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Prenatal alcohol-induced sex differences in immune, metabolic and neurobehavioral outcomes in adult rats

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Abstract

Prenatal alcohol exposure (PAE) can result in neurobehavioral anomalies, that may be exacerbated by co-occurring metabolic and immune system deficits. To test the hypothesis that the peripheral inflammation in adult PAE offspring is linked to poor glucose metabolism and neurocognitive deficits, pregnant Sprague-Dawley rats were exposed to ethanol vapor or ambient air during the latter half of gestation. We assessed, in adult offspring of both sexes, performance on a battery of neurocognitive behaviors, glucose tolerance, circulating and splenic immune cells by flow-cytometry, and circulating and tissue (liver, mesenteric adipose, and spleen) cytokines by multiplexed assays. PAE reduced both the ratio of spleen to body weight and splenic regulatory T-cell (Treg) numbers. PAE males, but not females exhibited an increase in circulating monocytes. Overall, PAE males exhibited a suppression of cytokine levels, while PAE females exhibited elevated cytokines in mesenteric adipose tissue (IL-6 and IL1α) and liver (IFN-γ, IL-1β, IL-13, IL-18, IL-12p70, and MCP-1), along with increased glucose intolerance. Behavioral analysis also showed sex-dependent PAE effects. PAE-males exhibited increased anxiety-like behavior while PAE-females showed decreased social interaction. PAE offspring of both sexes exhibited impaired recognition of novel objects. Multilinear regression modeling to predict the association between peripheral immune status, glucose intolerance and behavioral outcomes, showed that in PAE offspring, higher levels of adipose leptin and liver TNF- α predicted higher circulating glucose levels. Lower liver IL-1 α and higher plasma fractalkine predicted more time spent in the center of an open-field with sex being an additional predictor. Higher circulating and splenic Tregs predicted better social interaction in the PAE-offspring. Collectively, our data show that peripheral immune status is a persistent, sex-dependent predictor of glucose intolerance and neurobehavioral function in adult PAE offspring.

Keywords

Prenatal alcohol

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Appendix A. Supplementary data

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1. Introduction

Early life experiences may result in adverse health outcomes, including metabolic and immune system disorders and increased susceptibility to chronic diseases in adulthood (Barker et al., 1989, Barker et al., 1993). Prenatal alcohol exposure (PAE) is an example of common and significant adverse early life experience and it is acknowledged to result in a wide range of disabilities across the lifespan, collectively termed as Fetal alcohol spectrum disorders (FASD). It has been previously reported that about 18% of pregnant women consume alcohol during early gestation (Samhsa, 2013), and that exposure at the end of pregnancy is also high, accounting for more than 8% of births in statewide assessments (Bakhireva et al., 2017, Umer et al., 2020). The estimated prevalence of FASD is between 1.1 and 9.8% of school-aged children in the US (May et al., 2018), and up to 14–21% in other countries like South Africa (May et al., 2013).

Numerous clinical and pre-clinical studies demonstrate that PAE alters brain development and consequently results in neurological and behavioral deficits in adult offspring (Streissguth et al., 1990, Hamilton et al., 2010, Cullen et al., 2013, Mattson et al., 2013, Varlinskaya and Mooney, 2014, Marquardt and Brigman, 2016). Moreover, PAE triggers neuroinflammation (Drew and Kane, 2014, Bodnar et al., 2016, Noor and Milligan, 2018), and a link between inflammation and impaired cognitive function in adult PAE rodents has also been documented (Terasaki and Schwarz, 2016).

However, FASD is increasingly being recognized as a ‘whole-body’ disorder, rather than simply a brain-based disorder. Studies in both animal models and human populations have documented cardiovascular (Walton et al., 2019), renal (Gray et al., 2010), pulmonary (Gauthier and Brown, 2017) and metabolic dysfunction (Weeks et al., 2020) including impaired glucose tolerance (Gardebjer et al., 2015), with insulin insensitivity (Nguyen et al., 2019, Weeks et al., 2020). PAE has also been shown to alter the endocrine function within the hypothalamic–pituitaryadrenal axis (Weinberg, 1993, Weinberg et al., 2008) and to disrupt the developing immune system (Ahluwalia et al., 2000, Bodnar et al., 2016). Our previous studies showed that PAE reduced murine fetal cerebral blood flow (Bake et al., 2012), and that cerebrovascular blood flow deficits persisted into middle-aged adulthood, and were associated with poor neurological recovery in PAE offspring compared to control offspring after transient cerebrovascular ischemic stroke (Bake et al., 2017). These data collectively suggest that systemic adaptations to PAE may be a contributory factor to brain dysfunction in adults with PAE, and moreover, increase the damage resulting from adult-onset disease.

Systemic inflammation and immune dysfunction may constitute both a co-morbid condition as well as a contributory factor in both neurobehavioral and other systemic dysfunction including glucose intolerance in adult PAE offspring. There is a well-established linkage between pro-inflammatory states, metabolic dysregulation and cognitive performance in both human studies and animal models of PAE (Takeda et al., 2010, Butcher et al., 2014, Esser et al., 2014, Feinkohl et al., 2015, Kraig et al., 2018), which suggests that the state of the peripheral immune system is at least a predictor and may also be biologically linked

to neurocognitive dysregulation in FASD. To test this hypothesis, we utilized a rat model of PAE to determine changes in resident populations of immune cells and cytokines in spleen and in circulation, in adult offspring. To better understand the inflammation status in metabolically relevant organs, a panel of cytokines was also assessed in mesenteric adipose tissue and liver. We also assessed and neurocognitive deficits through a panel of tests for anxiety, memory and social interaction related behavioral domains in adult offspring. Additionally, we used multiple regression statistical models to identify predictive relationships between circulating and tissue mediators of inflammation with neurobehavioral and glucose intolerance in PAE offspring. Our data point to strong sex-specific effects of PAE on adult offspring, and further show that tissue cytokines predict glucose tolerance as well as performance on behavioral measures of anxiety. Moreover, a sub-population of tissue and circulating immune cells, regulatory T-lymphocytes (Tregs), were significant predictors of social interaction preferences in PAE offspring. Collectively, these data indicate that peripheral immune states in adult PAE offspring are related to both neurobehavioral indices and glucose intolerance.

2. Methods

2.1. In house breeding and multiple binge ethanol exposure

All procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and approval, and all animal procedures conform to ARRIVE guidelines (McGrath et al., 2010). Adult Sprague-Dawley breeders were purchased from Envigo laboratories, acclimated to the housing condition for one week, to minimize the contribution of transportation and other stressors, before being subjected to overnight, in-house timed-mating. The start of the mating period was defined as gestational day 0 (GD0). Pregnant females were identified by the presence of a vaginal plug and subsequently, by weight gain of ≥10 g by GD8. All offspring used in the study were raised from our in-house breeding program.

2.2. Exposure paradigm

Sprague-Dawley rats were time-mated and the females (2 rats/cage) were exposed to alcohol vapor (95%, ACS grade, Acros Organics, NJ # 61511) at a flow rate of 10 mL/min for 1 h daily for 12 days starting at gestational day (GD) 8. Each exposure episode occurred between 9:00–10:00 am, i.e., near the start of the subjective night, so as to not interfere with feeding behavior during the rat's subjective day. The cumulative exposure period encompassed key events in the growth of two important and interrelated systems, the brain (neurogenesis, migration and neuronal differentiation, (Workman et al., 2013), and immune system (the shift in hematopoiesis from the gonado-mesonephric region to the fetal liver and spleen and then to thymus and bone marrow (Landreth, 2002, Kuper et al., 2016). The control animals were handled the same way and they received ambient air for the same duration as the alcohol vapor. Animals were monitored for breathing difficulties or behaviors indicative of discomfort during exposure and returned to the home cages in the vivarium every day. The exposure paradigm did not result in obvious respiratory distress or other behavioral signs of distress, but did result in diminution of postural reflexes, but without loss of righting reflex, with effects disappearing within thirty to sixty minutes following

cessation of exposure. Blood ethanol concentration (BEC) was determined from tail-vein samples collected within 10 min after cessation of each exposure episode. Alcohol vapor exposure resulted in average BEC of 299 ± 52 mg/dL (across the entire treatment period) in dams used in the study. These levels well within those recorded in persons with alcohol use disorders (Adachi et al., 1991), and moreover, it should be noted that rats metabolize ethanol relatively quickly, with levels decreasing by ~0.4 mg/dL/min from peak BECs achieved (Livy et al., 2003). All animals were maintained on a 12 h light–dark cycle at 19–22 °C with ad libitum access to a commercially available rodent chow (diet # 8604, Envigo laboratories) and water. Maternal body weight was recorded daily throughout the treatment period. The average weight gain (body weight difference from GD0 through GD 20) in ethanol group (40 ± 5 g, relative to weight at GD0) was not significantly different from the control group (47 ± 11 g, $p > 0.55$). The binge pattern of alcohol exposure during subjective night did not alter food consumption during the animal's subjective day ($p > 0.25$). The litters were culled n = 10/ litter by postnatal day 3. After culling, the average sex ratio in both control and PAE litters were similar. The observed male-to-female ratio is 0.94 for controls and 1.25 for the PAE group (t = 0.003, p = 0.99, ns). Pups were weaned at postnatal day 21 and pair-housed with littermates of the same sex in a standard rat cage.

2.3. Glucose tolerance test

Adult PAE and control rats (4 mo) of both sexes were fasted overnight (15 h) and were injected ip. with glucose (1 g/kg bwt). Animals were anesthetized with 3% isoflurane, blood samples were collected from tail snip at pre (baseline/fasting), 30, 60, 90, 120 and 180 min and centrifuged at 2000 g for plasma collection. Blood glucose levels were determined using a glucometer (Accu-chek, IN) and the accuracy was confirmed using a colorimetric kit (Roche diagnostics, IN).

2.4. Extraction of adipose and spleen

Animals (5 mo old) from the same litters as those used in glucose tolerance test were anesthetized with xylazine (12.8 mg/kg bwt) and ketamine (88 mg/kg bwt), decapitated and trunk blood (approximately 1 mL for immune cell isolation) was collected into 0.5 M EDTA coated tubes. Liver, spleen and mesenteric adipose tissues were excised and processed for immune cell analyses.

2.4.1. Isolation of lymphocytes from spleen and blood—Lymphocytes were isolated from spleen by mechanical dispersion. Tissue was minced and gently ground between the rough sides of frosted glass slides, pelleted at 300 × g for 5 min, and resuspended in 10 mL of erythrocyte lysis buffer (TONBO biosciences, Cat#TNB-4300-L100) at room temperature for 10 min. Similarly, 1 mL of freshly collected blood was incubated in 14 mL of erythrocyte lysis buffer for 15 min to remove red blood cells. After removing red blood cells, cells were filtered through a 40-μm cell strainer to remove debris before proceeding with flow cytometry analysis.

2.4.2. Flow cytometry analysis—Splenic and blood cells were stained with fluorescence-tagged antibodies to detect cell lineages. CD45 (eBioscience, Cat#48–0461-82) was used as a pan lymphocyte marker. B cells were detected with antibodies against B220

(eBioscience, Cat#17–0460-80) and CD45RA (Invitrogen, Cat#MA5–17480); neutrophils were detected with antibodies against CD43 (Biolegend, Cat#202812) in combination with high side-scatter; T cells were detected with antibodies against CD3 (eBioscience, Cat#11–0030-82), CD4 (eBioscience, Cat#12–0040-82), or CD8 (eBioscience, Cat#17–0084-82); Treg FoxP3 (eBioscience, Cat#17–5773-80) and CD25 (eBioscience, Cat#12–0390-80); monocytes were detected with antibodies against CD11b (eBioscience, Cat#50–0110-80) in combination with low side-scatter; macrophage subtypes were detected with antibodies against CD11b and MAC HIS36 (eBioscience, Cat#12–0660-82) in combination with low side-scatter. Briefly, for 1 million cells isolated from spleen or blood, extracellular markers were initially stained for 30 min at RT in the dark. Next, LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (Invitrogen, Cat#L34959) was used following manufacturers recommendations. Finally, intracellular markers were stained using eBioscience™ FOXP3/Transcription Factor Staining Buffer Set (Invitrogen, Cat#00–5523-00) following manufacturers recommendations. Data were collected using the Beckman Coulter® Gallios 2/5/3 Flow Cytometer with analysis stopping after 50,000 live cell events and were analyzed using Kaluza software (Beckman Coulter, Brea, CA).

2.5. Multiplexed cytokine profiling

There is very little information available on the effect of PAE on the peripheral immune milieu in adults. Therefore, we sought to determine the impact of PAE on cytokines from plasma, and spleen from male and female offspring. Additionally, cytokines were measured in liver, a major source of inflammatory mediators and homeostatic control of the immune system (Robinson et al., 2016), and mesenteric adipose tissue which reflects and regulates gut inflammation due to its anatomical and vascular contiguity with the small intestine (ileum), and regulates metabolic homeostasis (Eder et al., 2019).

Plasma and tissue expression of a panel of rat cytokines and chemokines was measured using a multiplexed magnetic bead immunoassay (Millipore Corp. MA) following manufacturer's instructions and our previous protocols (Panta et al., 2019). Adipose, liver and spleen tissues were homogenized in RIPA lysis buffer, centrifuged at 15,000*g* for 15 min at 4 deg C, the supernatant was collected and stored at − 20 deg C. Briefly, the 96 well plate was blocked with assay buffer for 10 min and decanted. Standards and samples (100 μg) were added into appropriate wells, followed by addition of premixed beads, and incubated at room temperature for 2 h on a plate shaker. Wells were washed twice, 25 μl of detection antibody was added, incubated for 1 h and followed by 30 min incubation with 25 μl of streptavidin–phycoerythrin per well. After 2 washes, beads were resuspended in 150 μl of sheath fluid and a minimum of 50 beads per analyte was analyzed in a Bio-Plex suspension array system (Bio-Rad Laboratories, CA). The analytes in those samples that went below the lower detection limit were excluded. Cytokine/chemokine levels were normalized to total protein content. Total protein was determined using a commercially available colorimetric kit (Pierce BCA protein assay kit, Thermofisher, CA). The following cytokines and chemokines were assessed: Interleukin-1-alpha (IL-1α), IL-4, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-12-p-70, interferon-gamma (IFN- γ), monocyte chemoattractant protein-1 (MCP-1), Interferon gamma-induced protein 10 (IP-10), keratinocytes-derived chemokine (KC,) tumor necrosis factor-alpha (TNF-α), Regulated upon Activation, Normal T Cell

Expressed and Presumably Secreted (RANTES), macrophage inflammatory protein 1-alpha (MIP1-α), macrophage inflammatory protein 1-beta (MIP1-β), macrophage inflammatory protein 2 (MIP-2), granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxin, leptin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fractalkine, lipopolysaccharide-induced CXC chemokine (LIX).

Behavioral analysis: The animals were used for behavioral analysis 2 weeks after completion of the glucose tolerance test. The sequence of behavioral testing was Open Field followed by Social Interaction and Novel Object Recognition Task. In general, both control and ethanol treated animals were assessed on the same day for a particular behavioral test. All testing was performed between 9 am and 1 pm to minimize the contribution of circadian cycles to behavioral variations, and males and females were tested on separate days to minimize the contribution of odorant cues.

Open Field Test: The anxiety-like behavior was tested using an open field arena in a plexiglass chamber 16″ x16″ (Kinderscientific, CA). The arena is divided into 2 zones, the inner zone and the outer zone. The animals (4.5 mo old) were placed on the outer zone facing the sidewall and each rat was given 60 min to explore the arena with room lights turned off. The time spent in the inner zone was calculated using the Motormonitor Host software (Kinder Scientific, CA) to determine anxiety-like behavior. This software counts the time in the center when half of the body with both the forepaws are in the center zone.

Social interaction test: A three-chambered Plexiglass box was used for the assessment of social interaction (Panta et al., 2019, Panta et al., 2020). Test animals were habituated in the apparatus for 15 min. For testing, a conspecific, untreated adult same-sex stranger rat was placed within a plastic mesh cylinder in one of the end chambers, while the test rat was placed back in the middle chamber and allowed to explore for 10 min. The time spent in each chamber was recorded, and sociability was scored as the total time (in seconds) spent by the test rat in the chamber with the stranger rat.

Novel object recognition task (NORT): The object recognition memory was tested as described in (Panta et al., 2019, Panta et al., 2020). The PAE and control animals were habituated in a plexiglass chamber (16″ × 16″); the rats were trained with two identical objects (odor-free plastic animal toys), (A + A) placed in opposite corners of the chamber, for 10 min. Animals were returned to the home cage and were tested after an hour. For testing, animals were placed into the chamber with two objects in the same location as before, one that was previously available (A) and the other that was novel (B). The 5 min trials were video recorded for the analysis. The amount of time spent exploring the novel object was determined from these recordings by an investigator blind to the experimental condition. Exploration of an object was defined as the animal's snout directed to the object, sniffing or touching the object with its snout at a distance <2 cm to the object and/or touching it with the nose. The discrimination index was calculated as follows: DI: [*time spent with novel object*]-[*time spent with familiar object*]/[*time spent with novel object]* + *[time spent with familiar object*].

2.6. Ingenuity pathway analysis

Ingenuity Pathway Analysis was conducted on cytokines of each assessed tissue separately (plasma, spleen, liver, adipose) and also subdivided by sex to determine any sex differences. IPA's core analysis workflow was used to identify top canonical pathways that were potentially perturbed by PAE.

2.7. Statistical analysis

For all analysis, group mean ± SEM are reported. Group differences were determined by a two-way ANOVA (sex × treatment (PAE vs. control), with planned comparisons. For GTT and open-field data, a two-way repeated measures (time as repeated variable) ANOVA was used. All group differences were considered significant at $p < 0.05$. For any quantitative analysis, no more than 2 animals/sex from each litter was used for any single measurement. Statistical tests were performed with Prism GraphPad (GraphPad, San Diego, CA). Hierarchical clustering analysis using Pearson correlation and multiple linear regression models were constructed using R statistical software (R Core Team, 2017). For correlation and regression analyses, the litter, rather than an individual offspring, was the unit of analysis. Correlation analysis was performed in PAE animals to determine the variables that are most closely correlated with worse behavioral (NORT, Social interaction) and physiological (GTT) outcomes. Variables were initially z-score normalized across all samples to ensure equal weighting of predictors before proceeding with analysis. This correlation analysis was not adjusted for multiple comparisons as it was only used as a screening tool to select variables best suited as predictors in multilinear regression models.

3. Results

3.1. Prenatal ethanol vapor exposure and persistent effect on spleen weight in adult rats

To determine the long-term consequence of PAE on the growth and metabolism of young adult males and females (4–5 mo), we measured the body weight, the weight of the liver and spleen. PAE did not alter offspring body weights in either males or females (Fig. 1a; $F_{(1,31)} = 0.01962$, $p = 0.8895$). Since males and females differ in body weight, we normalized the liver and spleen weights to body weight. Interestingly, as shown in Fig. 1b and 1c, the normalized liver weight remained unaltered with PAE ($F_{(1,31)} = 2.537$, $p = 0.1214$); however, the normalized spleen weight significantly decreased in the PAE group compared to the controls (main effect of treatment, $F_{(1,31)} = 9.096$, $p = 0.0051$) suggesting that there is a persistent organ-specific effect on the spleen. Additionally, the two way ANOVA shows that female spleens are larger than males ($F_{(1,31)} = 28.66$, $p = 0.0001$), regardless of prenatal exposure status.

3.2. Prenatal ethanol vapor exposure elevates glucose intolerance in females but not in males

Glucose administration elevated blood glucose levels in both males and females with and without PAE. A two-way repeated measures ANOVA (time as repeated measure) showed that glucose levels were elevated at 30, 60, 90 and 120 min after glucose injection ($F_{(5,120)} = 16.7$, $p = 0.0001$) and gradually decreased over time in all groups. There was no significant

difference between control and PAE males at the timepoints observed in this study ($F_{(5,120)}$ = 0.37, p = 0.54, *ns*). However, as shown in Fig. 1d, the repeated measure analysis showed an interaction effect ($F_{(5,126)}$ = 2.85, p = 0.01) in females. The glucose levels were significantly elevated in PAE females from 30 min through 180 min compared to control females, indicating poor glucose metabolism.

3.3. Persistent effects of prenatal ethanol vapor exposure on splenic and circulating immune cell populations

To determine the impact of PAE on peripheral immune organs/cells in young adults (5 mo), we assessed the immune cell populations by flow cytometry.

Tregulatory cells (*Tregs*): A representative flow cytometry plot of Tregs in circulation is shown in Fig. 2a. There was no difference in circulating CD3+ T cells ($F_{(1,35)}$ = 0.1928, p = 0.6633, *ns* Fig. 2b) and Treg cells ($F_{(1,35)}$ = 2.660, p = 0.1119, *ns* Fig. 2c) due to treatment. There were, however, significant sex differences. While there was an increase in circulating CD3+ cells in females compared to males ($F_{(1,35)}$ = 10.770, p = 0.0023, Fig. 2b), circulating Tregs in females were decreased ($F_{(1,35)}$ = 10.720, p = 0.0024, Fig. 2c). A representative flow cytometry plot of Tregs in spleen is shown in Fig. 2d. Although the splenic CD3+ T cell population ($F_{(1,35)}$ = 0.03058, p = 0.8622, *ns* Fig. 2e) was unaffected with PAE, the splenic Tregs showed a significant decrease ($F_{(1,34)}$ = 4.731, p = 0.0367, Fig. 2f) in the PAE adults. This suggests that whereas splenic CD3+ cell numbers were unchanged, the proportion of Tregs in the CD3+ T cell population were decreased in this tissue. Additionally, a decrease in splenic Tregs in females was observed ($F_{(1,34)}$ = 7.300, p = 0.0107, Fig. 2f) though no difference was seen in splenic CD3+ T cells due to sex ($F_{(1,35)}$ = 0.7914, p = 0.3797, *ns* Fig. 2e).

Monocytes: A representative flow cytometry plot for spleen monocytes is shown in Fig. 3a. There was no change in the monocyte population in spleen due to PAE (Fig. 3b, $F_{(1,35)}$ = 0.05562, p = 0.8149, *ns*) or sex ($F_{(1,35)}$ = 1.784, p = 0.1903, *ns*). A representative flow cytometry plot for circulating monocytes is shown in Fig. 3c. As shown in Fig. 3d, while there was a significant increase in circulating monocyte population in PAE-males (interaction effect, treatment × sex, $F_{(1,35)}$ = 6.967, p = 0.0123, Fig. 3c), the pattern was reversed in PAE-females.

Other Immune Cells: We also measured helper T cells (CD4+), cytotoxic T cells (CD8+), B cells, and neutrophils in circulation and in the spleen as well as resident macrophages of the spleen. We found no differences due to PAE, though we did observe a number of significant sex differences in these immune cell populations (documented in Supplementary Table 1).

3.4. PAE alters cytokine profile in treatment- and sex-specific manner in peripheral tissues

The cytokine profiling and hierarchical clustering analysis using Pearson correlation (scale bar, red indicates higher and blue lower cytokine levels) revealed the biosignature of differential expression patterns based on sex and on *in utero* exposure status (Fig. 4).

Each column represents the average of a treatment group, and each row represents a tissue cytokine/chemokine that has been z-scored. Four clusters were identified (cytokines with similar expression patterns are grouped and color-coded). The first cluster, shown in blue, is composed of adipose and liver cytokines, and best defines PAE females which show an overall increase in these cytokines compared to control females and males. The second cluster, shown in purple, is the smallest and is composed of cytokines from the spleen (MIP-2, GM-CSF, Eotaxin) as well as leptin from the adipose and plasma. This second cluster shows that control males have an increase in cytokines/ chemokines compared to the control and PAE females. The third cluster outlined in red is composed of predominantly spleen cytokines and some overlap with plasma, liver, and adipose cytokines as well. This third cluster best defines the pattern in control males, overall, these cytokines are increased compared to PAE males and both groups of females. The fourth cluster, shown in green, is composed of a combination of adipose, liver, and plasma cytokines and best represents PAE males which shows an overall decrease in these cytokines compared to control males and both groups of females. IPA analysis was conducted on cytokines from the spleen and liver because these were the organs for which cytokines were the most significantly differentially regulated (19 for spleen and 18 for liver vs. 2 for plasma and 4 for adipose, $p < 0.05$). For both organs, “IL-17 Signaling” was a top canonical pathway identified as potentially perturbed (spleen p = 4.10E-37, liver p = 3.94E-39) with the related pathway of “Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-12A and IL-17F” (spleen p = 1.93E-36, liver p = 8.35E-40) also identified as significant in both organs. These pathways were also common between groups when subdividing by sex.

PAE alters inflammatory cytokines in adult mesenteric tissue and livera: Multiplex cytokine assay in adipose and liver shows a sex and treatment specific effect of a group of cytokines as shown in Fig. 4 (the cluster outlined in blue). In adult liver, PAE caused a significant decrease in GM-CSF, IL-6, IL-17A and fractalkine (Fig. 5a–d, main effect of treatment) in both males and females. Additionally, PAE-males showed reduction of more cytokines including IL-2, leptin and LIX (Fig. 6a–c, interaction effect). However, in PAE-females, there was a significant elevation (interaction effect) of a group of cytokines including IFN- γ, IL-1β, IL12-p70, IL-13, IL-18 and MCP-1 as shown in Fig. 7a–f (Supplementary Table 2 for adipose and liver depicts the detailed list of cytokines and chemokines with p and F values). Similarly, as shown in Fig. 8 a–c, there was a significant increase in IL-1α, IL-6, IL-12p70 (interaction effect, treatment × sex) and a significant decrease in VEGF (Fig. 8d) in the adipose tissue from adult PAE females compared to PAE males, with ethanol only uncovering sex differences after PAE.

PAE suppresses cytokines and chemokines in spleen and plasma from adult males not in adult females: Cytokine expression analysis as represented in the heatmap (Fig. 4, cluster outlined in purple) shows an immunosuppressive phenotype in PAE-males (Supplementary Table 3 shows the p and F values for each analyte). Briefly, EGF, Fractalkine, G-CSF, IFN- γ, IL-10, IL-13, IL-17A, IL-18, IL-1 α, IL-1β, IL-4, IP-10, Leptin, MIP-1 α and RANTES are significantly reduced (interaction effect, treatment × sex) in spleen of PAE-males. The plasma cytokine assay showed that fewer analytes were altered by PAE. There is a significant elevation of EGF due to PAE (Fig. 9a; main effect

of treatment) and a significant decrease of RANTES in PAE adults (Fig. 9b; main effect of treatment).

3.5. PAE and behavioral phenotypic differences in adulthood

PAE is an important modifier of adult behaviors (El Shawa et al., 2013), possibly by regulating the inflammatory mediators in brain and in circulation (Raineki et al., 2017). In this study, we sought to determine how prenatal alcohol vapor affects anxiety, social behaviors and recognition memory in adults.

PAE increases anxiety-like behavior in males to a greater extent than females: To assess anxiety, 4 mo. old PAE and control animals were placed in an open field apparatus for 60 min and the proportion of time spent in the center of the field is graphed in 10 min time bins. A 2-way repeated measures ANOVA (time as repeated measure) showed that males overall spent more time in the inner zone as compared to females. As shown in Fig. 10a, there was overall effect of time (over the 60 min testing period ($F_{(3.52)}$=5.136; p = 0.035), which was further analyzed by determining the slope of the (time) curve. In control males (−0.038) and females (−0.1072) the slope was not significantly different from 0 (p = 0.725, p = 0.256 respectively). Both PAE groups however had slopes that were significantly different from 0, with males displaying a greater deviation from 0 (−0.399, p = 0.0017) compared to females (−0.2038; p = 0.0007).

PAE diminishes social interaction in females but not males: The social interaction test is widely used to measure depressive behavior in rodents. The 4.5 mo old PAE and control animals were allowed to freely explore the 3-chamber apparatus and the time spent in the chamber containing the same-sex stranger rat was used to determine the social behavior of the test rat. The 2-way ANOVA showed that there is a significant interaction effect where PAE females spent significantly less time with the conspecific rat compared to the control females and the ethanol males (interaction effect, $F_{(1,34)}$ = 10.87, p = 0.002, Fig. 10b), indicative of less motivation to interact with a stranger rat or poor social preference in PAE females.

PAE reduces object recognition memory in adult males and females: PAE is known to cause deficits in cognition. In the present study, we used the novel object recognition task to determine the persistent effects of PAE on cognitive behavior in adults. As shown in Fig. 10c, PAE animals (irrespective of sex) showed significantly less preference to the novel object than the familiar object as indicated by low discrimination index ($F_{(1,35)}$ = 4.916, p = 0.0343).

3.6. Multilinear regression modeling to predict behavioral and physiological outcomes in PAE animals

Correlation analysis was performed in PAE animals to determine the variables that are most closely correlated with worse behavioral (NORT, Social interaction) and physiological (GTT) outcomes. Variables were initially z-score normalized across all samples to ensure equal weighting of predictors before proceeding with analysis. This correlation analysis was

then used to select variables best suited as predictors in multilinear regression models. All regression models are summarized in Supplementary Tables 4–10.

First, we identified good predictors of increased fasting glucose levels (Fig. 11a and b). For 3 month-old PAE animals, BEC of the dam was determined to be a good predictor ($F_{(1,14)}$ = 6.265, p = 0.0253, R^2 = 0.3092) with increased BEC of dam predicting a lower level of fasting glucose (Fig. 11a). However, while BEC of dam was a good predictor in 3 month old animals it was not in 5 month old PAE animals ($F_{(1,13)}$ = 0.6208, p = 0.4449, R^2 = 0.04557; data not shown). Instead the sex (males) of the animals ($F_{(1,9)}$ = 8.216, p = 0.0.0132, R^2 = 0.3834) and elevated leptin in the mesenteric adipose ($F_{(1,9)}$ = 6.978, p = 0.02684, R^2 = 0.4367) are shown to predict higher fasting glucose levels (Fig. 11b). Combined into a joint model, male sex and adipose leptin is more predictive ($F_{(2,8)}$ = 6.532, p = 0.02081, R^2 = 0.6202) of the fasting glucose. Predictors of glucose tolerance of 5 month old PAE animals were of additional interest (Fig. 11c). Area under the curve (AUC) of glucose blood levels was calculated for up to 180 min post-glucose injection with a smaller AUC representing a smaller peak and quicker return to baseline while a larger AUC represents a larger peak and longer time to return to baseline. Male sex predicted smaller AUC and therefore a quicker return to glucose homeostasis ($F_{(1,13)}$ = 9.68, p = 0.008265, R^2 = 0.4268). Higher levels of TNF- α in the liver predicted a slower return to baseline ($F_{(1,9)}$ =21.2, p =0.001300, R^2 =0.7020). Combining offspring sex and liver TNF- α into the same model improved the model ($F_{(2,8)}$ = 16.99, p = 0.001319, R^2 = 0.8094) with better predictive outcomes.

Subsequently, we identified good predictors of decreased anxiety-like behavior (or increased impulsivity), as determined by more time spent in the center of the open-field (Fig. 12a). Similar to the findings from the open-field test (Fig. 10a) we found that males (coded as 1) are more likely to spend longer time in the center of the open arena compared to females (coded as 0; $F_{(1,14)}$ = 8.196, p = 0.0125, R^2 = 0.3693). Additionally, we found that increase in normalized spleen weight ($F_{(1,10)}$ = 12.86, p = 0.0050, R^2 = 0.5626) and increase in liver IL-1 α ($F_{(1,10)}$ = 7.266, p = 0.02248, R^2 = 0.4208) predict a decrease in the time spent in center while increase in plasma fractalkine predicts more time spent in the center ($F_{(1,8)}$ = 7.042, p = 0.0291, R^2 = 0.4682). Furthermore, when all four predictors are combined to create a model, the overall model improved ($F_{(4,3)}$ = 18.85, p = 0.01856, R^2 = 0.9613), suggesting that the prediction of outcome is strengthened or that anxiety-like behavior could be mediated by a combination of these individual variables.

In the next set of regression analyses, we identified predictors of sociability, defined as increased time spent in the social chamber of the social interaction test (Fig. 12b). We found that increased Treg population of the spleen ($F_{(1,10)}$ = 7.863, p = 0.0187, R^2 = 0.4402) as well as increased circulating population of Tregs ($F_{(1,10)}$ = 30.35, p = 0.0003, R^2 = 0.7522) were good predictors of increased time spent in the social chamber with the conspecific rat for both young adult males and females. The combined model of splenic Tregs and circulating Tregs are more robust ($F_{(2,9)}$ = 28.87, p = 0.000122, R^2 = 0.8651).

Finally, we were interested in the BEC of dam as a sex-dependent predictor of behavioral outcomes in PAE animals. In the NORT, increased BEC of dam predicted lower NORT differential index in males (F(1,3) = 29.47, p = 0.0123, R^2 = 0.9076) but did not in females

($F(1,3) = 0.03562$, $p = 0.8623$, $R^2 = 0.01173$, Fig. 13a). In the social interaction test, increased BEC of dam predicted less time spent in the social chamber in females ($F_{(1,6)} = 15.73$, $p = 0.0074$, $R^2 = 0.7239$) whereas in males it did not ($F_{(1,6)} = 1.320$, $p = 0.2943$, $R^2 = 0.1804$, Fig. 13b). This suggests that biological sex is a crucial modifier of PAE-dependent deficits in adult behavior.

4. Discussion

Prenatal alcohol exposure has been shown to cause abnormal brain development and behavioral disorders in childhood, but the biological factors that promote the long-term consequences remain poorly characterized. In this study, we found that in females, spleen weight and splenic Tregs were reduced due to PAE, whereas circulating monocytes were elevated in PAE males. Moreover, male PAE offspring exhibited a general suppression in levels of circulating cytokines, but in female PAE offspring, we observed an elevation in inflammatory cytokines, particularly in tissues like mesenteric adipose and liver, that share enteric circulation. The present study also identified novel and sex-specific relationships between metabolic, immune, and behavioral domains as a result of PAE. For example, multilinear regression analyses revealed that high levels of adipose-derived leptin predicted high fasting glucose levels preferentially in PAE males, whereas high liver TNF-α levels were a strong predictor of impaired glucose metabolism preferentially in PAE females. In relation to behavioral outcomes, elevated circulating fractalkine and decreased liver IL-1α were predictive of decreased anxiety preferentially in PAE males. In contrast, in both PAE females and males, higher circulating and splenic Tregs were predictors of improved social behavior. These data suggest that basal immune-system function was altered in a sex-specific manner and that PAE also resulted in sex-specific effects on relationships between immune system, metabolism and behavior.

PAE in this study, from GD8 to GD19 in the rat, encompassed the development of two organs, the brain and immune system, that integrate allostatic responses to adverse life events ranging from psychosocial adversity to infectious and inflammatory disease (Danese and McEwen, 2012, Danese and Lewis, 2017). For the immune system, this exposure window encompasses the shift in hematopoiesis from the gonado-mesonephric region to fetal liver and spleen and then, to thymus and bone marrow (Landreth, 2002, Bertrand et al., 2006, Kuper et al., 2016). This period also brackets the period of neurogenesis and brain growth (Workman et al., 2013), and importantly, initial innervation of the developing thymus (Leposavić et al., 1992) and spleen (Ackerman et al., 1989, Bertrand et al., 2006, Anagnostou et al., 2007).

In rodents, PAE is an established cause of inflammation in the neonatal brain (Kane et al., 2011, Tiwari and Chopra, 2011, Noor and Milligan, 2018) and that pro-inflammatory state persists in the adult brain (Drew et al., 2015, Cantacorps et al., 2017). Increased brain inflammation in turn is associated with adverse neurobehavioral outcomes including anxiety and depression (Calcia et al., 2016, Kim and Won, 2017, Michopoulos et al., 2017, Rooney et al., 2020), which are significantly more prevalent in persons with FASD than in the general population (Himmelreich et al., 2020). Although these studies illustrate the adverse effects of developmental alcohol exposure on the neuroimmune response, the allostatic

adaptations of peripheral immune organs to PAE and their relation to neurobehavioral outcomes in adult offspring are unclear. Cytokines control immune cell proliferation, survival, and polarization (Frankenstein et al., 2006, Altan-Bonnet and Mukherjee, 2019). Consequently, the cytokine milieu of immune tissue compartments may provide insight into the persistent adaptations of those tissues to PAE. Therefore, we profiled both cytokines and cells in key immune organs in adult PAE offspring. We also examined immune alterations in mesenteric adipose tissue and liver because of their unique shared role as reservoirs for immune cells and mediators of gut-linked systemic inflammation (Meza-Perez and Randall, 2017, Stamataki and Swadling, 2020).

In our study, PAE males exhibited a significant, across-the-board decrease in cytokine levels in circulation and in immune organs compared to control males and females. It is possible that this cytokine suppression reflects ongoing and persistent inflammation, a hypothesis that is supported by several studies in children and adults with FASD, which have documented higher rates of acute and persistent systemic infections (Johnson et al., 1981, Gauthier et al., 2004, Linneberg et al., 2004, Libster et al., 2015). In contrast, adult PAE females exhibited elevation of pro-inflammatory cytokines and chemokines in mesenteric adipose tissue, including IL-1 α, IL-6, and IL-12p70. The female-specific induction of inflammatory mediators in this specific adipose tissue may be linked by the enteric portal vasculature to both gastro-intestinal and hepatic inflammation (Lautt, 2009). This hypothesis is consistent with our observation that liver cytokines, IL-13, IFN- γ, IL-18, IP-10, and IL-12p70, were also elevated in PAE females compared to the controls.

The portal circulatory link between mesenteric adipose tissue and liver also influences glucose metabolism (Sabio et al., 2008, Foster et al., 2011, Rytka et al., 2011). PAE has been documented to elevate the risk for metabolic disease, including glucose intolerance in adults (Chen and Nyomba, 2003, Probyn et al., 2013), and insulin resistance in females (Nguyen et al., 2019). Although insulin resistance is a major mechanism for glucose intolerance, inflammation also contributes to impaired glucose metabolism (Tsalamandris et al., 2019). For instance, activation of a JNK1 (c-Jun N-terminal Kinase), a stress-response in adipose tissue, results in IL-6 secretion into hepatic portal circulation, and insulin resistance (Sabio et al., 2008). Similarly, removal or transplantation of visceral adipose tissue influences glucose tolerance, due to linkage to portal venous circulation (Foster et al., 2011, Rytka et al., 2011). In the present study, PAE-females experienced glucose intolerance in the glucose challenge test compared to the control females, an outcome that may be linked to mesenteric adipose and hepatic elevations in inflammatory cytokines like IL-1v, IL-6, and IL-12p70, and to hepatic upregulation of IFN- γ, and IL-13 that modulate glucose intolerance and insulin resistance (Darkhal et al., 2015, Sestan et al., 2018). Hepatic and mesenteric adipose levels of the pro-angiogenic factor VEGF, were also elevated in PAE females, an effect that is also related to insulin resistance (Wei et al., 2013, Wu et al., 2014). TNF- α originating from Kupffer cells of the liver contributes to insulin resistance by al α ering phosphorylation of proteins involved in insulin signaling mechanism in hepatocytes (Taeye et al., 2007). The adipokine leptin also contributes to insulin resistance by impairing the interaction between the hormone and its receptor, and subsequent downstream signaling in adipocytes (Müller et al., 1997, Walder et al., 1997, Pérez et al., 2004). This supports our findings that elevation of these cytokines, TNF- α in the liver and leptin in the mese α teric adipose, predicted

altered glucose metabolism in our PAE animals. Although the mechanistic relationship between the inflammatory markers and insulin resistance was not tested in the present study, our data support the hypothesis that the dysregulation in the immune system contributes to glucose intolerance in PAE females. Furthermore, the prolonged inflammatory milieu in the mesenteric adipose tissue could lead to impairment in the intestinal/gut barrier, and eventually, neuroinflammation and behavioral dysregulation.

The link between PAE and neurobehavioral deficits, including deficits in attention, cognition, memory and adaptive behaviors, is well established (Streissguth et al., 1990, Mattson et al., 2001, Crocker et al., 2009, Green et al., 2009, Mattson et al., 2011, Lynch et al., 2015). Pre-clinical studies in animal models have largely replicated these findings (for example (Hellemans et al., 2008, Cullen et al., 2013)). In our study, we observed important sex-specific effects of PAE on distinct behavioral domains. For example, PAE males exhibited increased anxiety-like behavior, while PAE reduced social behavior in PAE females, but not in males. Finally, PAE resulted in cognitive impairment in both sexes as determined by the ability to recognize novel objects, a non-spatial task. In the case of social behavior, our findings are consistent with the report that gestational alcohol exposure decreased social motivation in female offspring (Varlinskaya and Mooney, 2014), though others have documented reduced social investigation in PAE males rather than females (Hamilton et al., 2010), perhaps attributable to lower levels of exposure or strain differences. In our study, regression models also identified important sex differences in the relationship between maternal BEC and behavior. Higher maternal BECs resulted in male offspring that were more susceptible to learning and memory impairment, while female offspring were more susceptible to deficits in domains related to mood disorders and depression. These differences in behavioral outcomes highlight the importance of offspring sex as a variable when evaluating the long-term consequences of PAE in adults.

Regression models also identified plasma fractalkine (CX3CL1) as predictor of time spent in the center of an open field, with lower plasma levels of fractalkine predicting more anxiety-like behavior (spending time in the periphery). In the central nervous system, the receptor for fractalkine, CX3CR1, is restricted to microglial cells and, when stimulated, suppresses microglia activation (Lauro et al., 2015). Fractalkine-Cx3CR1 signaling has been shown to regulate synaptic plasticity and cognitive function, playing a neuroprotective role. Additionally, fractalkine has been implicated in anxiety- and depressive-like behaviors resulting from maternal stress in a restraint stress model, with a persistent decrease in brain fractalkine expression adult rat offspring (Ślusarczyk et al., 2016), and diminished anxiety behavior in adult offspring after intracerebral ventricular injection of fractalkine (Ślusarczyk et al., 2016). Additionally, in the same regression model, we found that higher levels of liver IL-1 α predict anxiety-like behavior and time spent in the periphery of the open field. IL-1 α is similar to IL-1β in that both act on the same receptors, triggering the same pathways, except IL-1 α is membrane bound, acting in a paracrine manner, while IL-1β is secreted, acting as an endocrine molecule (Gabay et al., 2010). This means it is important to focus on the paracrine consequences of IL-1α in the liver, as it was this form that was predictive of anxiety-like behavior. Relevant to our model, IL-1 α induces production of TNF-α and IL-6 (Kim et al., 2013), and it has been posited that TNF-α and IL-6 mediate the transduction of liver inflammation to neurobehavioral changes (D'Mello and Swain, 2011).

Furthermore, administration of TNF-α and IL-6 to healthy volunteers resulted in transient mood depression and anxiety (Eisenberger et al., 2010). Collectively, this suggests that our model implicating fractalkine and IL-1 α as predictors of anxiety-like behavior may have an underlying mechanistic linkage that needs further assessment.

Importantly, PAE resulted in a significant decline in Treg populations in spleen, in both males and females, along with a significant decrease in spleen-to-body weight ratios and no changes in other measured immune cell populations of the spleen (e.g. helper T cells, cytotoxic T cells, B cells, neutrophils, monocytes, macrophages). Additionally, pathway analysis identified Th17 cells as a specific target of PAE. It is likely that the loss of splenic Tregs and potentially Th17 cells is a direct outcome of PAE, which in our model occurred during the period of thymic maturation (Landreth, 2002, Kuper et al., 2016). Concurrent dysregulation of Tregs and Th17 cells might suggest an overall dysregulation of the ability of T helper cell differentiation. This is an important outcome since the early and abundant appearance of Tregs in fetal life is linked to the development of peripheral immune tolerance for self (Burt, 2013). Consequently, decreased numbers of Tregs may predict the development of autoimmune disease and inflammation. While these data and other studies (Noor et al., 2020) suggest that Treg dysregulation may mediate some inflammatory effects of PAE, further studies using *ex vivo* stimulation models will be needed to uncover changes in T-cell function that are not captured by assessing cell population changes. In this present study, regression models showed that higher levels of Treg cells in circulation and in spleen predicted increased levels of social interaction in both male and female PAE offspring. While this model may have identified associative relationships between a lymphocyte sub-population and behavior, the literature suggests a causal linkage. For instance studies have documented a smaller Treg population (Li et al., 2010) and a pro-inflammatory profile (Calcia et al., 2016, Kim and Won, 2017) in patients with Major Depressive Disorder (MDD). The causal linkage is shown by cell-transfer studies, where T-cells transferred from mice stressed in a chronic social-defeat paradigm into stress-naïve mice reduced anxiety- and depressive-like behavior, as well as the expression of several pro-inflammatory cytokines (Brachman et al., 2015), while depletion of Tregs in mice resulted in an increase in depressive-like behavior as well as an increase in pro-inflammatory cytokines (Kim et al., 2012).

Collectively, our findings suggest that PAE-mediated alterations in peripheral immune organs and cytokine profiles are associated with decreased glucose metabolism and neurobehavioral impairment in adult offspring. Moreover, sex differences in PAE-linked cytokine expression, and the suppression of splenic Tregs, may elevate the risk for autoimmune disorders and explain variations in FASD-associated behaviors. These data are consistent with a number of previous studies that link immune system dysfunction to neuropathy and to adverse behavioral outcomes due to PAE in animal models (Pascual et al., 2017, Raineki et al., 2017, Sanchez et al., 2017, Noor et al., 2020), and to adverse neurodevelopmental outcomes in children with FASD (Bodnar et al., 2020). Moreover, they suggest either genetic sex and/or endocrine environment modify the relationship between immune system, metabolism and behavior as has been documented in other preclinical studies (Yao and Gregoire Nyomba, 2007, Bodnar et al., 2016, Nguyen et al., 2019). Future studies are needed to identify specific molecular mechanisms that link immune dysfunction

and glucose intolerance due to PAE to long-term neurobehavioral impairments in adults with FASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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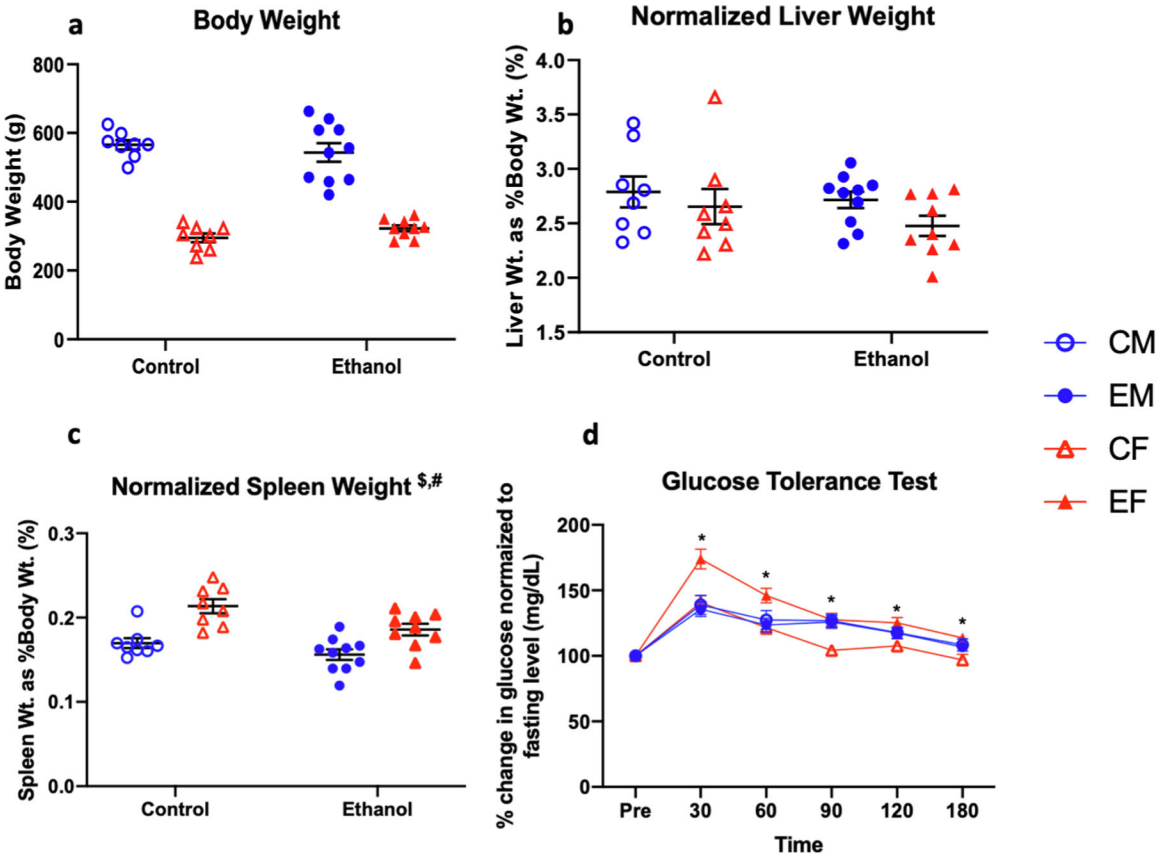

Fig. 1.

Effect of PAE on body mass, organ weights and glucose tolerance. (a) PAE did not affect the body mass in adult male and female rats. (b) PAE did not have an effect on liver weight (normalized to animal body weight) in both males and females. (c) PAE resulted in a reduction of spleen weight normalized to the body weight (treatment effect, $p = 0.005$). (d) PAE caused glucose intolerance in adult females as indicated by higher glucose levels at all time points compared to control females. CM = control male, EM = ethanol male, CF = control female, EF = ethanol female. Sample size n = 8–10 in each group; $^{\#}$, main effect of sex, $^{\$}$, main effect of treatment *$p < 0.05$; **, $p < 0.001$.

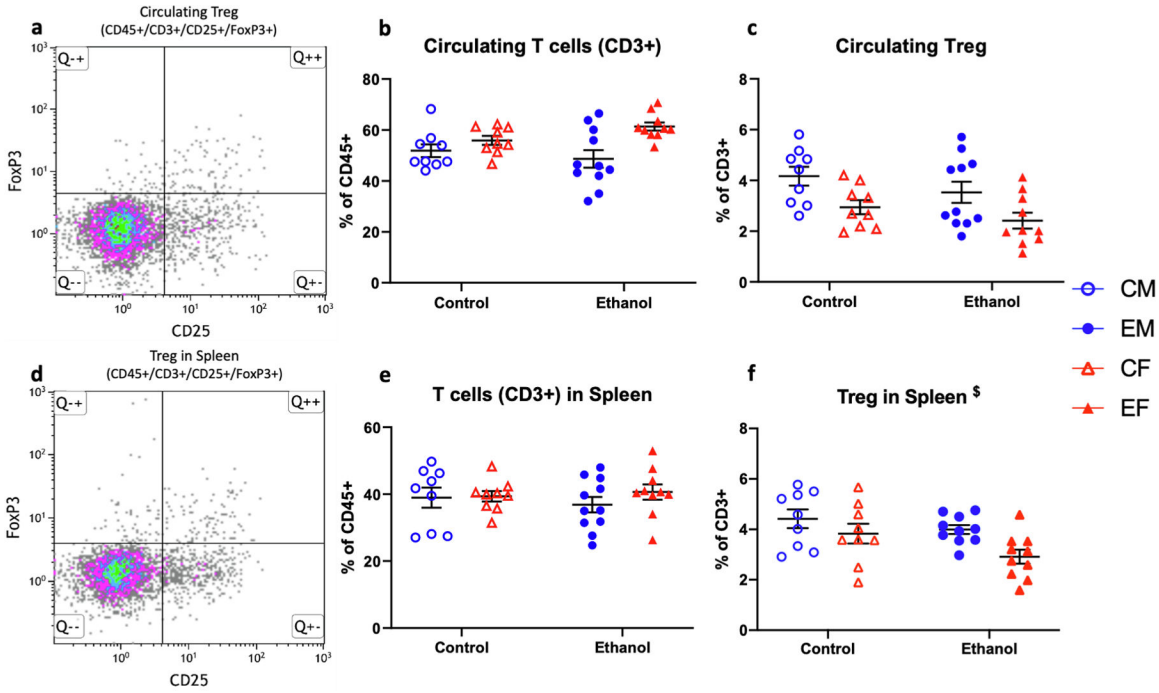

Fig. 2.

Effect of PAE on CD3+ T cell and Treg populations in circulation and in the spleen. (a). Representative flow cytometry plot of Tregs in circulation. (b&c). There was no difference in the circulating CD3+ T cell or Treg populations in PAE animals compared to the control groups. (d). Representative flow cytometry plot of Tregs in the spleen. (e). PAE had no effect on CD3+ T cell population of the spleen compared to control animals. (f). PAE reduced the Treg population of the spleen (treatment effect, $p = 0.04$) in both male and female offspring compared to controls. Sample size n = 8–10; $, main effect of treatment, $p < 0.05$.

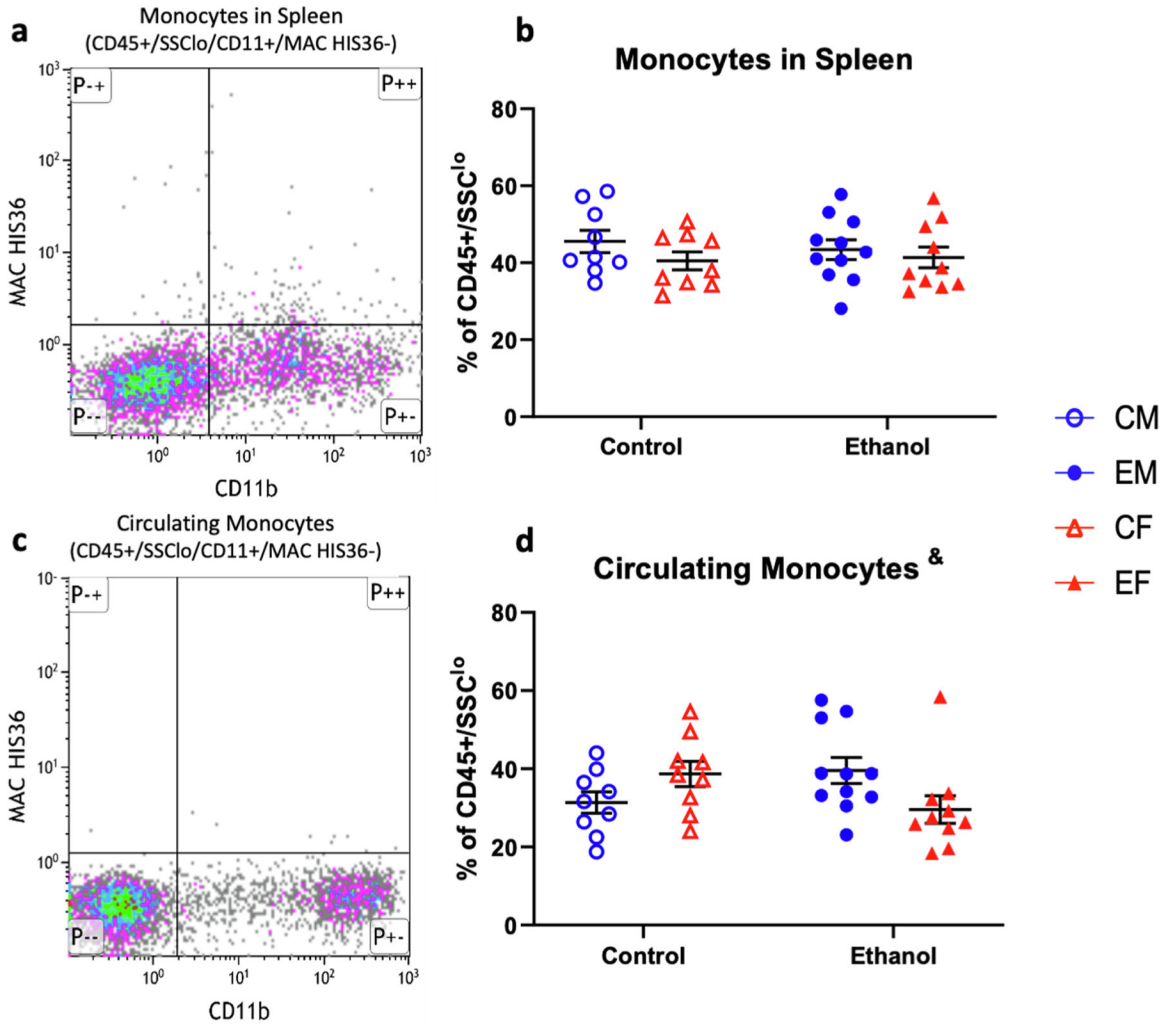

Fig. 3.

Effect of PAE on monocyte populations in circulation and spleen. (a). Representative flow cytometry plot of monocytes in the spleen. (b). The monocyte population in the spleen was not altered due to PAE compared to control animals. (c). Representative flow cytometry plot of monocytes in the circulation. (d). PAE increased circulating monocytes in males and decreased in females (an interaction effect, treatment × sex, $p = 0.01$). Sample size n = 8–10; &, interaction effect.

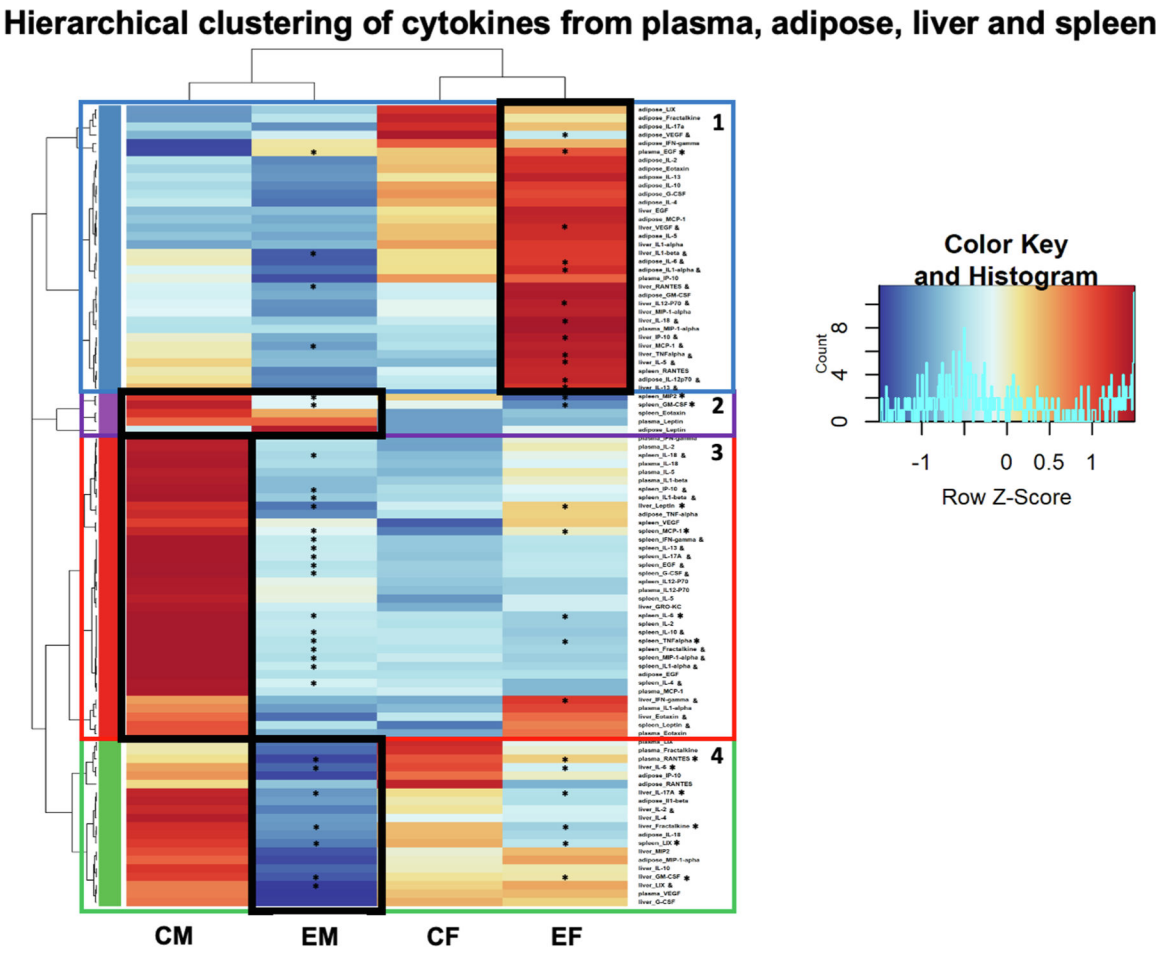

Fig. 4.

Global cytokine profiles of control and PAE rats. Heatmap of cytokines from plasma, adipose, liver, and spleen. Hierarchical clustering using Pearson correlation was used to identify unique cytokine clusters, cytokines with similar expression levels are grouped and color-coded (1. Blue, 2. Purple, 3. Red, 4. Green). The first cluster (blue) is composed of adipose and liver cytokines shows higher expression of these cytokines in PAE females compared to PAE males and the controls. The fourth cluster (green) composed of plasma, liver, and adipose cytokines, shows an overall decrease in PAE males. The third cluster and the second cluster represent the elevated levels of cytokines in control males compared to the ethanol males and the control females. MC = male control, ME = male ethanol, FC = female control, FE = female ethanol. Each group is the average of all animals within that group (n = 8–10) and cytokine expression has been Z-scored to allow comparison. *, main effect of treatment; &, interaction effect; $p < 0.05$.

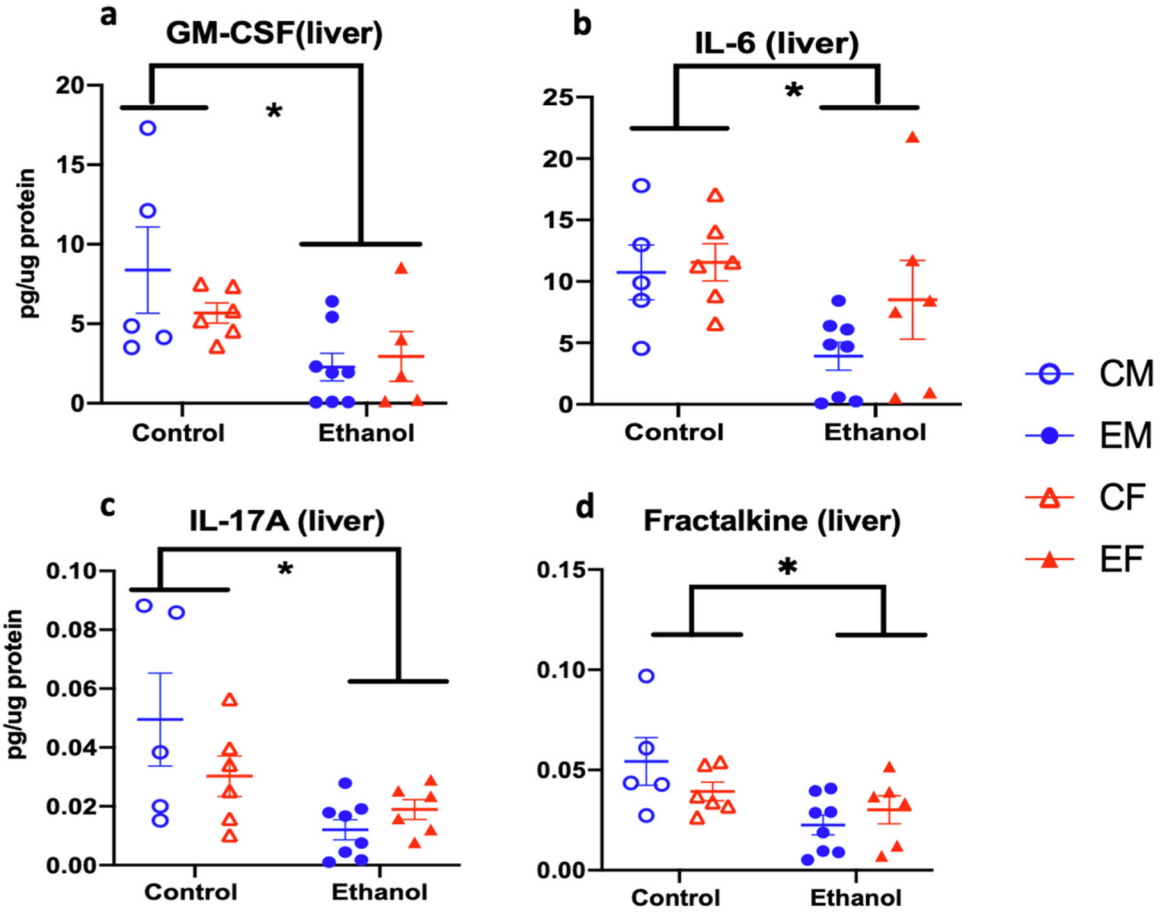

Fig. 5.

Effect of PAE on liver cytokines in adult offspring. There was a main effect of treatment on a group of cytokines resulting in decreased levels of GM-CSF (a), IL-6 (b) and IL-17A (c) and fractalkine (d) in the liver of both PAE males and females. Sample size n = 5–8; *: $p < 0.05$.

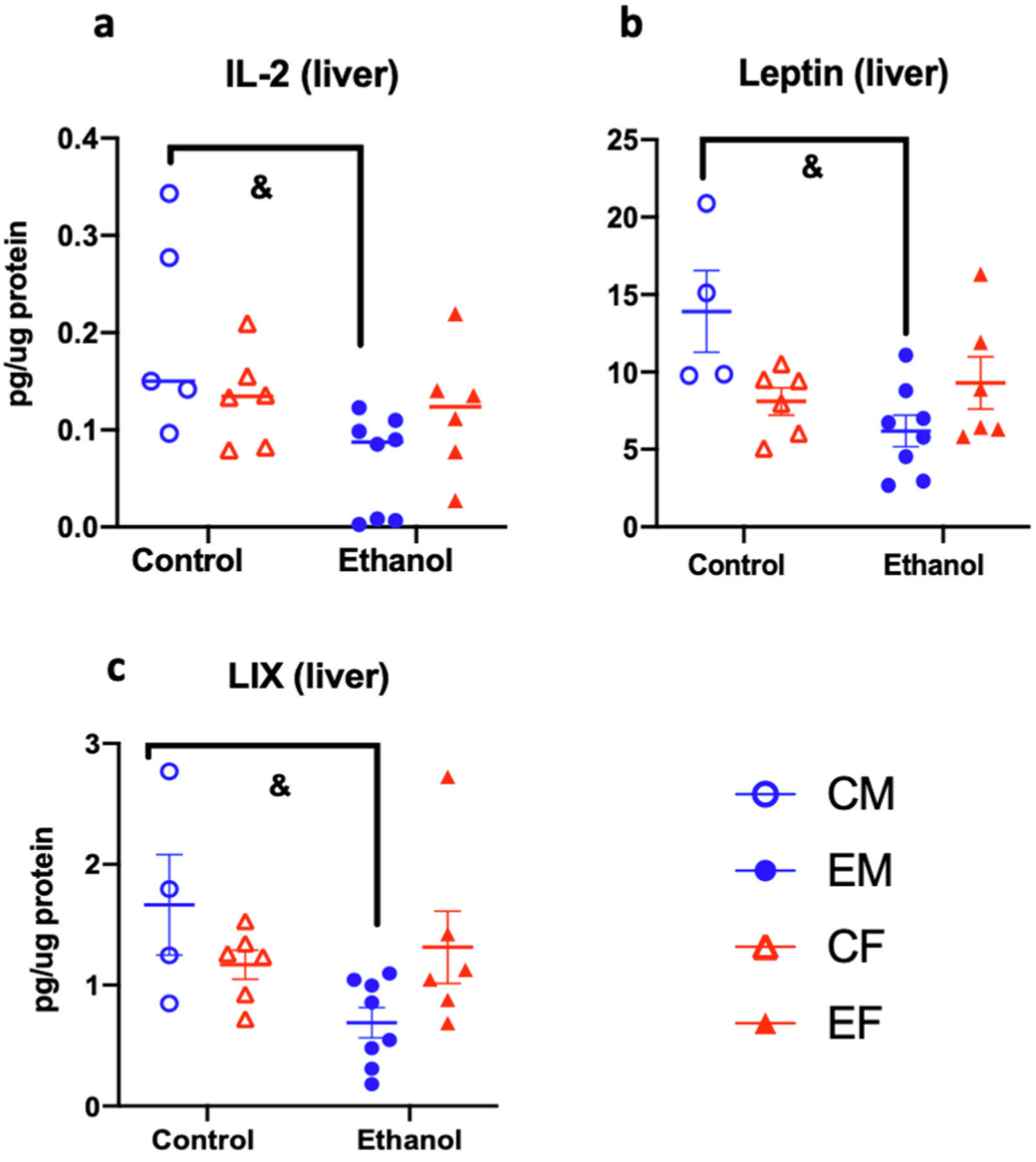

Fig. 6.

Effect of PAE on liver cytokines specific to males. There was an interaction effect (PAE × Sex), resulting in decreased levels of IL-2 (a), leptin (b) and LIX (c) in the liver of PAE males. Sample size n = 5–8; &: interaction effect, *: $p < 0.05$.

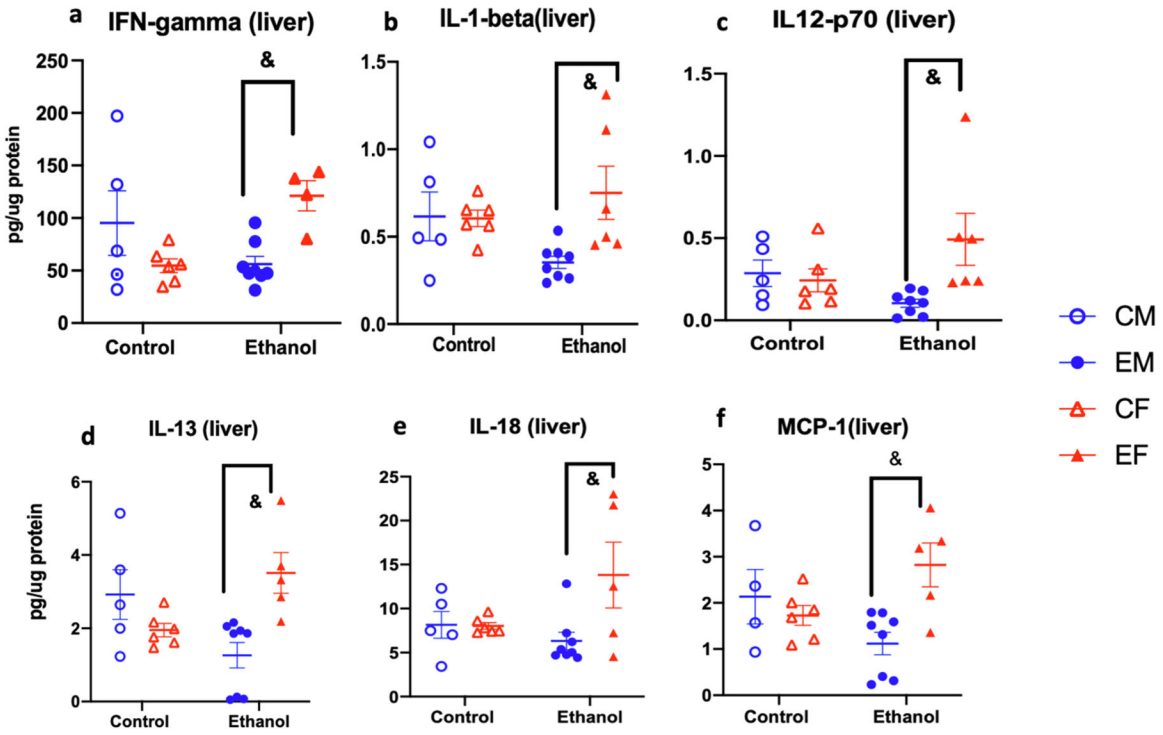

Fig. 7.

Effect of PAE on liver cytokines specific to females. There was an interaction effect (PAE × Sex), resulting in increased levels of IFN- γ (a), IL-1β (b), IL-12p70 (c),IL-13 (d), IL-18 (e), MCP-1 (f) in the liver of PAE females. Sample size n = 5–8; &: interaction effect, *: $p < 0.05$.

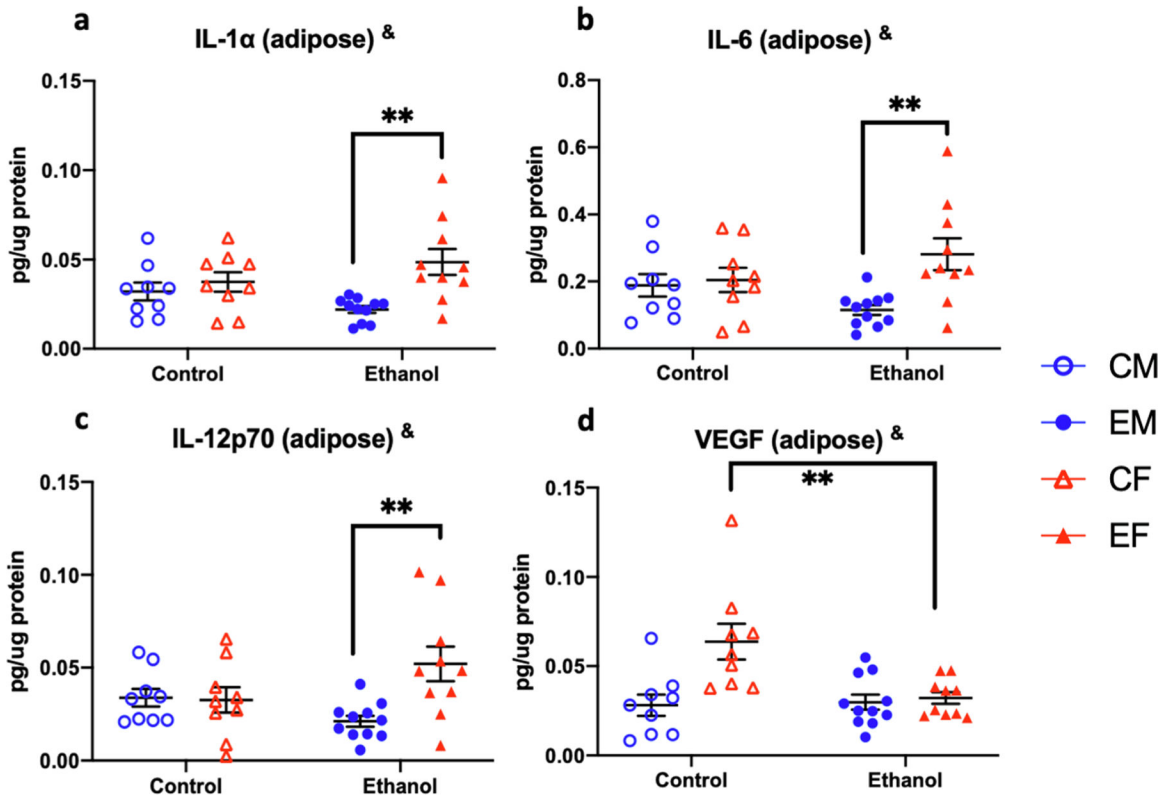

Fig. 8.

Effect of PAE on mesenteric adipose cytokines. There was an interaction (PAE × Sex) on IL-1 α (a), IL-6 (b), and IL-12p70 (c) in adipose tissue. Post-hoc analyses revealed PAE females have increased levels of IL-1 α, IL-6, and IL-12p70 compared to PAE males. (d). An interaction effect (PAE × sex) was observed in VEGF in adipose tissue also. Post-hoc analyses showed PAE females had decreased levels of VEGF compared to control offspring. Sample size n = 8–10; $^{\#}$, main effect of sex, $^{\&}$, interaction effect, **, $p < 0.05$.

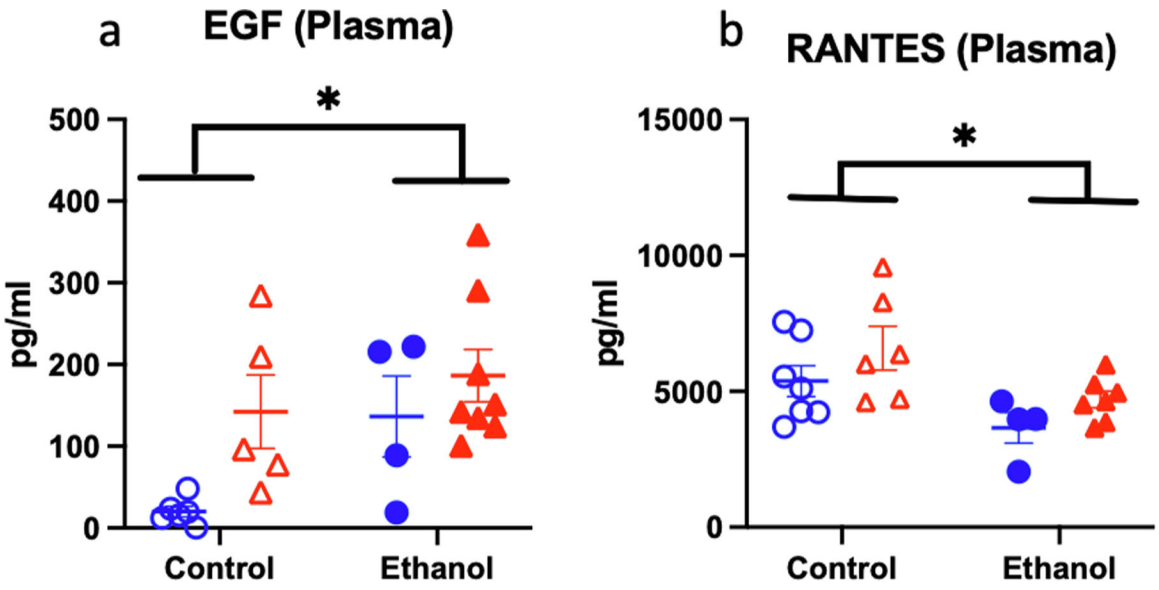

Fig. 9.

Effect of PAE on plasma cytokines. (a) EGF was significantly increased in both PAE-males and females (main effect of treatment) (b) RANTES was significantly decreased by PAE in both males and females. Sample size n = 4–6/group *: main effect of treatment, $p < 0.01$.

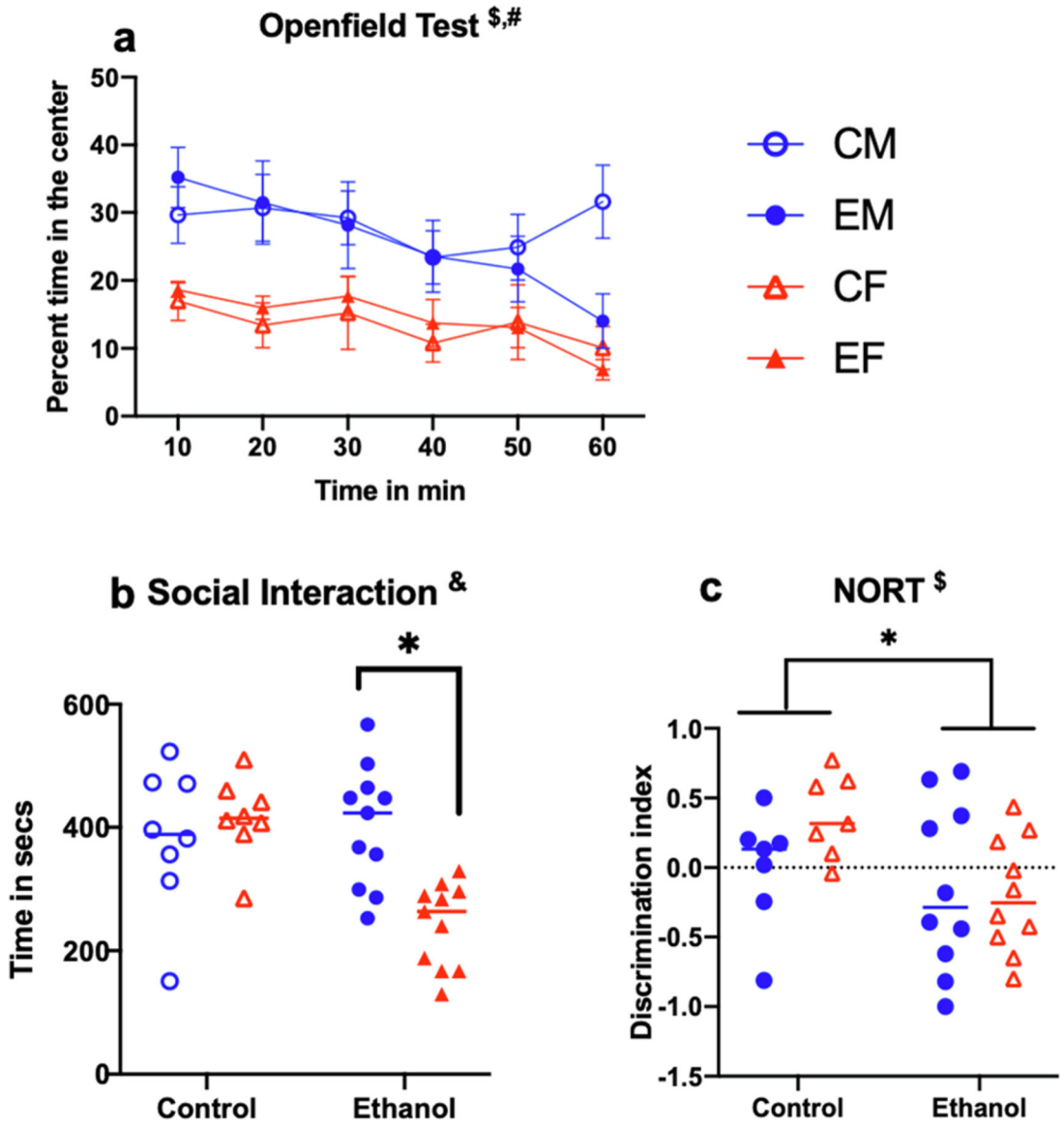

Fig. 10.

Effect of PAE on neurocognitive outcomes. a. Open-field analysis showed that males spent more time in center. The effect of time was further examined by assessing the slope of the curve (change in % time spent in the center over time). This analysis indicated that the slope for control males and females was not significantly different from 0. However, the slope in both the PAE groups was significantly different from 0, with a more pronounced deviation in PAE males. b. Social interaction test revealed significantly less preference for an interaction with a stranger rat in the PAE females test (interaction effect, treatment × sex). c. Novel object recognition test indicates that PAE animals have significant impairment in the cognitive ability to discriminate the novel object compared to the control animals (main effect of treatment). Sample size n = 8–10; $, main effect of treatment, #, main effect of sex &, interaction effect, * $p < 0.05$.

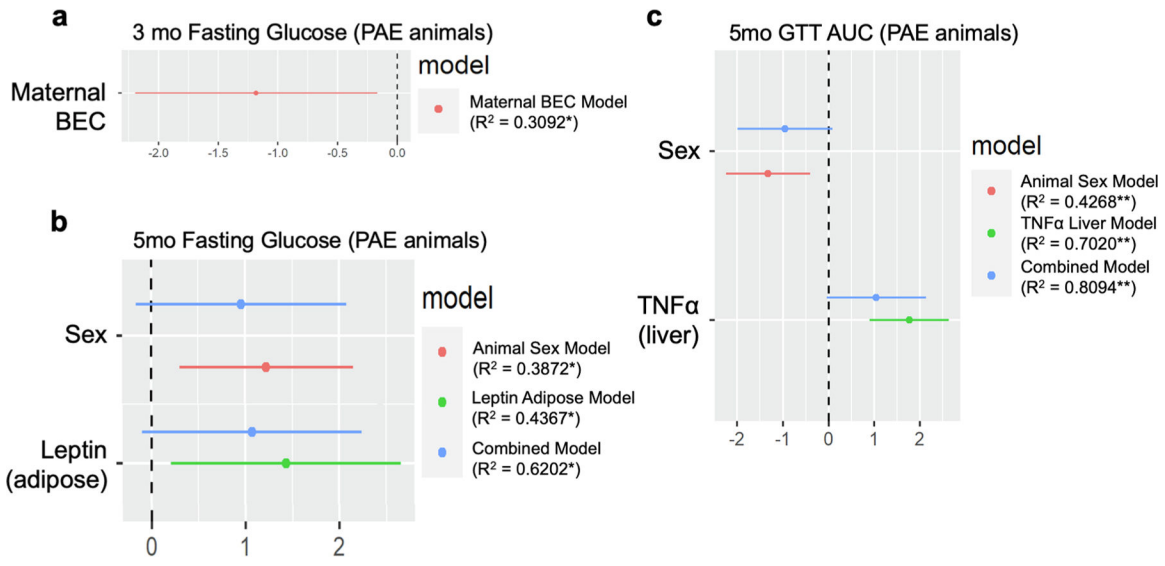

Fig. 11.

Multilinear regression models to predict physiological outcomes in PAE animals. (a). Higher maternal BEC predicted lower fasting glucose of PAE animals at 3mo. (b). Male sex (female coded 0, male coded 1) and higher leptin levels in mesenteric adipose tissue predicted higher fasting glucose levels of PAE animals at 5mo. (c). Female sex and higher TNF- α levels of the liver predicted a longer time to recover from glucose bolus in PAE animals at 5 mo. Sample size n = 6–10.

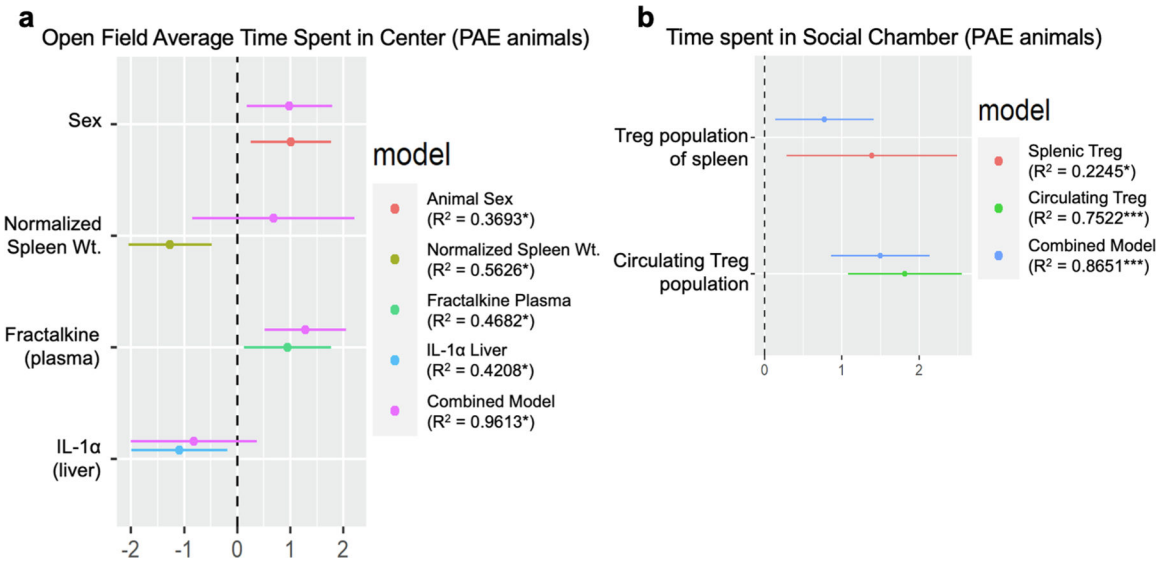

Fig. 12.

Multilinear regression models to predict behavioral outcomes in PAE animals. (a). In females, lower spleen weight, lower levels of plasma fractalkine, and higher liver IL-1 α predicted less time spent in the center of the open-field and more anxiety-like behavior in PAE animals at 5mo. (b). Lower Treg populations in the spleen and in circulation predicted less time spent in the social chamber, with a conspecific, and more depression-like behavior in PAE animals at 5mo. Sample size n = 6–10.

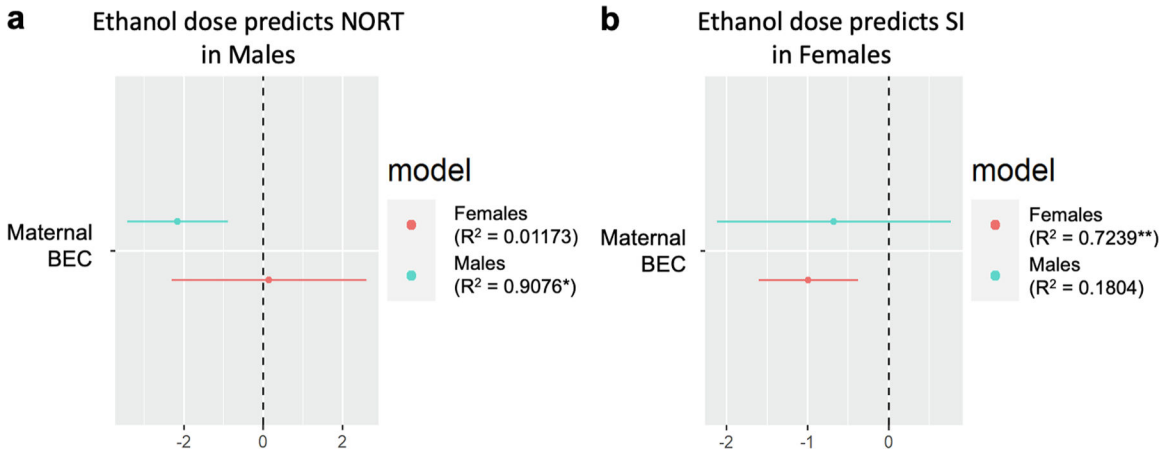

Fig. 13.

Multilinear regression models reveal sex differences in behavioral outcomes in PAE animals due to ethanol dose. (a). Higher maternal BEC predicted lower NORT DI in PAE male offspring at 5 mo but is not predictive in females. (b). Higher maternal BEC predicted less time spent in the social chamber in PAE female offspring at 5mo but was not predictive in males. Sample size n = 6–10.