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Maternal CD8⁺ T cell depletion alleviates intrauterine inflammation-induced perinatal brain injury

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Abstract

Problem—We investigated the mechanisms by which CD8⁺ T cell trafficking in placenta contributes to perinatal brain injury by studying effects of maternal CD8⁺ T cell depletion (DEP) in a mouse model of intrauterine inflammation (IUI).

Method of Study—Maternal CD8⁺ T cells were depleted with anti-CD8⁺ antibodies. IUI was induced with lipopolysaccharide (LPS). DEP was confirmed using flow cytometry. Preterm birth rate was evaluated. Offspring neurologic sequelae were assessed by Nissl staining, immune arrays, confirmatory individual TaqMan[®] gene assays, and neurobehavioral tests.

Results—DEP did not significantly prevent LPS-induced preterm birth, but improved neurobehavioral performance ($p < 0.001$) and increased cortical neuronal density ($p < 0.05$) in LPS-exposed pups compared to controls. These changes were associated with decreased CCL3 and CXCL10 and increased CCL5 in DEP LPS-exposed mice.

Conclusions—We demonstrate that DEP reduces perinatal brain injury following IUI. This supports a role for maternal CD8⁺ T cell trafficking in placenta in mediating perinatal brain injury separate from preterm birth mechanisms.

Graphical Abstract

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INTRODUCTION

Preterm birth is a significant challenge in perinatal medicine, conferring an increased risk for perinatal acute and chronic neurologic morbidity and constituting up to 70% of perinatal mortality.^{1–5} Intrauterine inflammation (IUI) is strongly associated with preterm birth, with clinical evidence of inflammation in up to 40% of preterm births.^{1,6,7} Furthermore, exposure to IUI during prenatal development is a known risk factor for adverse neurodevelopmental outcomes in offspring, including cerebral palsy, autism spectrum disorder, and schizophrenia.^{8–10}

While perinatal brain injury and preterm birth are both associated with IUI, evidence suggests that the two processes are regulated by divergent mechanisms.^{11,12} For instance, children born at term after threatened preterm labor were more likely to have mild neurodevelopmental delay, compared with children who were born at full term without threatened preterm labor.¹³ Different cytokines and chemokines may be responsible for these divergent mechanisms.^{11,12} Apart from this knowledge, however, these mechanisms are not fully understood. Successful pregnancy is dependent on maintenance of fetomaternal tolerance, mediated by maternal immune cells in the placenta.¹⁴ T cells comprise 5–20% of decidual lymphocytes, and, of these, the majority are CD8⁺. These decidual CD8⁺ T cells play cytotoxic roles, but can also contribute to immune suppression through memory T cells and may serve functions not yet known.¹⁵ On the other hand, increased infiltration of CD4⁺ and CD8⁺ T cells from maternal circulation, in response to pro-inflammatory cytokine secretion during inflammation, is implicated in chronic chorioamnionitis, although the function of the T cells is still largely unknown. In turn, acute maternal chorioamnionitis is often linked to cases of preterm birth.^{16,17}

Bizargity et al. found that lipopolysaccharide (LPS)-induced preterm birth was not prevented in a model of T and B cell-deficient Rag1^{-/-} mice. In fact, T cell deficiency worsened the preterm birth effect of LPS in this model. The effect was partially rescued by reconstituting the mice with CD4⁺ T cells; and regulatory T cells (Tregs) were suggested to play a role in mediating LPS-induced preterm birth. This study, however, did not examine the role of CD8⁺ T cells in prematurity-associated perinatal brain injury.¹⁸

Our group previously reported an increase of CD8⁺ T cell trafficking in placenta with exposure to IUI (LPS) and its association with perinatal brain injury.¹⁹ Therefore, the objective of our current study was to specifically examine the role of maternal CD8⁺ T cells in the pathogenesis of perinatal brain injury subsequent to IUI. We hypothesized that while maternal CD8⁺ T cell depletion would not have an effect on preterm birth, it would decrease perinatal brain injury in the mouse model of IUI.

MATERIALS AND METHODS

Animal model of IUI

All animal care and treatment procedures were approved by the Animal Care and Use Committee of the Johns Hopkins University. Timed pregnant CD-1[®] mice were obtained from Charles River Laboratories (Wilmington, MA, USA), and an established model of IUI was used as previously described.^{12,20–25} Briefly, on embryonic day (E)17, dams received 25 µg LPS (from *E. coli* O55:B5; Sigma-Aldrich, St. Louis, MO, USA) in 100 µL phosphate buffered saline (PBS) (pH 7.4, Gibco, ThermoFischer Scientific, Waltham, MA, USA) or 100 µL PBS vehicle via intrauterine injection between the first and second gestational sacs of the right uterine horn. A total of 92 dams were randomly assigned to five treatment groups: PBS, LPS, CD8⁺ T cell depletion (DEP) + PBS, DEP + LPS, and nonsurgical control. Preterm birth was defined as delivery of the litter before E18.5.

CD8⁺ T cell depletion and testing

The CD8⁺ T cell depleted mouse model was created by intraperitoneal injection of 200µg anti-mouse CD8α antibody (clone 53–6.72, Bio X Cell, West Lebanon, NH, USA) at E14 and E16, as used previously.^{26,27} On E17, maternal blood and placentas were collected six hours after LPS injection. One tenth volume 0.1M ethylenediaminetetraacetic acid (made from EDTA, J.T. Baker #8993-01, Phillipsburg, NJ, USA) was added to blood for prevention of coagulation, followed by lymphocyte isolation with LSM[®] lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA) according to manufacturer's protocol. Placentas were ground and digested for 24 hours by Collagenase D (No. 11088866001, Roche, Indianapolis, IN, USA) at 37°C, followed by lysis of red blood cells using ammonium citrate potassium lysis buffer (Quality Biological Inc., Gaithersburg, MD, USA). After washing and centrifugation, the cells were ready for flow cytometry.

Flow cytometry

Flow cytometry was used to analyze CD8⁺ T cells in the maternal blood and placenta. Isolated lymphocytes were stained with surface markers in 2% fetal bovine serum in PBS for 30 minutes at 4°C in the dark. Antibodies used were anti-mouse CD45⁺ (clone 30-F11,

ThermoFischer Scientific, Waltham, MA, USA), anti-mouse CD3⁺ (clone 17A2, BioLegend, San Diego, CA, USA), anti-mouse CD4⁺ (clone RM4-5, BD, Franklin Lakes, NJ, USA) and anti-mouse CD8 α or its isotope (clone 2.43, TONBO Biosciences, San Diego, CA, USA). For blocking experiments, cells were incubated with unlabeled anti-CD8⁺ antibodies (clones YTS169.9 and 53-6.72, Bio X Cell, West Lebanon, NH, USA) in fluorescence activated cell sorting (FACS) buffer for 20 minutes at 4°C in the dark prior to surface marker staining. Data were acquired on a BD FACSCaliburTM flow cytometer with Cellquest software (BD Bioscience, San Jose, CA, USA) and analyzed with FlowJo version 10.1 (FlowJo, LLC, Ashland, OR, USA).

Prewaning behavior analysis in offspring

Neuromotor tests were performed at postnatal days (PND) 5, 9, and 13 to assess the impact of maternal CD8⁺ T cell depletion on neurodevelopment in offspring. Negative geotaxis and cliff aversion tests were performed as previously described.²⁴

Histochemistry of fetal brains

Brains were collected on E17 six hours after surgery and fixed in 4% paraformaldehyde overnight at 4°C. Fixed tissues were immersed in 30% sucrose until saturated and then cryosectioned (20 μ m thickness). Routine Nissl staining was performed to evaluate the morphological change of cortical neurons in fetal brains. All photographs used for quantification were taken with Zeiss AxioPlan 2 Microscope System (Jena, Germany) attached to a Canon EOS Rebel Camera (Tokyo, Japan) through a 100-objective lens. Neurons were counted (field of view) based on Nissl staining using Image J (v1.48, <http://imagej.nih.gov/ij/>, National Institute of Health, Bethesda, MD, USA) on 10 randomly chosen fields in the frontal cortex. The neurons were identified by a large cell body, or perikaryon, containing a large, pale nucleus with a prominent dark nucleolus. The experiments were repeated five times for all groups.

Gene expression in placentas

Quantitative polymerase chain reactions (qPCR) were completed based on protocols described by Manangeeswaran et al.²⁸ Placentas were collected six hours after surgery and fresh frozen on dry ice, followed by long-term storage at -80°C. For each placenta, 2 μ g of RNA were used for complementary (c)DNA synthesis in a 40 μ L reaction, using BioRad iScriptTM cDNA Synthesis Kit (No. 170-8891, Bio-Rad, Hercules, CA, USA). A TaqMan[®] Mouse Immune Array v2.1 (No. 4365297, ThermoFischer Scientific, Waltham, MA, USA) was used to evaluate 96 immune markers. To carry out this array, 100 μ L of 2x iTaq Super Mix (Bio-Rad, Hercules, CA, USA, No. 172-5134) were diluted with 60 μ L of water and the 40 μ L of the prepared cDNA. Array analyses were performed with the Quant Studio 12K Flex Real-Time PCR System (ThermoFischer Scientific, Waltham, MA, USA) for 40 cycles. The ThermoFischer Connect cloud system (Waltham, MA, USA) was used for data storage. Afterwards, individual TaqMan[®] (ThermoFischer Scientific, Waltham, MA, USA) gene assays were run for confirmatory analysis. The primers used were CCL3 (Catalog Number Mm.PT.58.29283216), Actin (Cat. No. Mm.PT.39a.1), GADPH (Cat. No. Mm.PT.391.1), TNF (Cat. No. Mm.PT.58.12575861), IL1 β (Cat. No. Mm.Pt.58.44004828), CCL5 (Cat. No. Mm01302427-m1), CXCL10 (Cat. No. Mm00445235 m1), and 18S RNA (Cat. No.

1411057). CCL3, Actin, GAPDH, TNF, IL1 β were all from Integrated DNA Technologies (Coralville, IA, USA), and CCL5, CXCL10, and 18S RNA came from Applied Biosystems (ThermoFischer Scientific, Waltham, MA, USA).

Statistical analyses

Categorical data were analyzed using either Chi-squared test or Fisher's exact test. Continuous data were tested for normality and outliers were identified using Grubb's test. Student's t-test and One-Way Analysis of Variance (ANOVA) were used for parametric data. Mann-Whitney U and Kruskal-Wallis tests were used for non-parametric data. Repeated measures of littermates for neurodevelopment tests were analyzed using generalized estimating equations (SPSS, IBM Analytics, Armonk, NY, USA). Gene expression data were normalized to the geometric mean of six reference genes and analyzed by One-Way ANOVA using qBase+ software (Biogazelle, Marlborough, MA, USA). All other analyses were performed in Prism 5 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Validation of CD8⁺ T cell depletion and flow cytometry antibodies

In the presence of depleting antibodies, CD8⁺ antigen may become masked, preventing detection by flow cytometry. We tested different clones of depletion and detection antibodies to find compatible pairs to allow accurate reporting of CD8⁺ T cell depletion. White blood cells prepared from CD8⁺ T cell non-depleted (non-depleted) mice were stained with CD3⁺, CD4⁺, CD8⁺, and CD45⁺ antibodies in the presence or absence of blocking CD8⁺ antibodies. T cells were identified based on morphology (Figure 1A) and CD3⁺ staining. Frequency of CD8⁺ T cells and median fluorescence intensity (MFI) of CD8⁺ T cells was determined in the absence of blocking antibodies (Figure 1B). When blocked with anti-CD8⁺ clone 53-6.72, CD8⁺ T cells had a 10-fold reduction in MFI, but were still easily identified compared to T CD8⁻ cells (Figure 1C). When blocked with clone YTS169.9, no CD8⁺ T cells were identified (Figure 1D). We used the combination of clone 53-6.72 for maternal CD8⁺ T cell depletion and clone 2.43 for detection by flow cytometry, to assess CD8⁺ T cell depletion in peripheral blood of mice, as done previously.²⁹ The frequency of CD8⁺ T cells in CD8⁺ T cell-depleted (depleted) mice was reduced 80% compared to non-depleted control mice (Figure 2).

Maternal CD8⁺ T cell depletion did not prevent inflammation induced preterm birth

To determine if CD8⁺ T cells alone contribute to preterm birth, we depleted CD8⁺ T cells and induced preterm delivery by intrauterine LPS administration. Our results show that maternal CD8⁺ T cell depletion, despite a slight decrease, did not significantly reduce the preterm birth rate (Table I).

Maternal CD8⁺ T cell depletion prevented cortical neuronal injury

While CD8⁺ T cell depletion did not affect the preterm birth rate, we sought to determine if CD8⁺ T cells played a role in perinatal brain injury. On E17, six hours after administration of intrauterine LPS, we collected fetal brain tissue from depleted and non-depleted dams. Neurons were identified by Nissl staining and changes in neuronal organization and number

were assessed. In intrauterine LPS-exposed fetal brains, there were evidently dead neurons in the cortex with rounded cell shape and condensed nuclei. In maternal CD8-depleted fetal brains with or without LPS exposure, the morphology of neurons was similar to that of the PBS control (Figure 3A). Nissl counting demonstrated that there was a significant decrease of neurons in the LPS group compared to both the PBS control group ($p < 0.001$, ANOVA with Bonferroni post hoc test) and the maternal CD8⁺ T cell-depleted LPS group ($p < 0.05$, ANOVA with Bonferroni post hoc test) (Figure 3B).

Maternal CD8⁺ T cell depletion prevented adverse neuromotor outcomes in offspring exposed to IUI

A total of 23 litters were tested. Similar to our previous studies, LPS-exposed pups displayed slower performance on neuromotor activity compared to PBS controls on the negative geotaxis and cliff aversion tests. Maternal CD8⁺ T cell depletion significantly improved neuromotor behavior (Figure 4).

qPCR confirms that positive effects of CD8⁺ T cell depletion on improved neurobehavioral outcomes may be due to changes in levels of inflammatory molecules

Immune arrays demonstrate that significant changes in other inflammatory molecules were observed, primarily in chemokines and receptors (36.6%) and in pro-inflammatory cytokines (37.0%). All other groups showed significance in $< 30.0\%$ of changes (Table II). The confirmatory results from single-target qPCR showed a significant difference in the levels of chemokines CCL3, CXCL10, and CCL5 between placentas of depleted and non-depleted mice. With CD8⁺ T cell depletion, levels of CCL3 and CXCL10 are decreased in placentas from LPS-exposed mice, whereas levels of CCL5 are increased (Figure 5A). Although not significant, we saw similar decreasing trends in levels of tumor necrosis factor (TNF) and interleukin (IL)-1 β in placentas from LPS-exposed depleted and non-depleted mice (Figure 5B).

DISCUSSION

For the first time, our study demonstrates that depletion of maternal CD8⁺ T cells prevents fetal cortical neuronal injury following LPS-induced IUI. In addition, we determine that maternal CD8⁺ T cell depletion results in neuromotor improvement in the IUI-exposed neonates. Moreover, maternal CD8⁺ T cell depletion does not confer harm to the fetal cortical neurons nor to neuromotor behavior of the neonate in the absence of IUI. However, maternal CD8⁺ T cell depletion does not reduce preterm birth in this model.

Previously, our group has completed research in the field of immunoperinatology and investigated extensively how the maternal immune system and immune response may impact neonatal outcomes and potentially prevent long term sequelae to the fetus.^{19,21,29} We reported the effects of maternal N-acetyl L-cysteine dendrimer nanoparticle (DNAC) on preterm birth and perinatal brain injury in the same model of IUI.¹⁹ The administration of DNAC significantly reduced the preterm birth rate by decreasing placental inflammation. DNAC was effective at preventing preterm labor, but also improved the performance of offspring on neurobehavioral tests. We also noticed that in our mouse model of IUI, there

was an increase in the levels of CD8⁺ T cell infiltration into the placenta. DNAC treatment prevented this increase in T cell trafficking, and we hypothesized that this prevention contributed to the improvement of fetal neurodevelopment.¹⁹ Additionally, recently Wang et al. suggested that regulatory CD4⁺ T cells, via adoptive transfer, could act to decrease IUI caused by LPS, thereby improving the response in the fetal brain.³⁰ Collectively, these data support that an optimal balance of CD4⁺ Tregs to CD8⁺ effector T cells at the feto-maternal interface is associated with normal fetal brain development and alterations of this balance may result in perinatal brain injury. We thus decided to perform this current study to learn more about the mechanism by which infiltration of CD8⁺ T cells contributes to perinatal brain injury, and the effects of a blockade of this mechanism.

CD8⁺ T cells are extremely important to adaptive immunity. Comprised of both cytotoxic effector and memory T cells, they effectively kill other cells by leading to the production of cytokines and other cytotoxic molecules, if the cell expresses the antigen that they are specific to. These cytokines and molecules include TNF- α , interferon- γ , IL-1 β , perforin, and granzymes.^{31,32} Other groups demonstrate that CD8⁺ and CD4⁺ T cells comprise approximately 3% of leukocytes in the E8.5 decidua, but the function of decidual T cells is unknown. A study by Erlebacher et al. supports the notion that there is a lack of fetal/placental specificity for decidual CD8⁺ T cells.¹⁴ This is further corroborated by the findings of Tilburgs et al.¹⁵

Our group previously showed, utilizing mathematical models, that TNF-7agr; but not IL-1 β is associated with preterm birth.³³ Our previous studies showed that blockage of IL-1R did not prevent preterm birth despite successfully preventing cortical brain damage.¹¹ Other researchers demonstrated a clear role of IL-1 β in uterine inflammation and preterm birth which was attenuated with administration of non-competetive IL-1R modulator.³⁴ Of note, CD8⁺ T cells express IL-1R, and increases in IL-1 leads to enhanced effector function and trafficking of CD8⁺ T cells.³⁵ In addition, elevated TNF- α and IL-1, as shown by Bjarnadottir et al, can inhibit a subgroup of CD8⁺ T cells with suppressor function.³⁶ In the current study, we detected a trend of decreased TNF- α and IL-1 β , in IUI exposed CD8⁺ T cell depleted dams compared to non-depleted dams, although did not reach statistical significance.

CCL3, also known as macrophage inflammatory protein-1 α , aids in chemotaxis of various immune cells, including neutrophils, macrophages and lymphocytes, and elevated levels of CCL3 are associated with neuroinflammation of the offspring in other maternal immune activation models.³⁷ CXCL10 is a known chemoattractant for activated T cells, and is also involved in the creation of effector T cells and transportation to locations of tissue inflammation. It then binds to CXCR3, a G-protein coupled receptor, which is found on activated T cells and additionally plays a role in T cell function.³⁸ In placenta, CXCL10 is a marker for chronic inflammation and can be detected in fetal blood when associated with fetal inflammatory response syndrome.³⁹ CCL5, or RANTES, contributes to the recruitment of the peripherally induced-Tregs to the placenta, especially under inflammatory conditions, possibly as a natural response to infection.^{40,41} In our study, LPS exposure led to increases in CCL3 and CXCL10 but CD8⁺ T cell depletion prior to LPS exposure resulted in decreases in these chemokines. As expected under inflammatory conditions, we detected

increased expression of CCL5 in both depleted and non-depleted dams compared to controls. Our results suggest that restoring the dysregulation of Treg/CD8⁺ T cells at the fetomaternal interface by maternal CD8⁺ T cell depletion and limiting inflammation can improve neurodevelopmental outcomes.

CD4⁺/CD8⁺ ratios were found to be important in multiple areas of medicine and were even involved in the prognosis and treatment of human immunodeficiency virus (HIV), in particular. For HIV patients, it is known that a low CD4⁺/CD8⁺ ratio correlates with a worse prognosis. In a study by Mussini et al., it was also noticed that patients with lower CD4⁺/CD8⁺ ratios were more likely to experience non-acquired immune deficiency syndrome (AIDS)-defining events, including cardiovascular events, hepatic events, and acute kidney injuries. The researchers suggested that perhaps this association occurred due to persistent activation of the immune system, and that the CD4⁺/CD8⁺ ratio could act as a marker for this.⁴² Serrano-Villar et al. found similar results in their cohort study. In their patients, a low CD4⁺/CD8⁺ ratio was associated with increased CD8⁺ T cell activation, as well as other inflammatory markers. This decreased ratio – similar to levels typically found in older individuals – was also linked to immunosenescence. Patients with a lower CD4⁺/CD8⁺ ratio were again found to be at increased risk for non-AIDS-defining events, but they hypothesized that this may have different implications depending on the initial levels of CD4⁺ T cells. Those patients with lower levels of CD4⁺ T cells were more likely to be impacted by immunodeficiency, while the harm in individuals with higher levels of CD4⁺ T cells was likely caused by inflammation.⁴³ Both research teams agreed that the CD4⁺/CD8⁺ ratio in patients with HIV could serve as an important clinical tool for prognosis and recommendations for treatments or therapies.^{42,43}

On the other hand, this lowered CD4⁺/CD8⁺ T cell ratio has a positive effect in diagnoses of various types of cancer. It has been shown that increased intratumoral infiltration of T cells correlates with better prognoses and longer survival rates in women with ovarian cancer.⁴⁴ Furthermore, in a group of women who survived for 60 months or longer, the ratio of CD4⁺/CD8⁺ T cells in intraepithelial cells was significantly lower than that of women surviving less than 18 months.⁴⁵ Sato et al. also found that with a high ratio of CD8⁺/Treg cells, the risk of death from epithelial ovarian cancer decreased by almost 70%.⁴⁶ Decreased ratios of CD4⁺/CD8⁺ T cells were also shown to improve colorectal cancer survival rate.⁴⁷

Many researchers have demonstrated that a high level of CD8⁺ T cells improves cancer survival rate by causing CD8⁺ T cell infiltration of tumors and limiting tumor progression by immunomodulation, whereas infiltration of Tregs leads to local immunosuppression and resultant tumor progression.⁴⁸ On the contrary, in mice and humans, development of a healthy fetoplacental barrier, sustaining a healthy pregnancy, and having normal neurodevelopmental outcomes require ongoing adjustments to the maternal immune system, favoring immune-tolerance. In healthy pregnancies, the subtypes and the functional states of CD8⁺ effector and effector memory T cells differ between peripheral blood and placenta.⁴⁹ It is additionally possible to prevent some adverse pregnancy outcomes by reconstitution of CD4⁺ T cells.¹⁸ We propose that alterations to this balance, such as greater levels of CD8⁺ T cells that infiltrate the placenta, increase perinatal brain injury in instances of IUI. The role of activation, differentiation, migration, and trafficking of CD8⁺ T cells have been shown in

ovarian cancer and other tumors. Through our mouse model of IUI, we demonstrate that IUI increases trafficking of CD8⁺ T cells to the placenta, which in turn increases perinatal brain injury,¹⁹ and that maternal CD8⁺ T cell depletion rescues this phenotype. The subtypes and functional states of these placenta trafficking CD8⁺ T cells will require further study.

A potential worthwhile preventative method for perinatal brain injury caused by heightened CD8⁺ T cell ratios, and CD8⁺ T cell infiltration into the placenta, could be through maternal immunomodulation. This way, treatment can be performed on the mother, without needing to cross the placenta to the fetus at all, thus keeping the fetus safe from perinatal brain injury and any adverse effects of treatment. Indeed, in a 2014 study, our group reported that administration of mesenchymal stem cells (MSC) was able to block both preterm birth and perinatal brain injury, perhaps due to a regulation of cytokines, particularly IL-10. Maternal administration of MSCs was found to increase production of IL-10, possibly inhibiting CD8⁺ T cell activation and infiltration into the placenta.²⁹

As previously mentioned, Bizargity et al. demonstrated that LPS-induced preterm birth could not be prevented in T and B cell deficient Rag1^{-/-} mice and only worsened the preterm birth effect of LPS in the model.¹⁸ They did not report neurodevelopmental outcomes in this study. Using the findings in the current study, further studies should investigate the specific mechanisms that CD4⁺ T cells and Tregs play in mediating LPS-induced preterm birth as Bizargity et al. propose. The research by Bizargity et al. suggested an immunological component to the mechanism of preterm birth while the current study demonstrates that CD8⁺ T cells are not involved in this component. Although the reduction in preterm birth rate that we observed was not statistically significant, there was a reduction of approximately 20%, which may be clinically significant. More research will be needed to investigate upon this further. Additionally, as our study for the first time presents evidence for a mechanism of perinatal brain injury mediated by CD8⁺ T cells, we intend to perform further research into the specific subtypes of CD8⁺ T cells that may be involved.

We speculate that alterations of maternal CD8⁺ T cell proportions, changes in their activation, suppression patterns, and trafficking between peripheral blood and fetomaternal interface – due to IUI – may be associated with perinatal brain injury and adverse neurodevelopmental outcomes in the neonate. There is an urgent clinical need for safe, effective, and timely maternal immune-modulatory therapies to prevent these adverse neonatal outcomes associated with IUI. Clinically, accurate diagnosis of the exact timing of IUI is very challenging and at times not possible. Since maternal CD8⁺ T cell depletion confers protection from IUI-induced perinatal brain injury and does not have adverse fetal and neonatal effects in the absence of IUI, we suggest that it may be possible to translate CD8⁺ T cell immune-modulatory treatments universally to all at-risk pregnant women in the future as a preventative measure.

CONCLUSIONS

In conclusion, we demonstrate that maternal CD8⁺ T cells mediate perinatal brain injury, but not preterm birth, in our mouse model of IUI. This study provides evidence that maternal depletion of CD8⁺ T cells improves neurobehavioral outcomes of offspring, prevents cortical

neuronal injury, and underscores the implications of the maternal immune response to IUI. Maternal CD8⁺ T cell depletion does not, however, reduce the rate of preterm delivery. Such evidence suggests that CD8⁺ T cell mediation of perinatal brain injury is separate from preterm birth mechanisms. These studies highlight the importance of the field of immunoperinatology. Clinically, treating adverse maternal immune responses through immunomodulation would potentially alleviate adverse neonatal outcomes, thus preventing irreversible changes on the fetal brain, without direct intervention to the fetus. Current findings will guide our future studies to focus on the specific mechanism of how CD8⁺ T cells effect neurobehavioral outcomes and the specific subtypes involved.

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Figure 1. Validation of anti-CD8 depletion and flow cytometry antibodies

Peripheral blood cells were incubated with depletion antibodies before surface staining and flow cytometry analysis to ensure compatibility of antibodies to monitor depletion.

Lymphocytes were selected based on morphology on side and forward scatter (A), then sequentially gated on CD3⁺, CD4⁺, CD8⁺ T cells. Samples stained with anti-CD8 clone2.43 without blocking antibodies had normal distribution of CD4⁺ and CD8⁺ T cells (B).

Blocking surface staining with anti-CD8 clone53-6.72 decreased median fluorescence intensity of CD8⁺ T cells but not frequency compared to non-blocked (C). Blocking with anti-CD8 cloneYTS169.9 eliminated clone2.43 staining completely (D).

Figure 2. Maternal systemic CD8⁺ T cell depletion

CD8⁺ T cell depletion with clone 53-6.72 significantly reduced the number of peripheral CD8⁺ T-cells compared to non-depleted controls ($p < 0.001$; Student's T-test; Depleted: 6.1 ± 1.3%, $n = 18$; Non-depleted: 31.1 ± 2.1%, $n = 7$). Data are reported as mean ± standard error.

Figure 3. CD8⁺ T-cell depletion prevents cortical neuron injury after intrauterine lipopolysaccharide (LPS) exposure

(A) Nissl staining was performed on fetal brains collected six hours after LPS exposure. Ten fields were chosen at random from the frontal cortex and neurons were quantified. (B) LPS exposure significantly reduced the number of neurons/field compared to phosphate-buffered saline (PBS) controls (***p*<0.001, analysis of variance (ANOVA) with Bonferroni post hoc test). Fetal brains from CD8-depleted dams (DEP) with LPS exposure had significantly more neurons than LPS alone (**p*<0.05, ANOVA with Bonferroni post hoc test).

Figure 4. Maternal CD8⁺ T cell depletion prevents neuromotor deficits after lipopolysaccharide (LPS) exposure *in utero*

Offspring neurodevelopment was assessed at postnatal days (PND) five, nine, and 13.

Maternal CD8⁺ T-cell depletion prior to LPS exposure significantly improved offspring performance on (A) the cliff aversion test and (B) the negative geotaxis test (*p<0.05, **p<0.01, ***p<0.001 compared to LPS; one-way analysis of variance with Bonferroni multiple comparison test). Phosphate-buffered saline (PBS): n=6, 60 pups; LPS: n=3, 24 pups; PBS+CD8⁺ T cell depletion (DEP): n=8, 92 pups; LPS+DEP: n=9, 56 pups; non-surgical control: n=6, 66 pups.

Figure 5. Changes in inflammatory molecules occur due to maternal CD8⁺ T cell depletion Mice were treated with lipopolysaccharide (LPS) to induce intrauterine inflammation. Placentas were collected, and quantitative polymerase chain reactions were performed. Maternal CD8⁺ T cell depletion prior to LPS exposure (A) significantly decreased levels of chemokines CCL3 and CXCL10, and increased levels of CCL5. Levels of tumor necrosis factor and interleukin-1β were decreased (B), similarly to CCL3 and CXCL10, though not significant (*p<0.05, **p<0.01, ***p<0.001; analysis of variance; phosphate-buffered saline (PBS) (n=5), LPS (n=7), PBS+CD8⁺ T cell depletion (DEP) (n=7), LPS+DEP (n=5)).

Table IMaternal CD8⁺ T-cell depletion does not affect preterm birth rate

Treatment Group (Number of litters)	Preterm Delivery n (%)
Phosphate-buffered saline (PBS) (3)	0 (0.0)
Lipopolysaccharide (LPS) (11)	8 (72.3)
LPS+CD8 ⁺ T cell depletion (DEP) (29)	15 (51.3)
PBS+DEP (15)	0 (0.0)

Fisher's exact test, $p > 0.05$

Table II

Gene expression profile.

Relative quantification: fold change to reference group (PBS) Endogenous control: Actinβ, 18S and GAPDH						
Fold change				P value		
Sr. No.	Gene Symbol	LPS	LPS+DEP	PBS+DEP	LPS	PBS+DEP
<i>Chemokines and receptors</i>						
1	CCL2	6.229	9.67	0.895	0.002	0.001
2	CCL3	31.077	29.335	0.94	0	0
3	CCL5	17.027	22.654	0.443	0	0.001
4	CCL19	1.319	1.465	1.653	0.41	0.287
5	CCR2	1.094	1.095	0.741	0.737	0.74
6	CCR4	0.952	1.478	1.624	0.913	0.415
7	CCR7	0.614	0.63	0.752	0.21	0.257
8	CXCL10	49.586	33.85	0.711	0	0
9	CXCL11	34.82	47.914	1.03	0.001	0.001
10	CXCR3	0.906	0.616	0.818	0.896	0.514
<i>Pro-inflammatory cytokines</i>						
11	IL1α	4.562	6.397	1.279	0.001	0.001
12	IL1β	9.604	5.932	1.031	0	0.001
13	IL2Ra	0.37	0.463	0.231	0.183	0.296
14	IL6	12.393	15.541	0.924	0.005	0.003
15	IL17	1.451	1.062	0.501	0.648	0.941
16	IL18	2.016	2.852	2.489	0.029	0.013
17	TNF	5.417	2.837	0.354	0.006	0.051
18	NOS2	0.773	0.612	0.754	0.218	0.07
19	PTGS2	1.74	1.535	0.849	0.099	0.232
<i>Th1 cytokines</i>						

Relative quantification: fold change to reference group (PBS) Endogenous control: Actin β , 18S and GAPDH						
Sr. No.	Gene Symbol	Fold change				P value
		LPS	LPS+DEP	PBS+DEP	LPS	
20	IL15	1.489	1.995	1.63	0.096	0.059
21	IL12 α	1.14	0.886	0.934	0.758	0.821
22	IL12 β	2.81	4.534	1.195	0.063	0.012
23	IFNG	0.019	0.014	0.015	0.333	0.299
24	IL2	0.752	-	0.594	NaN	NaN
25	TBX21	0.579	0.503	1.037	0.22	0.275
<i>Th2 cytokines</i>						
26	IL4	1.324	3.018	-	0.596	0.128
27	IL5	0.6	-	0.788	NaN	NaN
28	IL9	-	-	-	NaN	NaN
29	IL10	2.22	2.509	1.343	0.124	0.103
30	IL13	0.161	0.9	-	0.442	7.57E-01
<i>Immune cell surface antigens</i>						
31	CD19	0.292	-	1.085	0.398	NaN
32	CD28	0.459	0.565	0.462	0.161	0.334
33	CD34	0.674	0.751	0.648	0.216	0.344
34	CD38	0.571	0.874	0.586	0.351	0.821
35	CD3E	0.253	0.369	0.565	0.03	0.046
36	CD4	0.908	0.86	0.877	0.837	0.787
37	CD40	2.006	2.727	0.916	0.053	3.40E-02
38	CD40LG	0.93	1.734	-	NaN	NaN
39	CD68	1.053	1.226	1.082	0.832	0.551
40	CD80	2.08	2.109	0.796	0.103	0.184
41	CD86	2.196	2.96	1.383	0.013	0.014
42	CD8 α	0.535	1.272	0.975	0.179	7.99E-01
43	PTPRC	1.205	1.11	0.535	0.426	0.761

Relative quantification: fold change to reference group (PBS) Endogenous control: Actin β , 18S and GAPDH						
Sr. No.	Gene Symbol	Fold change				P value
		LPS	LPS+DEP	PBS+DEP	LPS	
44	ICOS	0.328	0.294	0.239	0.221	0.205
45	H2-Ea	0.412	0.853	0.702	0.289	0.864
46	H2-E β 1	2.279	5.176	11.629	0.647	0.277
47	CTLA4	1.422	2.006	0.692	0.606	0.38
<i>Endothelium molecules</i>						
48	ECE1	0.638	0.413	0.537	0.198	0.077
49	EDN1	1.599	1.416	1.399	0.334	0.498
50	SELE	0.753	0.644	0.825	0.38	0.207
51	SELP	3.535	3.843	0.419	0.012	0.014
52	VCAM1	1.783	1.462	0.899	0.205	0.41
53	VEGFa	1.044	0.915	1.137	0.891	0.807
54	AGTR2	0.893	0.62	3.249	0.801	6.83E-01
<i>Transcription factors and regulators of transcription</i>						
55	SMAD3	1.263	1.178	1.182	0.637	0.772
56	SMAD7	0.834	0.651	0.84	4.04E-01	0.189
57	SOCs1	4.538	4.015	2.543	0	0.002
58	SOCs2	2.331	3.251	1.872	0.006	0
59	STAT1	0.873	0.989	0.725	5.44E-01	0.965
60	STAT3	1.175	1.419	0.845	0.422	0.07
61	STAT4	1.065	1.29	1.097	0.813	0.43
62	NF- κ B1	1.692	1.408	0.805	0.001	0.233
63	NF- κ B2	1.829	1.616	0.543	0.107	0.234
64	CSF1	1.562	1.247	0.748	0.015	3.83E-01
65	CSF2	2.586	2.69	0.632	0.031	0.031
66	CSF3	28.596	17.664	1.225	0	0
67	IL3	-	0.04	0.018	NaN	0.118

Relative quantification: fold change to reference group (PBS) Endogenous control: Actin β , 18S and GAPDH						
Sr. No.	Gene Symbol	Fold change				P value
		LPS	LPS+DEP	PBS+DEP	LPS	
68	IL7	0.66	1.114	0.514	0.432	0.862
<i>Apoptosis related</i>						
69	BAX	0.994	1.239	1.285	0.977	0.409
70	BCL2	0.66	0.854	0.962	0.02	0.648
71	BCL2L1	1.273	1.216	1.423	0.498	5.86E-01
72	FAS	2.343	3.161	2.526	0.274	0.157
73	FASL	0.221	0.274	0.207	0.141	0.198
74	LT α	-	2.044	-	NaN	NaN
75	STAT6	0.886	0.867	0.894	0.494	5.72E-01
<i>Anti-inflammation</i>						
76	TGFB1	0.731	0.61	0.51	9.40E-02	0.021
77	HMOX1	0.802	0.746	0.61	0.452	3.83E-01
<i>Cytotoxic molecules</i>						
78	GZMB	0.237	0.313	0.243	0.204	0.307
79	PRF1	0.231	0.207	0.138	0.206	0.191
<i>Others</i>						
80	B2M	0.831	1.154	1.153	3.67E-01	0.631
81	FN1	1.628	1.52	1.184	0.49	0.592
82	GUSB	0.726	0.73	0.725	0.178	0.196
83	HPRT1	0.502	1.245	0.57	0.172	0.663
84	SKI	0.95	0.766	0.905	0.805	0.477
85	TFR3	0.966	1.292	1.11	0.915	0.398
86	C3	1.065	0.602	0.577	0.926	0.441
87	COL4a5	0.84	1.184	1.24	0.58	0.534

Relative quantification: fold change to reference group (PBS) Endogenous control: Actin β , 18S and GAPDH									
Fold change									
Sr. No.	Gene Symbol	LPS	LPS+DEP	PBS+DEP	LPS	LPS+DEP	PBS+DEP	LPS	P value
88	IKBKB	0.916	0.822	0.823	0.746	0.596	0.649		
89	LRP2	0.924	1.448	2.116	0.908	0.603	0.306		
90	PGK1	0.914	0.99	0.93	0.736	0.977	0.784		
91	TNFRSF18	0.889	0.991	1.588	0.716	0.969	0.19		
92	CYP1A2	1.447	2.122	3.154	6.32E-01	0.385	2.65E-01		
93	CYP7A1	-	-	-	NaN	NaN	NaN		

NaN = not a number – undetectable results, Sr. No. = serial number, phosphate buffered saline (PBS) (n=5), lipopolysaccharide (LPS) (n=7), LPS+CD8 depletion (DEP) (n=7), PBS+DEP (n=5).