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Placental Macrophages: A Window Into Fetal Microglial Function in Maternal Obesity

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Abstract

Fetal placental macrophages and microglia (resident brain macrophages) have a common origin in the fetal yolk sac. Yolk-sac-derived macrophages comprise the permanent pool of brain microglia throughout an individual's lifetime. Inappropriate fetal microglial priming may therefore have lifelong neurodevelopmental consequences, but direct evaluation of microglial function in a living fetus or neonate is impossible. We sought to test the hypothesis that maternal obesity would prime both placental macrophages and fetal brain microglia to overrespond to an immune challenge, thus providing a window into microglial function using placental cells. Obesity was induced in C57BL/6J mice using a 60% high-fat diet. On embryonic day 17.5, fetal brain microglia and corresponding CD11b+ placental cells were isolated from fresh tissue. Cells were treated with media or lipopolysaccharide (LPS). Tumor necrosis factor-alpha (TNF-α) production by stimulated and unstimulated cells was quantified via ELISA. We demonstrate for the first time that the proinflammatory cytokine production of CD11b+ placental cells is strongly correlated with that of brain microglia (Spearman's $\rho = 0.73$, $p = 0.002$) in the setting of maternal obesity. Maternal obesity-exposed CD11b+ cells had an exaggerated response to LPS compared to controls, with a

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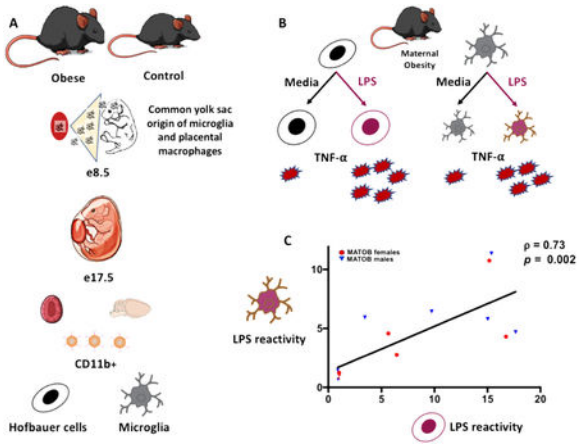
5.1-fold increase in TNF-α production in placentas (p=0.003) and 3.8-fold increase in TNF-α production in brains (p=0.002). In sex-stratified analyses, only male obesity-exposed brains and placentas had significant increase in TNF-α production in response to LPS. Taken together, these data suggest that maternal obesity primes both placental macrophages and fetal brain microglia to overproduce a proinflammatory cytokine in response to immune challenge. Male brain and placental immune response is more marked than female in this setting. Given that fetal microglial priming may impact neuroimmune function throughout the lifespan, these data could provide insight into the male predominance of certain neurodevelopmental morbidities linked to maternal obesity, including cognitive dysfunction, autism spectrum disorder, and ADHD. Placental CD11b+ macrophages may have the potential to serve as an accessible biomarker of aberrant fetal brain immune activation in maternal obesity. This finding may have broader implications for assaying the impact of other maternal exposures on fetal brain development.

Graphical abstract



Keywords

maternal obesity; fetal brain; microglia; placenta; Hofbauer cells; inflammation

1. Introduction

One in three women in the United States now starts pregnancy obese (Deputy, Dub, and Sharma 2018), with slightly lower but comparable rates of pre-pregnancy obesity in Europe and around the world in developed settings (WHO 2014). Human epidemiologic studies have identified long-term neurodevelopmental morbidities in offspring born to obese mothers, including an increased risk of autism spectrum disorder, cognitive deficits, attention deficit hyperactivity disorder (ADHD), anxiety and depression, and disordered eating (Edlow 2017a). Obesity is a state of chronic low-level immune activation (Gregor and Hotamisligil 2011). Pregnancy may augment this low-grade metabolically-induced inflammation, as normal pregnancy is associated with increased levels of certain pro-inflammatory cytokines (Christian and Porter 2014). In addition, levels of circulating pro-inflammatory cytokines such as IL-6 and CRP increase in pregnancy with increasing maternal BMI (Aye et al. 2014; Friis et al. 2013; Christian and Porter 2014). In normal

pregnancy, the placenta is relatively protected from maternal peripheral inflammation, with both maternal decidual and placental macrophages typically maintained in an anti-inflammatory state to enable immune tolerance of the fetus (Bolton and Bilbo 2014). This placental immune quiescence is likely deranged in the setting of maternal obesity, with activation of pro-inflammatory pathways in the placenta and histopathological evidence of placental inflammation described in the literature (Aye et al. 2014; Bar et al. 2012; Challier et al. 2008). Animal model studies have also demonstrated brain inflammation in the offspring of obese females, with developmental neuroinflammation identified as an important candidate mechanism underlying postnatal cognitive and behavioral morbidities in offspring of obese dams (Hanamsagar and Bilbo 2016; Bilbo and Tsang 2010; Edlow et al. 2016; Kang et al. 2014; White et al. 2009). However, relatively little is known about how placental inflammation might correlate with fetal brain inflammation in the setting of maternal obesity.

In considering the potential impact of maternal obesity on fetal brain inflammation, the logical starting point is to assess the functional impact on microglia, the resident brain immune cells. Microglia function not only as immune sentinels, they also play a key role in normal brain development and brain homeostasis (Bilbo et al. 2018; Koyama and Ikegaya 2015; Bilimoria and Stevens 2015; Paolicelli et al. 2011; Rakic and Zecevic 2000; Streit 2001). Aberrant microglial activation and subsequent pro-inflammatory cytokine production *in utero* may result in abnormal development, with the developing brain particularly vulnerable to inflammatory disruption during critical windows (Bilbo and Schwarz 2009, 2012; Deverman and Patterson 2009;Urakubo et al. 2001; Yu et al. 2004; Rodier 1980).

Microglia are of particular interest in evaluating correlations between brain and placental inflammation, given that subpopulations of placental macrophages and brain microglia have a common origin in the fetal yolk sac (Ginhoux et al. 2010; Reyes, Wolfe, and Golos 2017; Takahashi et al. 1991). Fate-mapping studies have demonstrated that these primitive yolk-sac-derived macrophages colonize the developing brain as early as e 9.5 in rodents, entering the parenchyma via the blood stream and ventricles (Ginhoux et al. 2010). Microglia continue to proliferate throughout the first postnatal weeks in humans and rodents, and form a self-renewing pool that lasts throughout the lifespan, without contribution from peripheral hematopoietic cells in the periphery under normal conditions (Ajami et al. 2007; Ginhoux et al. 2010).

Given the embryonic origins of adult microglia, fetal exposure to inflammation–as may occur in the setting of maternal obesity—can have enduring consequences for microglial function across the lifespan. Aberrant microglial activation or priming has been implicated in a number of later-onset neurodevelopmental morbidities, including autism spectrum disorder, schizophrenia, obsessive compulsive disorder, and neurodegenerative conditions such as Alzheimer's or Parkinson's Disease (Bilbo et al. 2018; Bilimoria and Stevens 2015; Koyama and Ikegaya 2015; Perry and Teeling 2013). Despite the knowledge that maternal immune activation or inflammation in the setting of maternal obesity may be programming microglia to overrespond to immune challenge, there is no reliable way to directly assay the impact of maternal obesity on the developing brain. Unlike microglia, however, the placenta is accessible in pregnancy (via chorionic villus sampling) and immediately after delivery, so

if a subpopulation of placental cells could give information about microglial function in the setting of a maternal exposure such as obesity, this could inform evaluations of offspring risk for later development of neurodevelopmental morbidities. We therefore sought to determine whether placental macrophages could provide information about the function of brain microglia, and whether maternal obesity primed both placental macrophages and fetal brain microglia to overrespond to an immune challenge.

2. Materials and Methods

2.1 Animals and diet

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were placed on either a lard-based, high-fat diet containing 60% calories from fat (Research Diets D12492, New Brunswick, NJ), or a matched control diet containing 10% calories from fat (Research Diets D12450J), starting at 4-5 weeks of age. Diets were matched for protein, fiber, sucrose, and micronutrients (content information available at https://researchdiets.com). Dams were continued on their allocated diet for 12-14 weeks pre-breeding, and throughout pregnancy. Male C57BL/6J mice were fed the control diet. For females, obesity was defined as at least a 30% increase in weight compared to age-matched controls (Gallou-Kabani et al. 2007; Edlow et al. 2016). Mice were housed in same-sex groups of 4 throughout the pre-breeding feeding period, with *ad libitum* access to food and water. The colony was maintained at 22°C on a 12:12 hour light-dark cycle. Obese and lean females were paired with control males for breeding. Female breeders were examined daily during breeding, and the presence of a vaginal plug was defined as embryonic day 0.5 (e0.5). Males were separated upon observation of a vaginal plug. Females were weighed at pregnancy days 0 (P0), P10 and P15. All procedures were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

2.2 Tissue collection

On embryonic day e17.5, pregnant mice were euthanized with isoflurane followed by decapitation. E17.5 was selected as the timepoint for examination because the male testes begin to secrete testosterone at e18, while the ovaries remain quiescent in females. We thus selected a timepoint that would permit assessment of placental and brain immune function prior to sex hormone exposure, which would be imbalanced in male and female fetuses. Embryos were rapidly dissected from the uterine horns and placed in ice-cold phosphate buffered saline (PBS 1x). Theiler staging of embryos was performed to confirm embryonic day 17.5 at the time of sacrifice (Theiler 1989). Crown-rump lengths, fetal, and placental weights were recorded. Forebrains were rapidly dissected from skulls and corresponding/matched placentas were dissected from each amniotic sac. Sex genotyping was subsequently performed on tail snip DNA using real time PCR with specific probes for the *Sry* gene (Transnetyx, Cordova, TN). Only one to two fetuses per sex per litter were used for experiments.

2.3 Microglial and placental macrophage isolation

Isolated fresh forebrains and placentas were minced into small pieces using a sterile razor and homogenized in 5 mLs of enzyme digestion mix comprised of Hanks Balanced Salt

Solution without magnesium and calcium (HBSS, Gibco/ThermoFisher Scientific, Waltham, MA), 5% fetal bovine serum (FBS, ThermoFisher Scientific), Hepes 10M (Gibco/ ThermoFisher Scientific), collagenase A (2mg/mL, Roche, Indianapolis, IN), and DNaseI (Invitrogen, Carlsbad, CA). Tissue was incubated for 45 minutes in a 37°C water bath, and every 15 minutes samples were removed from the bath and passed through successively smaller glass Pasteur pipettes to ensure complete dissociation. Samples were then passed through a nylon filter and centrifuged at 1,200 rpm for 10 min at 4°C. Cell pellets were resuspended in 30% Percoll (GE Healthcare, Uppsala, Sweden) in 1X sterile phosphate buffered saline (PBS) prepared from isotonic Percoll (90% Percoll and 10% 10X PBS, Thermo Fisher Scientific, NY, USA). The 30% resuspension was then carefully underlayed with 70% Percoll in 1X PBS. Microglia and other mononuclear cells are known to accumulate at the interface of a 30%/70% Percoll gradient (Jin and Kim 2015). Samples were centrifuged at 1,400 rpm for 15 minutes at 23°C. Mononuclear cells were pipetted from the interface of the Percoll gradient, and centrifuged with a post-Percoll solution containing HBSS with calcium and magnesium, FBS, and Hepes 1M at 1200 rpm for 10 minutes at 4°C. Cells were then resuspended in MACS buffer and incubated with CD11b antibody-conjugated magnetic beads (MACS Miltenyi Biotec, San Diego CA) for 15 min at 4°C.

CD11b is a subunit of the complement receptor 3 complex (CD11b/CD18), also known as macrophage-1 antigen or Mac1. As an integrin family member, CD11b regulates leukocyte adhesion and migration, and may play a role in cell-mediated cytotoxicity, chemotaxis, and phagocytosis. CD11b is one of the most widely used microglial markers, and microglia are the only intra-parenchymal CNS cell type that express CD11b under normal conditions (Brandon 1995; Salter and Stevens 2017; Greter, Lelios, and Croxford 2015; Jeong et al. 2013). In the placenta, CD11b+ cells will be highly enriched for fetal macrophages, including both Hofbauer cells (fetal macrophages of yolk sac origin), and possibly fetal peripheral monocytes (Schliefsteiner et al. 2017; Sisino et al. 2013; Challier et al. 2008). After washing, cells were again centrifuged at 1200 rpm for 10 minutes at 4°C, resuspended in MACS buffer and then passed through nylon filters onto magnetic bead columns (LS columns with the MidiMACS separator, Miltenyi Biotec, San Diego CA) and CD11b positive (CD11b+) versus negative (CD11b−) populations were separated and placed on ice. Samples were centrifuged at 1200 rpm for 10 minutes at 4°C to isolate the final purified CD11b+ and CD11b− cells.

2.4 Ex vivo LPS stimulation

Purified CD11b+ and CD11b− cells were resuspended in neuro media, containing high glucose DMEM, L-glutamine, Pen Strep, N2 media supplement, sodium pyruvate, and 5μg/mL Forskolin. An aliquot of the cell suspension for each sample was treated with Trypan Blue in a 1:2 or 1:10 dilution, depending on cell density, and 10 μl of the treated cell suspension was applied to a standard hemacytometer with Neubauer rulings. Live cells were hand-counted in 3 large corner squares. Cell counts in the 3 quadrants were averaged for the final density determination. Total number of cells was calculated using the following formula: (average cell count over 3 quadrants) × (Trypan Blue dilution factor) × 10^4. Cells were plated in 96-well round-bottom plates and incubated with LPS (1250 ng/ml) or media

alone for 4 hours at 37°C, 5% CO2. The LPS dose was based on pilot dosing experiments, coupled with evaluation of the literature regarding response of rodent microglia to varying doses of LPS *ex vivo* (Frank et al. 2010; Frank et al. 2006; Njie et al. 2012; Turano, Lawrence, and Schwarz 2017; Williamson et al. 2011). We selected a dose at the highest end of the reported range for rodent microglia because studies reporting a dose-response for *ex vivo* treatment of microglia with LPS found that increasing the dose of LPS up to the range of 1000 ng/mL was associated with increased production of pro-inflammatory cytokines (Frank et al. 2010; Frank et al. 2006; Lee et al. 1993; Njie et al. 2012). In addition, there are limited data regarding response of fetal microglia to LPS (Cao et al. 2015; Lee et al. 1993; Schaafsma et al. 2017), but these data suggest that fetal microglia might have less robust production of pro-inflammatory cytokines in response to LPS (Cao et al. 2015) compared to neonatal or adult microglia (Frank et al. 2010; Frank et al. 2006; Njie et al. 2012; Turano, Lawrence, and Schwarz 2017; Williamson et al. 2011). The potential for decreased fetal microglial responsiveness to LPS provided additional rationale for selecting a high dose. For each condition, samples were run in duplicate. At the end of incubation, the plate was centrifuged at 2400 rpm for 5 minutes at 4°C to pellet cells. Supernatants were removed and frozen at −80°C until quantification of TNF-α production.

2.5 Quantification of TNF-α production by CD11b+ and CD11b− fetal brain and placental cells

Tumor necrosis factor alpha or TNF-α is a pro-inflammatory cytokine primarily secreted by activated macrophages. TNF-α has been demonstrated to be increased in maternal plasma and the fetal brain in the setting of maternal immune activation with Poly (I:C), and in the placenta, fetal plasma, and offspring brain in rodent models of maternal high-fat diet (Desai et al. 2013; Garay et al. 2013; Winther et al. 2018). TNF-α has been implicated in microglial regulation of synaptic maturation and plasticity (Salter and Stevens 2017), and increased serum and brain levels of TNF-α correlate with neurobehavioral morbidity in offspring (Xie et al. 2017; Winther et al. 2018).

TNF-α levels were quantified in the supernatant from each sample using a commercially available ELISA kit (Mouse TNF-alpha Quantikine ELISA kit, R&D Systems, Minneapolis, MN). TNF-α level for each sample was determined by averaging the replicates. Cytokine expression data were adjusted for sample cell counts using two different methods. For each sample, an “LPS reactivity ratio” was generated by dividing the mean TNF-α pg/mL signal in the LPS treatment wells by the mean TNF-α pg/mL signal in the media-treated wells. This ratio represents a fold-change increase in TNF-α after LPS treatment, and contains a built-in correction for the cellularity of the sample (for a given sample, the media-treated and the LPS-treated wells contained the same number of cells). For analyses evaluating the baseline production of TNF-α in the unstimulated/media condition, the raw TNF-α signal was adjusted to cell count per well for each sample, and data are expressed as pg/mL TNF-α produced per 100,000 cells.

2.6 Statistical analyses

Differences between obesity-exposed and control animals were determined using Student’s t-test (normally-distributed data) and Mann-Whitney testing (non-normally distributed data),

with significance defined as $p<0.05$. To help control for litter effects, only 1-2 fetuses per sex per litter were used for these experiments. In addition, we built a linear mixed effects model controlling for the random effect of dam, to evaluate the effect of maternal obesity on TNF-α production while accounting for any clustering by litter. Main effects and significant interactions between maternal obesity status and fetal sex on LPS reactivity of brain and placental cells were evaluated using 2-way ANOVA. Correlation between brain and placental cell reactivity to LPS was determined using Spearman's rank correlation. Given our own prior data demonstrating sex differences in embryo size and embryonic brain gene expression signatures in this model of maternal diet-induced obesity (Edlow et al. 2016; Edlow et al. 2017b), as well as the work of others demonstrating significant impact of fetal sex on placental structure, gene expression and function in the setting of obesity (Evans and Myatt 2017; Kim et al. 2014; Muralimanoharan et al. 2015), and impact of fetal sex on microglia in the developing brain (Hanamsagar et al. 2017; Schwarz, Sholar, and Bilbo 2012), we planned to evaluate for sex differences. A pre-specified sex-stratified analysis was therefore performed to evaluate for significant differences in LPS reactivity between obesity-exposed and control cells within the males and female study populations. Analyses were performed using GraphPad Prism (Prism 7, GraphPad Software, San Diego, CA) and StataIC (v.14, StataCorp LLC, College Station, TX). Data are expressed as mean ± SEM unless otherwise indicated.

3. Results

3.1 Diet-induced obesity model and study population characteristics

CD11b+ cells from brains and corresponding placentas were isolated from 18 control fetuses (9 females, 9 males) and 16 obesity-exposed fetuses (8 males, 8 females), reflecting 9 litters (N=4 obese, N=5 control, cells isolated from 1-2 fetuses/sex/litter). Dam pregnancy weight trajectories are depicted in Figure 1. Dam and fetus characteristics are depicted in Table 1. Obese dams were significantly heavier at breeding and at time of sacrifice on e17.5 than their lean counterparts. There were no statistically significant differences in litter size, fetal weight, or placental weight between study groups. Significantly more CD11b+ cells were isolated from brains of obesity-exposed fetuses compared to controls, per hemocytometer cell counts. There were no significant differences between groups in CD11b+ cells isolated from the placenta.

3.2 Responsiveness of microglia and placental CD11b+ cells to immune challenge in the setting of maternal obesity

Maternal obesity status did not significantly impact baseline TNF-α production by CD11b+ cells isolated from brains and corresponding placentas (Supplemental Figure 1). However, the pro-inflammatory response to LPS was significantly increased by maternal obesity, with maternal obesity priming CD11b+ cells in the brain and placenta to overrespond to LPS treatment compared to controls (Figure 2 A/B). In analyses combining males and females, maternal obesity-exposed CD11b+ cells had an exaggerated response to LPS compared to controls, with a 3.8-fold increase in TNF-α production in brains (p=0.002), and a 5.1-fold increase in TNF-α production in placentas (p=0.003), Figure 2A.

The effect of maternal obesity on TNF-α production by CD11b+ cells in brain and placenta remained significant, even after controlling for dam. For all analyses, TNF-α release was adjusted for cell count or to each subject's own untreated media baseline, so the increased responsiveness of maternal obesity-exposed CD11b+ cells to LPS was not attributable simply to increased cell numbers. In addition, the same priming effect was seen in the placenta as in the fetal brain, but the number of CD11b+ cells did not differ significantly between obese and lean groups in the placenta.

3.2.1 Sex differences in responsiveness of microglia and placental CD11b+ cells to immune challenge in the setting of maternal obesity—In the pre-specified sex-stratified analysis, only male obesity-exposed brain and placental cells had statistically-significant increases in LPS reactivity (5.5-fold increase in TNF-α production over controls in brain, p=0.008, 5.8-fold increase in TNF-α production over controls in placenta, p= 0.02, Figure 3B). Female cells displayed a similar trend but didn't achieve statistical significance (2.8-fold increase in TNF-α production over controls in brain, p=0.19, 4.6-fold increase in TNF-α production over controls in placenta, p=0.06, Figure 3B).

2-way ANOVA analyses (maternal obesity status × fetal sex) demonstrated a main effect of maternal obesity status on LPS reactivity of brain and placental CD11b+ cells ($F_{(1,25)}$ = 12.01, p=0.002 for fetal brain, $F_{(1,28)}$= 10.65, p=0.003 for placenta). For both brains and placentas, there was no significant main effect of fetal sex on LPS reactivity ($F_{(1,25)}$= 0.8, p=0.38 brain; $F_{(1,28)}$= 0.36, p=0.55 placenta), and the interaction terms between maternal obesity and fetal sex on LPS reactivity for both brain and placenta were nonsignificant ($F_{(1,25)}$= 1.25, p=0.27 brain, $F_{(1,28)}$= 0.32 p=0.58 placenta).

3.3 Placental CD11b+ cell reactivity is highly correlated with reactivity of brain microglia

To determine how correlated placental CD11b+ cell reactivity and microglial reactivity were in the setting of immune challenge, Spearman's correlation analysis was performed for brain and placenta values for each fetus. Only obesity-exposed fetal brains and placentas were included in the correlation, given the lack of significant response to LPS in control samples. Placental CD11b+ cell reactivity was strongly correlated with brain microglial reactivity in the setting of maternal obesity (Spearman's ρ= 0.73, p=0.002, Figure 3). This analysis suggests that the behavior of placental CD11b+ cells in response to immune challenge may serve as a reliable proxy for the behavior of brain microglia in the setting of maternal obesity.

3.4 Responsiveness of CD11b− brain and placental cells to immune challenge in the setting of maternal obesity

Production of TNF-α by the negatively-selected cell population in fetal brain and placental tissue (all CD11b− cells isolated from these tissues) was also evaluated by ELISA. For the majority of samples, TNF-α production by CD11b− brain and placental cells fell below the detectable range of the assay (<7.21 pg/mL). Analyses performed on the subset of fetal brain and placenta samples with TNF-α values above the limit of detection for the assay demonstrated no significant response to LPS in obesity-exposed or control CD11b− cells.

4. Discussion

Here we demonstrate for the first time that a subset of placental immune cells may provide insight into the behavior of microglia in the fetal brain. Specifically, we report that maternal obesity primes CD11b+ cells in both the placenta and fetal brain to overrespond to immune challenge with LPS, compared to controls. This effect was most pronounced in male fetal brains and placentas. There was a strong correlation between production of pro-inflammatory cytokine TNF-α by placental CD11b+ cells and fetal brain microglia. Given that the pool of microglia throughout an individual's lifespan is fetal in origin, maternal obesity-associated priming of microglia to overrespond to immune challenge has potential implications for understanding the increased risk for autism spectrum disorder, cognitive dysfunction, and other neurodevelopmental morbidities noted in offspring of obese women. It is difficult to identify which offspring may be most at risk for aberrant microglial activation in the setting of maternal obesity (and other disorders of maternal chronic immune activation), due to the inability to directly assay the developing brain in an ongoing pregnancy or after birth. These data represent an initial step toward the use of placental macrophages as a biomarker, accessible during pregnancy and at the time of birth, of fetal neuroimmune activation. If these placental macrophages and corresponding brain microglia can be further characterized in a variety of maternal exposure states, these data may have broader implications for assaying the impact not only of maternal obesity but other maternal exposures on fetal brain development.

A strength of this study is the novel use of placental macrophages to provide information about the behavior of fetal brain immune cells, and the demonstration of a strong correlation between the behavior of these two cell types in response to immune challenge. The common yolk-sac origin of fetal placental macrophages and microglia (Ginhoux et al. 2010; Ginhoux and Prinz 2015) inspired our examination of whether both cell types would behave similarly after *in vivo* exposure to maternal obesity, followed by a second *ex vivo* immune challenge (LPS administration). This "two-hit" model, in which maternal obesity may not in isolation change the behavior of fetal microglia and placental macrophages (as evidenced by the lack of baseline differences in TNF-a production between obesity-exposed and lean-exposed cells), but primes them to overrespond to a second immune challenge, may help explain why maternal obesity is a risk factor for neurodevelopmental morbidity, but not all offspring are affected.

Our finding that maternal obesity alone did not alter baseline levels of TNF-α production by fetal brain microglia or placental macrophages is consistent with the findings of two rodent models of maternal immune activation via polyinosinic:polycytidylic acid (Poly(I:C)), which did not find alterations in microglial TNF-α mRNA or protein expression at e16.5 (Pratt et al. 2013), and did not identify an effect on microglia at e17.5 even in the setting of repeated Poly(I:C) administration (Smolders et al. 2015). The absence of maternal obesity-induced baseline increase in TNF-α production by either microglia or placental macrophages in our study is also consistent with the absence of detectable fetal inflammation and limited placental inflammation observed in a rat model of maternal obesity induced by a cafeteria diet (Crew, Waddell, and Mark 2016). In contrast to these two models of maternal immune activation/inflammation, maternal LPS administration, whether one-time (O'Loughlin et al.

2017), or repeated (Schaafsma et al. 2017), was sufficient to increase TNF-α mRNA expression in embryonic brain at e17.5 and e18. It may be that maternal obesity is a more comparable exposure to Poly(I:C) than it is to LPS, and a second hit may be required to induce overexpression of pro-inflammatory cytokines by microglia in this setting. The two-hit model is well described in models of maternal immune activation, and has been implicated in neuropsychiatric outcomes ranging from anxiety to cognitive dysfunction and schizophrenia, among others (Estes and McAllister 2016; Debost et al. 2017; Bolton et al. 2013; Giovanoli et al. 2013; Williamson et al. 2011).

The absence of a significant response of control CD11b+ fetal brain or placental cells to LPS merits further discussion. In rodent models, *ex vivo* LPS challenge has been demonstrated to produce a pro-inflammatory response in control/wild-type adult microglia (Frank et al. 2010; Frank et al. 2006; Njie et al. 2012) and in postnatal day 4 microglia (Turano, Lawrence, and Schwarz 2017). However, the limited available data suggest that normal fetal microglia may have a less consistent pro-inflammatory response to LPS administration (Cao et al. 2015; Schaafsma et al. 2017; Lee et al. 1993). The few studies that have examined fetal microglial response to LPS report conflicting results: some report no detectable increase in TNF-α (Cao et al. 2015) or IL-1β (Lee et al. 1993) in control supernatants after LPS administration measured by ELISA, but increased TNF-α, IL-6, and IL-1β RNA after LPS administration has been detected via quantitative PCR (RTqPCR) or Northern blot (Lee et al. 1993; Schaafsma et al. 2017). These differing results may be partly attributable to different sensitivities of ELISA versus RTqPCR for low levels of cytokine expression (Amsen, de Visser, and Town 2009; Peinnequin et al. 2004). Examination of cytokine production via ELISA is a strength of this study. Many studies examining the impact of maternal immune activation on fetal and offspring microglial function have focused primarily on mRNA levels, and in the limited number of studies that have examined both message levels and protein, discrepancies have been noted between pro-inflammatory cytokine expression by mRNA versus protein.(Schaafsma et al. 2017; Pratt et al. 2013; Lee et al. 1993) Characterization of cytokine expression by ELISA is closer to phenotype.

The joint focus on fetal brain microglia and placental macrophages in the setting of maternal obesity is a unique contribution of our study. As the interface between the maternal and fetal environments and the source of fetal nutrition, the placenta has been implicated in developmental programming (Jansson and Powell 2007), including of the developing fetal brain (Hsiao and Patterson 2012; Bale 2016; Bronson and Bale 2016). While the placenta is involved in mediating immune protection of the fetus, these protective mechanisms can become deranged in the setting of inflammation or maternal immune activation, contributing to long-term programming of the fetal brain and offspring behavior in later life (Hsiao and Patterson 2012; Bronson and Bale 2014). In normal pregnancy, both maternal decidual and placental macrophages are maintained in an anti-inflammatory state to enable immune tolerance of the fetus (Bolton and Bilbo 2014). Human studies have demonstrated that maternal obesity is associated with accumulation of placental macrophages (Hofbauer cells) in the placenta, and accompanying evidence of increased inflammation (Aye et al. 2014; Bar et al. 2012; Challier et al. 2008; Roberts et al. 2011). Placental inflammation may in turn have direct effects on the developing fetal brain (Bronson and Bale 2014; Goeden et al. 2016). A proposed underlying mechanism is increased placental serotonin production in the

setting of inflammation, which inhibits neurogenesis and axon outgrowth in the developing forebrain (Bonnin et al. 2011; Goeden et al. 2016). Increased placental conversion of maternal l-tryptophan to serotonin may be mediated in part through IL-6 (Howell and Powell 2017), which has been demonstrated to be increased in the setting of maternal obesity (Christian and Porter 2014; Friis et al. 2013; Stewart et al. 2007). The relative contributions of maternal obesity compared to high-fat diet on placental and brain serotonin signaling may be difficult to disentangle: in a non-human primate model of maternal high-fat diet, fetal central serotonergic signaling was perturbed and offspring demonstrated increased anxiety-like behavior, independent of maternal obesity status (Sullivan et al. 2010).

The neurodevelopmental morbidities observed with increased frequency in offspring of obese mothers include not only mood disorders (anxiety and depression), but also autism spectrum disorder, developmental delay and/or disorders of cognitive function, ADHD, disordered eating, cerebral palsy, and schizophrenia in some studies (Brion et al. 2011; Crisham Janik et al. 2013; Edlow 2017a; Heikura et al. 2008; Hinkle et al. 2012; Huang et al. 2014; Krakowiak et al. 2012; Rising and Lifshitz 2005; Rodriguez et al. 2008; Tanda et al. 2013). Microglial number, activation state, and function has been implicated as potentially contributing to the majority of these pathologies (Koyama and Ikegaya 2015; Lenz and McCarthy 2015; Matcovitch-Natan et al. 2016; Sekar et al. 2016). The fetal origin of the permanent pool of microglia in the brain suggests that pregnancy and the perinatal period are key windows during which brain microglial function may be permanently altered.

While prior work has demonstrated that maternal high-fat diet is associated with chronic priming of microglia in offspring, and increased pro-inflammatory cytokine expression in the adult offspring brain in response to a bacterial challenge (Bilbo and Tsang 2010), this the first study to report microglial and placental macrophage priming by maternal obesity detectable even in fetal life. It is biologically plausible that maternal obesity could program macrophages to overrespond to immune challenge, given that maternal obesity has been shown to be associated with placental inflammation, fetal and neonatal peripheral inflammation, and offspring brain inflammation (Aye et al. 2014; Challier et al. 2008; Desai et al. 2013; Zhu et al. 2010; Bilbo and Tsang 2010; Kang et al. 2014).

The examination of both male and female fetal brains and placentas allowed us to investigate sex differences in response to immune challenge in the setting of maternal obesity. The finding that male brain and placental macrophages have a significantly increased response to LPS in maternal obesity is consistent with other studies that have demonstrated increased male brain vulnerability to early-life immune challenge (Schwarz, Sholar, and Bilbo 2012; Iwasa et al. 2010; Llorente et al. 2009; Rebuli et al. 2016; Ruggiero et al. 2018; Hilton, Nunez, and McCarthy 2003; Nunez, Alt, and McCarthy 2003; Walker, Nakamura, and Hodgson 2010), and may provide insight into the male predominance of certain neurodevelopmental morbidities linked to maternal obesity (autism spectrum disorders, developmental/cognitive delay, ADHD, and in some studies schizophrenia) (Bilbo, Smith, and Schwarz 2012; Lenz and McCarthy 2015; Edlow 2017a; Koyama and Ikegaya 2015). Recent studies have demonstrated that the placenta may transmit sex-specific signals to the developing fetal brain in the setting of maternal environmental perturbations, and that such signaling may be mediated in part via X-linked genedosage effects and sex differences in

placental inflammation (Bale 2016; Howerton et al. 2013; Bronson and Bale 2014). Differences in the male placenta may therefore render the developing male brain more vulnerable to insults such as maternal stress (Bronson and Bale 2014; Bale 2016). The finding of a more pronounced priming effect of maternal obesity on male brain and placental macrophages should interpreted with caution, however, given that significant differences between males and females were noted in the sex-stratified analyses only, while the interaction term on 2-way ANOVA did not achieve statistical significance. The absence of a statistically-significant response of female macrophages to LPS in the setting of maternal obesity may be because only males have a significant response, or may be because more female fetuses than 8-9/diet group are needed to see a statistically significant effect.

Our study was limited in its ability to fully characterize the CD11b+ placental macrophage population. While placental CD11b+ macrophages are fetal in origin, and therefore enriched for Hofbauer cells (resident fetal macrophages of yolk sac origin within the placental villus core) (Reyes, Wolfe, and Golos 2017), further characterization of the CD11b+ placental macrophages is an important area for future study. There are at least three possibilities for the identity of the CD11b+ cells isolated from the placenta: (1) Hofbauer cells (2) circulating blood monocytes of fetal origin, or (3) circulating blood monocytes of maternal origin (less likely but some contamination of placentas with maternal blood despite PBS rinsing cannot be ruled out). Distinguishing between these three possibilities via flow cytometry/fluorescence-activated cell sorting may be challenging, due to the inherent complexity of the mononuclear phagocyte system and significant phenotypic variability of placental macrophages which has been elucidated in recent years (Hume 2008a; Reyes, Wolfe, and Golos 2017). There has been increasing recognition of many shared markers between what are typically considered “resident” versus “inflammatory” macrophages, and recognition of more than 20 macrophage markers expressed by Hofbauer cells, most of which are gestational age-specific (Gordon and Taylor 2005; Hume 2008a, 2008b; Reyes, Wolfe, and Golos 2017). In addition, there may be some overlap between Hofbauer cells and peripheral fetal macrophages as pregnancy progresses. Hofbauer cells are exclusively of fetal yolk sac origin in early pregnancy, but during later stages of pregnancy, may originate from fetal monocytes recruited to the placenta (Kim et al. 2008; Kim et al. 2009; Takahashi et al. 1991; Seval, Korgun, and Demir 2007).

Similarly, while the brain CD11b+ macrophage population is highly enriched for microglia, we cannot rule out the possibility of some infiltrating immune cells from the periphery. Data are limited regarding the potential for infiltration of peripheral monocytes into the brain, as this is a more recent consideration. The original fetal pool of yolk-sac derived macrophages is the lifelong source of myeloid cells in the CNS; this microglial pool is self-renewing and its maintenance does not depend on circulating monocytes under normal conditions (Salter and Stevens 2017; Greter, Lelios, and Croxford 2015; Jeong et al. 2013; Bruttger et al. 2015; Hashimoto et al. 2013; Sheng, Ruedl, and Karjalainen 2015). There is controversy, however, regarding whether peripheral CD11b+ monocytes could contribute to the brain population under inflammatory conditions (Greter, Lelios, and Croxford 2015; Bruttger et al. 2015; Tay et al. 2017; Sevenich 2018; Wohleb et al. 2014), and whether the increased permeability of the blood brain barrier in fetal life might permit some influx of peripheral immune cells into the developing brain (Ginhoux and Prinz 2015; Saunders, Liddelow, and Dziegielewska

2012; Garay et al. 2013). Novel markers highly specific to microglia that can distinguish between microglia and other monocytes or macrophages present in the brain have been recently described (transcriptional repressor Spalt Like Transcription Factor 1 or Sall 1, and Transmembrane protein 119 or Tmem119).(Bennett et al. 2016; Buttgereit et al. 2016). However, the accuracy of these markers to distinguish microglia from other immune cells in the embryonic brain is unknown at this time (Smolders et al. 2018). Future experiments will therefore focus on additional characterization of CD11b+ cells in the developing brain and placenta via flow cytometry and/or transcriptional profiling, to better understand whether some of these cells could be infiltrating peripheral macrophages.

5. Conclusions

These experiments provide two novel insights into fetal immune function in the setting of maternal obesity. First, we demonstrate for the first time the potential for placental macrophages to provide information about the behavior of brain microglia, true for both obese and lean pregnancy. While further characterization of the CD11b+ cells in the placenta is needed, the fact that these cells behave similarly to brain microglia in response to immune challenge may have broader implications for assaying fetal brain development in a variety of maternal exposures. Second, we demonstrate that maternal obesity primes both placental macrophages and fetal brain microglia to overrespond to an *ex vivo* immune challenge. Sex-stratified analyses suggest that male placental macrophages and microglia have a stronger pro-inflammatory response in the setting of this priming. This observation may provide insight into the male predominance of neurodevelopmental morbidities observed with increased frequency in the setting of maternal obesity, such as autism spectrum disorder, cognitive dysfunction, and ADHD. All of these morbidities have been linked to microglial function, although the role of placental inflammation in these conditions has been less well-described. These experiments will form the foundation for an experimental program to investigate placental-brain crosstalk in maternal chronic inflammatory states, such as obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Reactivity of CD11b+ placental macrophages is highly correlated with that of fetal brain microglia
- Maternal obesity primes CD11b+ placental and brain macrophages to overrespond to immune challenge
- Male fetuses are more vulnerable to this priming effect of maternal obesity than females
- Placental CD11b+ cells may be an accessible biomarker of fetal brain immune activation in maternal obesity

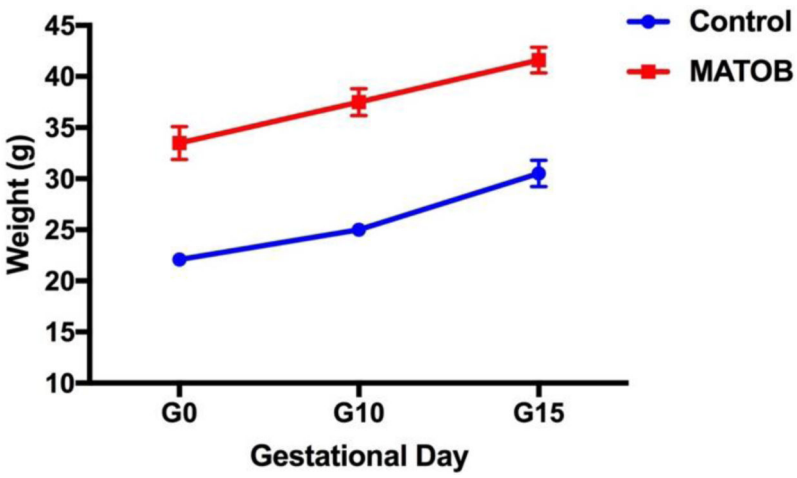

Figure 1: Pregnancy weight trajectory in obese and lean dams

G0: gestational day 0 (day of mating); G10: gestational day 10; G15: gestational day 15; MATOB: Maternal obesity

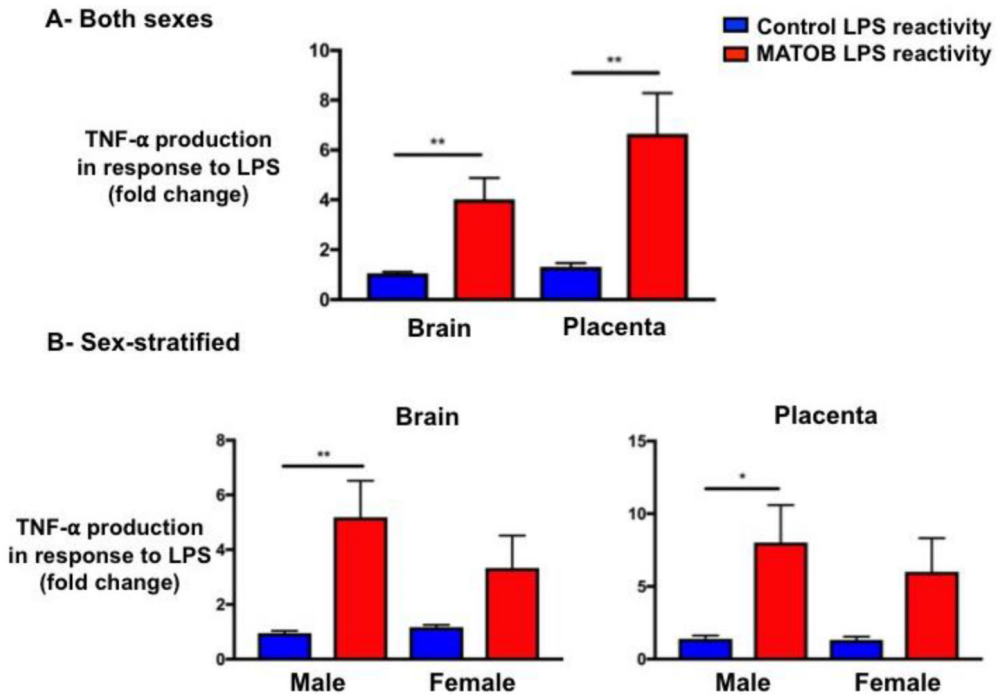

Figure 2: Responsiveness of microglia and placental CD11b+ cells to immune challenge in the setting of maternal obesity

Maternal obesity is associated with significantly increased pro-inflammatory response to LPS by both brain microglia and placental CD11b+ cells. Sex-stratified analyses demonstrate that only obesity-exposed males have a statistically significant increase in TNF-α production in response to LPS.

LPS: lipopolysaccharide; MATOB: Maternal obesity; **p<0.01; *p<0.05

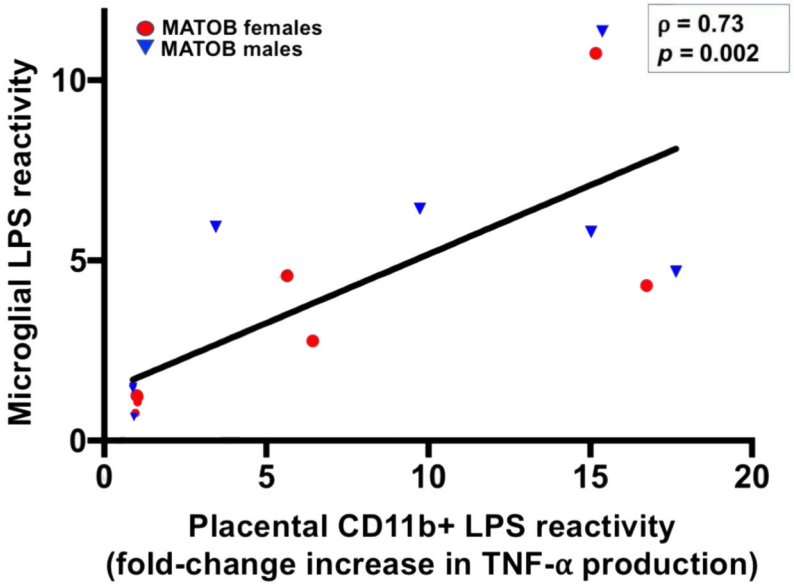

Figure 3: Response of fetal brain microglia and placental CD11b+ cells to immune challenge is highly correlated in the setting of maternal obesity

TNF-α production in response to LPS challenge by CD11b+ cells in the placenta and fetal brain is highly correlated. Axis values reflect fold-change increase in TNF-α production after LPS administration.

MATOB: maternal obesity, red circles (female) and blue triangles (male) represent matched fetal brains and placentas of obese dams.

LPS: lipopolysaccharide

Table 1:

Study population characteristics

Characteristic	Control	Maternal Obesity	*p*-value
Dam weight at breeding (g)*	20.60 ± 0.58	31.83 ± 0.94	0.02
Dam weight at sacrifice (g)	36.38 ± 1.13	42.97 ± 1.80	0.03
Litter size	8.8 ± 1.2	6.25 ± 1.49	0.24
Fetal weight (g)			
Male	0.96 ± 0.03	0.97 ± 0.03	0.93
Female	0.86 ± 0.04	0.91 ± 0.02	0.67
Both sexes combined	0.90 ± 0. 03	0.93 ± 0.02	0.57
Placental weight (g)			
Male	0.12 ± 0.01	0.11 ± 0.01	0.59
Female	0.11 ± 0.01	0.10 ± 0.01	0.19
Both sexes combined	0.12 ± 0. 01	0. 10 ± 0. 01	0.13
CD11b+ cells isolated from brain			
Male	$3.85 \times 10^5 \pm 0.63$	$7.42 \times 10^5 \pm 1.14$	0.02
Female	$4.65 \times 10^5 \pm 0.50$	$7.75 \times l0^5 \pm 0.81$	0.003
Both sexes combined	$4.25 \times 10^5 \pm 0.40$	$7.60 \times 10^5 \pm 0.66$	<0.001
CD11b+ cells isolated from placenta			
Male	$7.02 \times 10^5 \pm 1.54$	$8.32 \times 10^5 \pm 1.37$	0.47
Female	$7.21 \times 10^5 \pm 1.86$	$6.23 \times 10^5 \pm 0.76$	0.99
Both sexes combined	$7.171 \times 10^5 \pm 1.16$	$7.27 \times 10^5 \pm 0.80$	0.59
CD11b− cells isolated from brain			
Male	$9.13 \times 10^6 \pm 2.12$	$10.33 \times 10^6 \pm 1.30$	0.35
Female	$10.03 \times 10^6 \pm 1.56$	$14.52 \times 10^6 \pm 2.89$	0.24
Both sexes combined	$9.57 \times 10^6 \pm 1.28$	$12.56 \times 10^6 \pm 1.69$	0.16
CD11b− cells isolated from placenta			
Male	$4.42 \times 10^6 \pm 0.77$	$4.22 \times 10^6 \pm 0.36$	0.91
Female	$3.12 \times 10^6 \pm 0.60$	$4.90 \times 10^6 \pm 0.28$	0.03
Combined	$3.77 \times 10^6 \pm 0.50$	$4.58 \times 10^6 \pm 0.24$	0.09

*Data are expressed as mean ± SEM unless otherwise indicated