 $\pm$ 0.03	0.842 $\pm$ 0.04	0.247
Kidney	0.855 $\pm$ 0.03	0.899 $\pm$ 0.05	0.953 $\pm$ 0.04	0.850 $\pm$ 0.04	0.185
Brain	1.90 $\pm$ 0.06	1.86 $\pm$ 0.03	1.84 $\pm$ 0.04	1.86 $\pm$ 0.08	0.641
Pup Weight (grams)	2.53 $\pm$ 0.07	2.68 $\pm$ 0.06	2.57 $\pm$ 0.05	2.34 $\pm$ 0.08	0.01 *
Fetal Absorption (%)	0.05 $\pm$ 0.02	0.04 $\pm$ 0.02	0.04 $\pm$ 0.02	0.02 $\pm$ 0.01	0.762

* Pups born to HELLP + Orencia rats weighed significantly less compared to pups in the HELLP group (p=0.02).