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Hypertension, inflammation and T lymphocytes are increased in a rat model of HELLP syndrome

Kedra Wallace¹, Rachael Morris¹, Patrick B. Kyle², Denise Cornelius³, Marie Darby¹, Jeremy Scott³, Janae Moseley³, Krystal Chatman¹, and Babbette LaMarca³

¹Department of Obstetrics & Gynecology, University of Mississippi Medical Center, Jackson, MS, USA

²Department of Pathology & Clinical Chemistry, University of Mississippi Medical Center, Jackson, MS, USA

³Department of Pharmacology & Toxicology, University of Mississippi Medical Center, Jackson, MS, USA

Abstract

Objective—An animal model of hemolysis, elevated liver enzymes, low platelet count (HELLP) was used to determine if T lymphocytes accompany hypertension and increased inflammatory cytokines.

Methods—sFlt-1 (4.7 µg/kg/day) and sEndoglin (7 µg/kg/day) were infused into normal pregnant rats (HELLP rats) for 8 days.

Results—HELLP was associated with increased mean arterial pressure ($p = 0.0001$), hemolysis ($p = 0.044$), elevated liver enzymes ($p = 0.027$), and reduced platelets ($p = 0.035$). HELLP rats had increased plasma levels of TNF α ($p = 0.039$), IL-6 ($p = 0.038$) and IL-17 ($p = 0.04$). CD4⁺ and CD8⁺ T lymphocytes were increased.

Conclusion—These data support the hypothesis that T cells are associated with hypertension and inflammation.

Keywords

Angiogenic factors; CD4; CD8; HELLP syndrome; Hypertension; Inflammation; Preeclampsia; T lymphocytes

INTRODUCTION

Hemolysis-elevated liver enzymes-low platelet (HELLP) syndrome affects 10–20% of women with severe preeclampsia in the United States (1-3). Despite being one of the leading

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Correspondence: Kedra Wallace, PhD, University of Mississippi Medical Center, Department of Obstetrics & Gynecology, 2500 N. State St., Jackson, MS 39216, USA. Tel: 601 984 5396. Fax: 601 815 1440. kwallace2@umc.edu.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

causes of maternal death and a major contributor to maternal and perinatal morbidity, the mechanisms responsible for the pathogenesis of HELLP remain unclear (3-5). The symptoms associated with HELLP begin to appear in the third trimester and are characterized by abnormal laboratory values in multiple organs, indicating hepatic dysfunction/damage, microangiopathic hemolytic anemia, endothelium dysfunction, hepatic apoptosis and hypertension (6-10). Importantly, HELLP is also associated with increased inflammatory cytokines such as interferon gamma, tumor necrosis factor-alpha (TNF α), interleukin-6 (IL-6) and anti-angiogenic factors soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEndoglin) (11-15).

Various animal models have reported that either placental ischemia, adoptive transfer of placental ischemia-induced CD4⁺ T cells, and infusion of sFlt-1 or of TNF α into pregnant rats leads to hypertension, maternal endothelial dysfunction and inflammatory cytokine production (16-21). The reduced uterine perfusion pressure (RUPP) model of placental ischemia, an animal model of preeclampsia (17,18,20,22-24) was found not to imitate HELLP, indicating that placental ischemia alone is not responsible for the advent of HELLP syndrome (25). A study by Venkatesha et al. found that by overexpressing placenta derived sFlt-1 and sEndoglin in pregnant rats an animal model HELLP could be produced (11). Importantly, this study demonstrated that neither overexpression of sFlt-1 or sEndoglin alone into pregnant rats created a profile of HELLP, but rather the combination of these two placental derived factors was needed to create a profile similar to HELLP (11).

Recent data from both women and experimental animal models of hypertensive disorders during pregnancy define an imbalance in T cells, which are known producers of inflammatory cytokines and mediators of hypertension during pregnancy (20,26-29). We have recently shown that normal pregnant recipient rats of placental ischemia induced CD4⁺ T lymphocytes have increased mean arterial pressure, endothelial dysfunction, increased plasma levels of IL-6, sFlt-1, TNF α and increased production of agonistic autoantibodies to the angiotensin II type I receptor (AT1-AA) (20,21,30), all of which are present in women with preeclampsia and HELLP syndrome (14,15,29,31-36). Furthermore, preeclampsia is associated with a decrease in regulatory T cells (Treg) which are a subset of specialized CD4⁺ T cells which act to suppress inflammatory T cell responses (20,37). T helper 17 (Th17) cells, another subset of CD4⁺ T cells, secrete the inflammatory cytokine interleukin-17 (IL-17), which has been implicated in a number of autoimmune disorders as well as in preeclampsia (38-40). The decrease in Tregs and increase in Th17 cells in women with preeclampsia leads to an imbalance or a shift in the Treg/Th17 ratio, implicating these CD4⁺ subsets as having a role in the pathogenesis of preeclampsia (29,41,42). HELLP is associated with increased production of Th1 cytokines; however, it is unknown if Th17 cells are stimulated in HELLP. Therefore, this study was designed to test the hypothesis that Th1 lymphocytes and hypertension are increased in an animal model of HELLP syndrome.

METHODS

All studies were performed in 220–250 g timed-pregnant Sprague-Dawley rats (Harlan). Animals were housed in a temperature controlled room with a 12:12 light:dark cycle. All experimental procedures in this study were in accordance with the National Institutes of

Health guidelines for use and care of animals and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Effect of Chronic sFlt-1 and sEndoglin in Pregnant Rats

It has previously been shown that overexpression of sFlt-1 and sEndoglin into normal pregnant rats creates a biochemical profile (hemolysis-increased lactate dehydrogenase; elevated liver enzymes-increased alanine aminotransferase and low platelet count) of HELLP in pregnant rats (11). In the current study, we infused sFlt-1 and sEndoglin into normal pregnant rats (HELLP rats, $n = 16$) beginning on gestational day (GD) 12. sFlt-1 and sEndoglin (R&D Systems, Minneapolis, MN) were infused at a rate of 4.7 and 7 $\mu\text{g/kg/day}$, respectively, for 8 days (days 12–19 of gestation) via mini-osmotic pumps (model 2002; Alzet Scientific, Cupertino, CA) which were surgically inserted into the intraperitoneal cavity of normal pregnant rats. Mini-osmotic pumps containing sflt-1 (4.7 $\mu\text{g/kg/day}$) +sEndoglin (7 $\mu\text{g/kg/day}$) were surgically inserted into the intraperitoneal cavity of virgin rats ($n = 4$) for a period of 8 days to determine if a similar biochemical profile is seen in non-pregnant rats; these results were compared to virgin control rats ($n = 4$).

Measurement of Mean Arterial Pressure (MAP) in Chronically Instrumented Conscious Rats and Determination of Hemolysis, Liver Enzymes and Platelet Counts

On GD 18 HELLP and normal pregnant (NP) control rats ($n = 13$) underwent carotid artery surgery for MAP measurement (methods supplement). On GD19 MAP was measured (18,20,21,43), blood and tissues were collected for analysis. Whole blood was collected in EDTA treated tubes and processed for lactate dehydrogenase (LDH), platelet counts (Sysmex[®] XE-5000 from Sysmex America Inc., Lincolnshire, IL) and flow cytometry. The remaining blood was centrifuged at 3200 rpm for 10 min and alanine aminotransferase (ALT) levels were measured from plasma via a Roche c501 Cobas[®] Series (Roche Diagnostics, Indianapolis, IN).

Determination of Cytokine Production

Plasma was measured for IL-6, TNF α , IL-17, sFlt-1 and sEndoglin using commercial ELISA kits (R&D Systems) and assayed according to the manufacturer's protocol. Protein levels of sFlt-1 were measured in placentas (44) (1 placenta per rat, $n = 6$ rat/group) and a portion of the median lobe of the maternal liver ($n = 6$ /group). Placentas chosen were those closest to the average placental weight for that dam. Briefly, frozen tissue was placed in 1 mL of radioimmunoprecipitation lysis buffer containing protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA) and mechanically homogenized while on ice. The solution was centrifuged at 12 000 g for 20 min at 4 °C. The resulting protein concentration was measured by the bicinchoninic acid method (Pierce, Rockford, IL).

Determination of T and B Lymphocytes

Immediately after MAP measurement one placenta, a portion of the median maternal liver lobe, kidney, spleen, and plasma was collected for lymphocyte isolation. The kidney was minced with a razor blade while on ice, digested for 45 min at 37 °C in 2 mLs of 0.125 U DNaseI (Sigma, St. Louis, MO) and 1 mg/mL of collagenase IV (Invitrogen, Grand Islands,

NY). Afterwards, 2 mL's of RPMI1640 (Invitrogen) was added to the collagenase-tissue digestion, followed by centrifugation at 1600 rpm for 10 min. The remaining tissue was homogenized in RPMI1640 and the homogenate was strained using a 100 μ M cell strainer (Fisher, Pittsburgh, PA) followed by centrifugation at 1600 rpm for 10 min. The resultant single cell suspensions were layered over a Ficoll-Hypaque gradient (Accurate Chemical Corp., Westbury, NY) to allow density-gradient separation of lymphocytes from plasma, platelets, red blood cells and granulocytes, followed by centrifugation at 1600RPM for 25 min (20,23). Resulting lymphocytes were separated into individual tubes to be labeled for Treg (CD4⁺CD25⁺FoxP3⁺), Th17 (CD4⁺CD25⁺ROR γ ⁺), CD4⁺IL-17 A⁺, CD8⁺ T, or B cells (IgM or CD45R) at a concentration of 1×10^6 cells per tube. Staining procedures are described in the methods supplement and gating strategy is demonstrated in Supplemental Figure 1. All cells were analyzed using a Beckman Coulter Gallios Flow Cytometer equipped with a 488 nm blue laser and a 638 nm red laser, which is optimized for high sensitivity performance and has the capacity to acquire up to 25 000 events per second.

Statistical Analysis

All of the data are reported as mean $\pm$ standard error mean. Differences between NP and HELLP rats or virgin control rats and virgin + sFlt-1 + sEndoglin rats were analyzed using Student's *t*-test. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of Chronic Infusion of sFlt-1 and sEndoglin on HELLP Biochemistry and MAP

To create an animal model of HELLP syndrome, we chronically infused sFlt-1 and sEndoglin on day 12 of pregnancy into NP rats for 8 days. HELLP rats ($n = 16$) had significantly increased LDH ($p = 0.044$; Figure 1A), increased ALT ($p = 0.027$; Figure 1B) and significantly decreased platelet counts ($p = 0.035$; Figure 1C) compared to NP control rats ($n = 13$). To determine the dose of sFlt-1 to be infused, we administered either 3.7 (19,45) or 4.7 μ g/kg/day (44) of sFlt-1 and infused it in combination with 7 μ g g/kg/day of sEndoglin at either GD12 or GD14 via mini-osmotic pumps (19). sFlt-1 infused into NP rats via mini osmotic pumps at either dose (19,44,45) has previously been shown to increase MAP and lead to endothelial activation, both of which are associated with preeclampsia and HELLP (6,35,46-48). At total of 4.7 μ g/kg/day of sFlt-1 and 7 μ g/kg/day of sEndoglin infused into NP rats via mini-osmotic pumps on GD12 significantly increased LDH, ALT and decreased platelet levels compared to NP control rats, whereas the other doses did not lead to a significant reduction in platelets which is a key component of HELLP syndrome (Supplemental Table 1). No animals died in this study due to chronic infusion of sFlt-1 and sEndoglin.

HELLP rats had significantly higher MAP compared to NP control rats ($p = 0.0001$; Figure 1D). Rat pups from HELLP rats weighed less at birth compared to pups from NP control rats, but did not reach statistical significance ($p = 0.08$; Supplemental Table 2), neither was the number of fetal resorptions increased in litters from HELLP rats (3.7% resorption HELLP vs. 4.4% resorption NP; $p = 0.71$). There were no significant differences in placenta

($p = 0.67$), spleen ($p = 0.75$), kidney ($p = 0.54$) or liver ($p = 0.29$) weights between the two groups (Supplemental Table 2).

Virgin rats infused with sFlt-1 and sEndoglin did not have any statistically significant changes in LDH ($p = 0.26$), ALT ($p = 0.37$) or MAP compared to virgin control rats ($p = 0.54$; Supplemental Figure 2). However, platelets in virgin rats infused with sFlt-1 and sEndoglin were significantly decreased compared to virgin control rats ($p = 0.03$; Supplemental Figure 2).

Chronic Infusion of sFlt-1 and sEndoglin on Circulating TNF α , IL-6 and IL-17

We measured circulating levels of TNF α , IL-6 and IL-17 in plasma collected from NP control and HELLP rats to determine if HELLP rats had increases in inflammatory cytokines as seen in women with HELLP syndrome (15). TNF α ($p = 0.039$), IL-6 ($p = 0.038$) and IL-17 ($p = 0.04$) were significantly increased in HELLP rats compared to NP control rats (Figure 2).

Circulating sEndoglin ($p = 0.009$) was significantly increased in HELLP rats compared to NP control rats, and circulating sFlt-1 levels tended to increase in HELLP rats but did not reach statistical significance ($p = 0.09$; Supplemental Figure 3). In response to sFlt-1 and sEndoglin infusion, protein levels of sFlt-1 were significantly increased in the liver ($p = 0.02$) and placentas of HELLP rats compared to NP control rats ($p = 0.04$; Supplemental Figure 3). sEndoglin was significantly increased in the placentas of HELLP rats ($p = 0.01$) and tended to increase in the liver of HELLP rats compared to NP rats $p = 0.14$; Supplemental Figure 3).

Chronic Infusion of sFlt-1 and sEndoglin Increases CD4⁺ T Lymphocytes

In the current study, we sought to determine if T cells were increased in animals with HELLP syndrome, a severe form of preeclampsia. CD4⁺ T cells are significantly increased in the circulation ($p = 0.024$), spleen ($p = 0.009$), liver ($p = 0.036$), kidney ($p = 0.034$) and placenta ($p = 0.009$) of HELLP rats compared to NP control rats (Figure 3).

Recent studies have reported that women with preeclampsia have a decrease in the ratio of Treg/Th17 cells (28,29,41). To determine if infusion of sFlt-1 and sEndoglin into pregnant rats could alter this ratio, we measured Treg and Th17 cells. With the exception of the kidney ($p = 0.01$), Tregs were not significantly decreased in HELLP rats compared to NP rats (Supplemental Table 3). There was a trend for Th17 cells to increase in the circulation, liver, spleen, placenta and kidney for HELLP rats, but they did not reach statistical significance (Supplemental Table 3). The ratio of Treg/Th17 cells was decreased in the circulation of HELLP rats compared to NP control rats ($p = 0.05$; Figure 4A) and in the spleen ($p = 0.02$; Figure 4B). The ratio of Treg/Th17 cells was significantly decreased in the kidney ($p = 0.002$; Figure 4C) and liver ($p = 0.05$; Figure 4D) of HELLP rats compared to NP control rats while there was no significant difference ($p = 0.98$) in the Treg/Th17 ratio in the placenta between the HELLP and NP control rats (1.73 ± 0.56 , $n = 11$ in HELLP vs. 1.75 ± 0.52 , $n = 11$ in NP).

Chronic Infusion of sFlt-1 and sEndoglin Increases CD4⁺ IL-17 A⁺ T Lymphocytes

Since Th17 cells and the IL-17 which is secreted from Th17 cells are increased in response to infusion of sFlt-1 and sEndoglin, we also measured CD4⁺IL-17 A⁺ cells in this model. CD4⁺IL-17 A⁺ cells are significantly increased in the circulation ($p = 0.008$) and kidney ($p = 0.02$; Figure 5). While IL-17 A⁺ cells in HELLP rats were significantly increased in the placenta ($p = 0.03$) and the liver ($p = 0.05$), these same cells were not double positive for CD4⁺ T cells compared to NP control rats ($p = 0.16$, $p = 0.96$ respectively; Supplemental Figure 3). There was no significant difference in IL-17 A⁺ cells in the spleen ($p = 0.79$; $17.46 \pm 5.1\%$ HELLP vs. $15.26 \pm 5.5\%$ NP) or CD4⁺IL-17 A⁺ T cells ($p = 0.32$; $0.7 \pm .28\%$ HELLP vs. $0.32 \pm .18\%$ in NP) between HELLP and NP control rats.

Chronic Infusion of sFlt-1 and sEndoglin Increased Circulating, Hepatic and Placental CD8⁺ T Lymphocytes

CD8⁺ T cells were significantly elevated in the circulation ($p = 0.03$), liver ($p = 0.04$) and placentas ($p = 0.03$) of HELLP rats when compared to NP control rats (Figure 6). CD8⁺ T cells were increased in the spleens of HELLP rats ($14.39 \pm 4.6\%$) compared to NP control rats ($9.6 \pm 4.7\%$) but did not reach statistical significance ($p = 0.48$); likewise in the kidney ($1.06 \pm 0.26\%$ HELLP, vs. $1.19 \pm 0.32\%$ NP; $p = 0.74$).

Chronic Infusion of sFlt-1 and sEndoglin does not Increase B Cells

Chronic infusion of sFlt-1 and sEndoglin did not increase B cell production in the circulation (3.72 ± 2.6 HELLP vs. $3.59 \pm 1.7\%$ NP; $p = 0.96$), spleens (14.88 ± 2.6 HELLP vs. $12.58 \pm 3.6\%$ NP; $p = 0.66$), liver (4.97 ± 2.2 HELLP vs. 3.13 ± 2.7 NP; $p = 0.61$), kidneys (0.37 ± 0.1 HELLP vs. $1.13 \pm 0.6\%$ NP; $p = 0.18$) or placenta (8.45 ± 2.89 HELLP vs. $13.08 \pm 3.8\%$ NP; $p = 0.34$).

DISCUSSION

HELLP, a severe manifestation of preeclampsia, is associated with hemolysis, liver dysfunction, low platelet count, immune activation, and hypertension (6,10,12,13,49). Previous studies have demonstrated a role for T lymphocyte imbalance in preeclampsia (27-29), and studies from our lab have demonstrated that adoptive transfer of placental ischemia stimulated CD4⁺ T cells into NP rats elicit high blood pressure, endothelial dysfunction, AT1-AA production and an inflammatory cytokine response similar to what is seen in preeclamptic women (20,21,30). We have recently published data demonstrating increased inflammatory cytokines and increased production of the AT1-AA in women with HELLP (15), but it is unknown if an increase in Th1 lymphocytes exist in HELLP. Therefore, in the current study we sought to determine if inflammatory cytokines and lymphocytes are increased in an animal model of HELLP syndrome.

In the current study, we demonstrate that infusion of sFlt-1 and sEndoglin into NP rats leads to hemolysis, liver injury, decreased platelets and increased blood pressure, all of which characterize HELLP. Furthermore, infusion of sFlt-1 and sEndoglin into virgin rats decreased platelets but did not increase LDH, ALT or blood pressure. These results are similar to what was previously reported by Venkatesha et al., who reported vascular

damage, which can attribute to a decrease in platelets, in virgin rats injected with sFlt-1 and sEndoglin (11). It could be that the effects of placental derived sFlt-1 and sEndoglin on the maternal vessels are long-lasting causing damage even in the absence of a placenta, which would explain why some women do not develop HELLP until after pregnancy and have an increased risk for developing cardiovascular disease later in life (6,50-56). Studies by other investigators have demonstrated an increase in placental levels of IL-6, sEndoglin and TNF α , thereby suggesting that immune activation, or over activation, is a component of HELLP syndrome (12-14). In the current study, we demonstrate that HELLP rats have increased plasma levels of IL-6, IL-17 and TNF α and increased hepatic and placental levels of sFlt-1 and sEndoglin. We recently demonstrated that women with HELLP have increased circulating levels of IL-6, TNF α , sFlt-1 and sEndoglin (15); however, the presence of immune cells was not examined.

Although preeclampsia is associated with an increase in CD4⁺ T cells, it is unknown if the same is true for HELLP or if this could be detected in an animal model of HELLP. Infusion of sFlt-1 and sEndoglin into pregnant rats significantly increased CD4⁺ T cells compared to NP control rats, and importantly in the circulation, spleen and kidney decreased the Treg/Th17 ratio. HELLP did not suppress Treg cells, but did increase Th17 cells, thereby accounting for a decreased Treg/Th17 ratio in these animals. Previous studies have shown that women with HELLP do not have decreased Treg cells compared to NP women and non-pregnant women (57,58). Further studies demonstrated that not only were Treg cells from women with HELLP not decreased, but their suppressive activity was in the same range as women with healthy pregnancies; unlike Treg cells collected from women with preeclampsia (58), thus suggesting that nonfunctional Treg cells may not contribute to the immune activation in HELLP.

We cannot discount the potential role of CD8⁺ cells, which were increased in this model of HELLP and are also capable of secreting IL-17 (59,60), which may be another mechanism by which IL-17 was increased in the present model. Future studies will include measuring CD8⁺IL-17 A⁺ cells. As we only measured cytokines in plasma, it is important to recognize that T lymphocytes are not the only cell types responsible for the secretion of cytokines, and further studies are needed to determine the cellular source of these inflammatory cytokines and their role, in contributing to the pathophysiological symptoms in this model of HELLP.

Overall we conclude that elevated sFlt-1 and sEndoglin during pregnancy leads to a condition similar to that seen in women presenting with HELLP. These patients not only present with high blood pressure and elevated liver enzymes, LDH and decreased platelets but also increased TNF α and IL-6¹⁵. The current rat model mimicking HELLP indicates that hypertension in response to infusion of sFlt-1 and sEndoglin is associated with increased inflammatory cytokines, and increased CD4⁺ and CD8⁺ T cells. Studies examining various treatment options will be instrumental in better identifying new therapeutic options for women with HELLP. With the use of such animal models as that presented here, we hope to make great strides to develop treatment options to improve maternal and fetal health during pregnancies affected by HELLP.

The development of this animal model of HELLP and our results suggests a number of future research directions to both better define the mechanisms leading to the development of HELLP and to find potential therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

HELLP in rats is accompanied with hypertension. HELLP rats ($n = 16$) have increased lactate dehydrogenase (A), increased alanine aminotransferase (B), decreased platelet count (C), and increased mean arterial pressure (D) compared to NP (normal pregnant) control rats ($n = 13$). * $p < 0.05$ compared to NP control rats.

Figure 2. Circulating inflammatory cytokines are increased in HELLP rats. ELISA results from plasma collected from NP ($n = 10$) and HELLP rats ($n = 10$). TNF α (A), IL-6 (B) and IL-17 (C). * $p < 0.05$ compared to NP control rats.

Figure 3. Chronic infusion of sFlt-1 and sEndoglin increases CD4⁺ T cells. Circulating leukocytes, spleens, livers, kidneys and placentas were collected from NP control and HELLP rats. Panel (A) are representative flow cytometry plots of NP control and HELLP rats stained with anti-CD4. Bar graphs (B) demonstrate the averaged %CD4⁺ cells $\pm$ SEM indicating that CD4⁺ T cells are significantly increased in HELLP rats compared to NP control rats. * p < 0.05 compared to NP control rats.

Figure 4.

Chronic infusion of sFlt-1 and sEndoglin decreases the ratio of Treg/Th17 cells. The ratio of Treg to Th17 cells was determined from appropriately stained cells. The bar graphs represent the averaged data $\pm$ SEM for the ratio in the circulation (A), spleen (B), kidney (C) and liver (D) between NP control and HELLP rats. * $p < 0.05$, ** $p < 0.005$ compared to NP control rats.

Figure 5. Chronic infusion of sFlt-1 and sEndoglin is a stimulus for CD4⁺IL-17 A⁺ cells. Detection of CD4⁺IL-17 A⁺ cells in the circulation and kidney of NP control and HELLP rats (A). The bar graphs represent the averaged data $\pm$ SEM data (B). * p < 0.05 compared to NP control rats.

Figure 6. Chronic infusion of sFlt-1 and sEndoglin is a stimulus for CD8⁺ T cell imbalance. Circulating leukocytes, livers and placentas were collected from NP and HELLP rats. Panel (A) are representative flow cytometry plots of NP and HELLP rats stained with anti-CD8. Panel (B) shows the graphed results indicating that CD8⁺ T cells are increased in HELLP rats compared to NP rats. **p* < 0.05 compared to NP rats.