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Discrete role for maternal stress and gut microbes in shaping maternal and offspring immunity

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ABSTRACT

Psychosocial stress is prevalent during pregnancy, and is associated with immune dysfunction, both for the mother and the child. The gut microbiome has been implicated as a potential mechanism by which stress during pregnancy can impact both maternal and offspring immune function; however, the complex interplay between the gut microbiome and the immune system is not well-understood. Here, we leverage a model of antimicrobial-mediated gut microbiome reduction, in combination with a well-established model of maternal restraint stress, to investigate the independent effects of and interaction between maternal stress and the gut microbiome in shaping maternal and offspring immunity. First, we confirmed that the antimicrobial treatment reduced maternal gut bacterial load and altered fecal alpha and beta diversity, with a reduction in commensal microbes and an increase in the relative abundance of rare taxa. Prenatal stress also disrupted the gut microbiome, according to measures of both alpha and beta diversity. Furthermore, prenatal stress and antimicrobials independently induced systemic and gastrointestinal immune suppression in the dam with a concomitant increase in circulating corticosterone. While stress increased neutrophils in the maternal circulation, lymphoid cells and monocytes were not impacted by either stress or antimicrobial treatment. Although the fetal immune compartment was largely spared, stress increased circulating neutrophils and CD8 T cells, and antibiotics increased neutrophils and reduced T cells in the adult offspring. Altogether, these data indicate similar, but discrete, roles for maternal stress and gut microbes in influencing maternal and offspring immune function.

1. Introduction

Psychosocial stress is prevalent during pregnancy and is a risk factor for the development of perinatal mental health disorders, including anxiety disorders and major depressive disorder (Lancaster et al., 2010; O'Hara and Wisner, 2014). In addition to maternal psychiatric outcomes, psychosocial stress during pregnancy has been associated with offspring immune dysfunction later in life (Merlot et al., 2008), including increased risk of hospitalization with severe infectious diseases (Nielsen et al., 2011). Although the mechanisms underlying fetal programming effects of stress during pregnancy are not well defined, there is emerging evidence that a complex interplay between the microbiome, glucocorticoids, and the immune system contribute to these effects (Chen and Gur, 2019; Gur et al., 2015).

The microbiome consists of the genetic material of all commensal microbiota, including bacteria (bacteriome), fungi (mycobiome), archaea, and viruses, that colonize host tissues, and interacts bi-directionally with the host to shape immune function (Lambring et al., 2019). Specifically, the gut microbiome closely communicates with the immune system directly through interactions with the gut-associated

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lymphoid tissue (Rivera-Amill, 2014) as well as indirectly through microbial metabolites (Lambring et al., 2019). Although the microbiome generally remains stable, it can be altered by factors such as diet (David et al., 2014) and psychosocial stress (Bailey et al., 2011; Galley et al., 2015; Galley and Bailey, 2014; Knowles et al., 2008). This holds true during pregnancy, as previous work from our group and others have demonstrated that stress during pregnancy disrupts the maternal gut bacteriome (Antonson et al., 2020; Gur et al., 2019, 2017; Jašarević et al., 2017). Furthermore, studies have shown that maternal stress is associated with offspring gut bacterial composition, both clinically (Zijlmans et al., 2015) and in animal models (Bailey et al., 2004; Gur et al., 2017, 2019). These changes in the maternal gut bacteriome following stress exposure have also been associated with adverse offspring behavioral and neuroimmune outcomes later in life (Gur et al., 2017, 2019), suggesting that gut bacteria may play a role in stress-induced fetal programming.

In addition to this direct link between stress, the microbiome, and immune development, there is a host of evidence that perinatal microbes play a critical role in immune development aside from the context of stress. Indeed, studies using germ-free mice, which lack microbes on any mucosal surface, have shown that commensal microbes regulate various immune functions, including myelopoiesis, response to enteric or peripheral infections, and microglial maturation in the developing brain (Kennedy et al., 2018; Pronovost and Hsiao, 2019). Furthermore, studies using antibiotics to reduce the bacterial load within the maternal gut have shown that perinatal antibiotics treatment reduces elements of innate and adaptive immunity in the offspring (Champagne-Jorgensen et al., 2020; Deshmukh et al., 2014; Gonzalez-Perez et al., 2016). Clinically, perinatal antibiotics use has been associated with increased risk of severe infection (Miller et al., 2018) along with increased risk for developing immune-mediated atopic diseases (Lapin et al., 2015; Qu et al., 2021; Tsakok et al., 2013). Although the data on the impact of the maternal gut mycobiome on the offspring is more limited, there is one study using antifungals showing that disrupting the mycobiome early in life impacts offspring immune development as well (Moser et al., 2001).

Although prenatal stress and antibiotics treatment are well known to impact offspring immune development, the *interaction* between maternal stress and the microbiome in shaping offspring immune function has yet to be fully defined. Furthermore, most existing studies have used antibiotics alone, without accounting for the resulting increase in fungal abundance (Dollive et al., 2013; Samonis et al., 1993). Thus, in this study, we used our established mouse model of prenatal restraint stress in conjunction with a minimally absorbed broad-spectrum cocktail of antibiotics and antifungals to reduce the microbial load within the gastrointestinal tract to examine the interaction between maternal stress and the maternal gut microbiome on maternal and offspring immunity. Given our previous findings that maternal stress impacts the maternal and offspring bacteriome and is associated with offspring neuroinflammation, we hypothesized that stress would disrupt maternal and offspring immune compartments in a microbe-dependent manner. However, in contrast to our hypothesis, our results indicate that prenatal stress and antimicrobial treatment have largely distinct roles in shaping maternal and offspring immunity.

2. Methods

2.1. Animals and experimental design

Adult nulliparous female and male C57BL/6 mice were ordered from Jackson Laboratories (Bar Harbor, ME) and housed in conventional facilities at the Ohio State University. Mice were acclimated to the facilities for at least one week prior to breeding, and all mice were singly housed to prevent confounding effects of coprophagia by cohoused mice on microbiome-related outcomes.

For breeding, female mice were paired monogamously with male mice and checked for the presence of vaginal plugs each morning to indicate copulation. The morning of plug detection was designated embryonic day (E)0.5, at which point females were separated from males and returned to their home cage. Body mass was measured daily, and a weight gain of 1 g by E7.5 was used as confirmation of pregnancy.

On E7.5, pregnant mice (dams) were randomly assigned to receive an antibiotic cocktail through the drinking water or a vehicle control. The antimicrobial cocktail consisted of neomycin sulfate (Sigma Aldrich, St. Louis, MO, 1 mg/mL), vancomycin (Sigma Aldrich, St. Louis, MO, 0.5 mg/mL), meropenem trihydrate (Neta Scientific, Hainesport, NJ, 1 mg/mL), and nystatin (Sigma Aldrich, St. Louis, MO, 1 mg/mL), which were dissolved in reverse osmosis water and administered to the dams *ad libitum* in a water bottle. The vehicle control mice received reverse osmosis water in water bottles for the duration of the experiment. Cages were changed on E7.5 to prevent the mice from coprophagia of pretreatment fecal pellets. Water bottles and contents were replaced every 5 days until the end of treatment, either at tissue collection on E17.5 or until parturition. Water consumption was determined by measuring the weight of the water bottle at the start and end of treatment. Following parturition, all mice received reverse osmosis water.

On E10.5, mice in each condition were randomly assigned to undergo restraint stress or remain undisturbed as the non-stressed control group. As previously described (Antonson et al., 2020; Chen et al., 2020; Gur et al., 2017, 2019), the dams in the stress group were restrained in 50 mL conical tubes for 2 h each day between 9 a.m. and 12 p.m. from E10.5–E16.5.

In the first set of experiments, dams were euthanized on E17.5 for tissue collection (Group 1; Fig. 1A). In the second set of experiments, dams underwent parturition and pups were weaned on postnatal day (P) 28 and cohoused with same-sex littermates until tissue collection in adulthood (Group 2; Fig. 1A). The Group 1 timepoint included five cohorts of animals, and the Group 2 timepoint included three separate cohorts of animals.

All experiments were performed according to the principles outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Ohio State University.

2.2. Tissue collection

For Group 1, dams were euthanized on E17.5 using CO_2. Whole blood was collected by cardiac puncture into EDTA-coated tubes on ice for flow cytometry or non-coated tubes at room temperature for serum. Intestinal tissue, specifically, distal ileal tissue 1 inch proximal to the cecum and the distal half of the colon with contents removed, and spleens were dissected from the dams. The uterus was excised, and placentas and fetal brains were dissected. All tissues were frozen on dry ice and then stored at −80 °C until further processing. Fetuses were microdissected and identification of testes or a uterine horn within the abdominal cavity was used to determine fetal sex. The serum tubes were left at room temperature for 30 min to allow for clotting to occur, after which the tubes were centrifuged at 13,300 rpm at 4 °C for 15 min and serum was collected and stored at −80 °C.

For Group 2, adult offspring were euthanized at P84-97. Whole blood was collected by cardiac puncture into EDTA-coated tubes on ice for flow cytometry.

2.3. Quantitative Real-Time PCR

Total RNA was extracted from whole tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentration was determined using the Nanodrop One Spectrophotometer (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 2 μg of RNA per 20 μL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR reactions were performed using TaqMan Assays following the protocol of the Taqman Fast Advanced Master Mix (Applied Biosystems, Foster City,

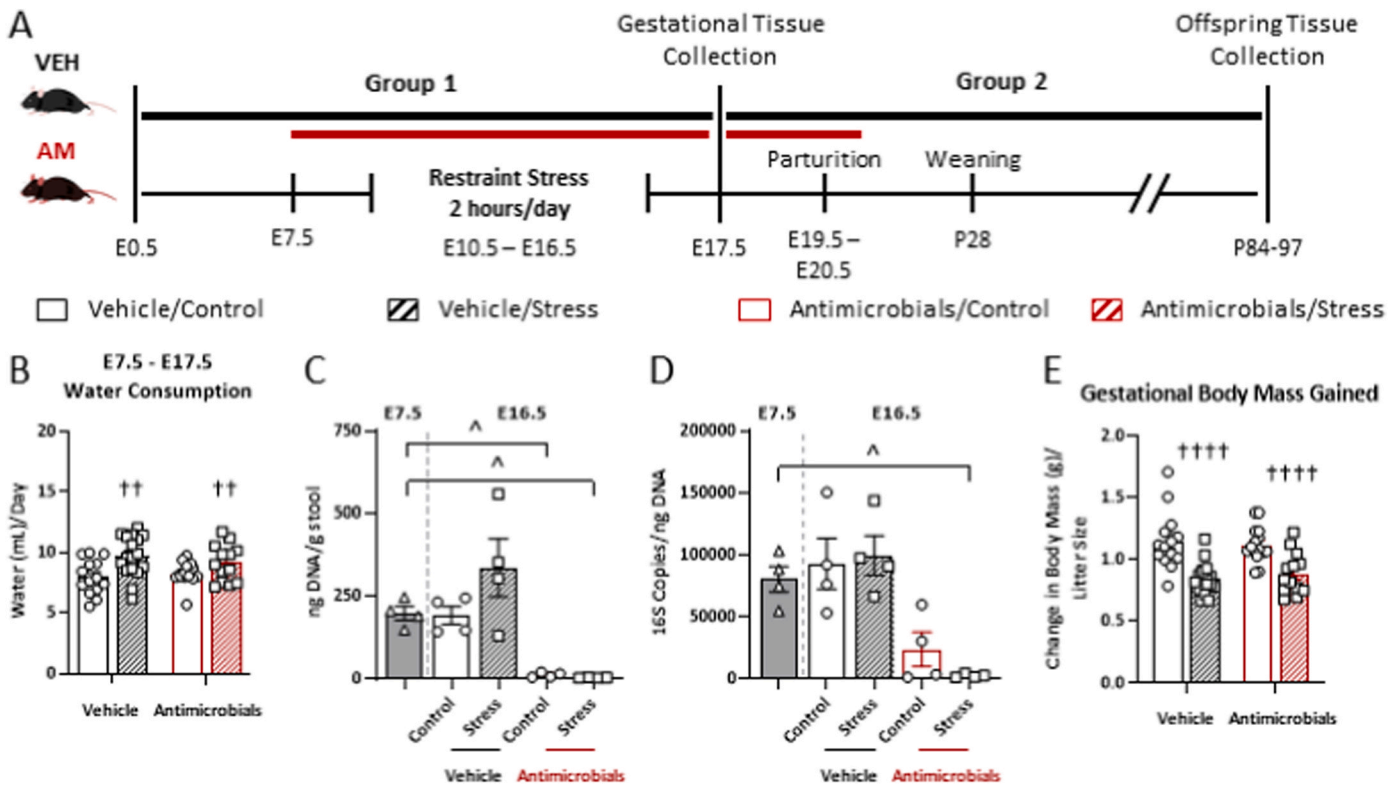

Fig. 1. Experimental design and validation. A. Pregnant C57BL/6 mice were administered antimicrobial treatment (AM) through drinking water or a vehicle control (VEH) from E7.5 until tissue collection on E17.5 for Group 1 or until parturition for Group 2. Within both groups, dams were randomly assigned to undergo restraint stress for 2 h daily from E10.5-E16.5 or remain undisturbed as the non-stressed control group. Maternal and fetal tissues were collected from Group 1 animals on E17.5. Group 2 animals underwent parturition and adult offspring were assessed for behavior at 10 weeks of age. B. Water consumption was increased during the treatment period by exposure to restraint stress, but was not impacted by antimicrobial treatment. Antimicrobial treatment decreased C. fecal DNA concentration normalized to weight of stool and D. fecal 16S copy number. E. Restraint stress, but not antimicrobials, restricted maternal gestational weight gain normalized to litter size. Bars represent mean ± SEM. B, E. n = 13–17 dams/group. Two-Way ANOVA: † indicates main effect of stress (††$p < 0.01$; ††††$p < 0.0001$). C, D. n = 4 dams/group. One-Way ANOVA: ^ indicates significant Tukey post hoc test (^ $p < 0.05$). E − embryonic.

CA) and using the QuantStudio 5 Real-Time PCR Systems machine (Applied Biosystems, Foster City, CA). The primers for the endogenous housekeeping genes and genes of interest are listed in Supp. Table 1. *Gapdh* was used as the endogenous housekeeping gene for intestine and brain tissues, while *Tbp* was used for placental tissues. qPCR data is presented as fold change compared to the vehicle control group, using the $2^{-\Delta\Delta Ct}$ method. For determining sex differences, data were compared to the female vehicle control group.

2.4. Flow cytometry

Flow cytometry was performed on whole blood as described previously (Antonson et al., 2020). Briefly, 50 μL of whole blood was aliquoted for analysis of myeloid cells and 50 μL was aliquoted for analysis of lymphoid cells. Samples were incubated with antibodies for 30 min at 4 °C, lysed with RBC Lysis Buffer (Tonbo Biosciences, San Diego, CA), washed with FACS Buffer (1% BSA and 2 mM EDTA in PBS), and fixed in 10% neutral buffered formalin. Cells were analyzed using a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA) using FlowJo 10 software. The gating strategy for identifying myeloid cell populations is depicted in Fig. 4a and the gating strategy for identifying lymphoid cell populations is depicted in Supp Fig 2.

For analysis of myeloid cells, the following antibodies were used: V450 CD45 (BD Bioscience, San Jose, CA; 1 μL), APC CD11b (eBioscience, San Diego, CA; 1 μL), PerCP-Cy5.5 Ly6G (BD Bioscience, San Jose, CA; 1 μL), and PE-Cy7 Ly6C (eBioscience, San Diego, CA; 1 μL). For analysis of lymphoid cells, the following antibodies were used: V450 CD45 (BD Bioscience, San Jose, CA; 1 μL), AF700 CD3 (BioLegend, San Diego, CA; 2 μL), APC CD4 (BioLegend, San Diego, CA; 1.5 μL), PE CD8 (BioLegend, San Diego, CA; 1 μL), PerCP-Cy5.5 CD19 (BioLegend, San Diego, CA; 1.5 μL), and LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen, Carlsbad, CA; 1 μL). For single-stain compensation controls, UltraComp eBeads Compensation Beads (Invitrogen, Carlsbad, CA) were used. For the LIVE/DEAD stain compensation control, 1 μL of stain was added to 50 μL whole blood pooled from all samples. Whole blood was also pooled from all samples for an unstained control.

2.5. ELISA

Corticosterone was measured in maternal serum and offspring plasma using the Cayman Chemical Corticosterone ELISA kit (Ann Arbor, MI) following the manufacturer's protocol. All samples and standards were assayed in duplicate.

2.6. 16S rRNA sequencing and bacterial PCR

Fresh fecal samples were collected from all mice on E7.5, prior to initiation of treatment, and on E16.5, immediately following restraint stress. Fecal samples were frozen and stored at −20 °C until further processing. DNA was extracted from fecal samples using the Qiagen QIAmp PowerFecal Pro DNA Kit (Hilden, Germany), per manufacturer's protocol. DNA was quantified using the Nanodrop One Spectrophotometer (Invitrogen, Carlsbad, CA).

For bacterial PCR, sample-derived standards were used, as previously described (Galley et al., 2014). Briefly, 2 μL of each sample was pooled to create a representative template for PCR to generate standards

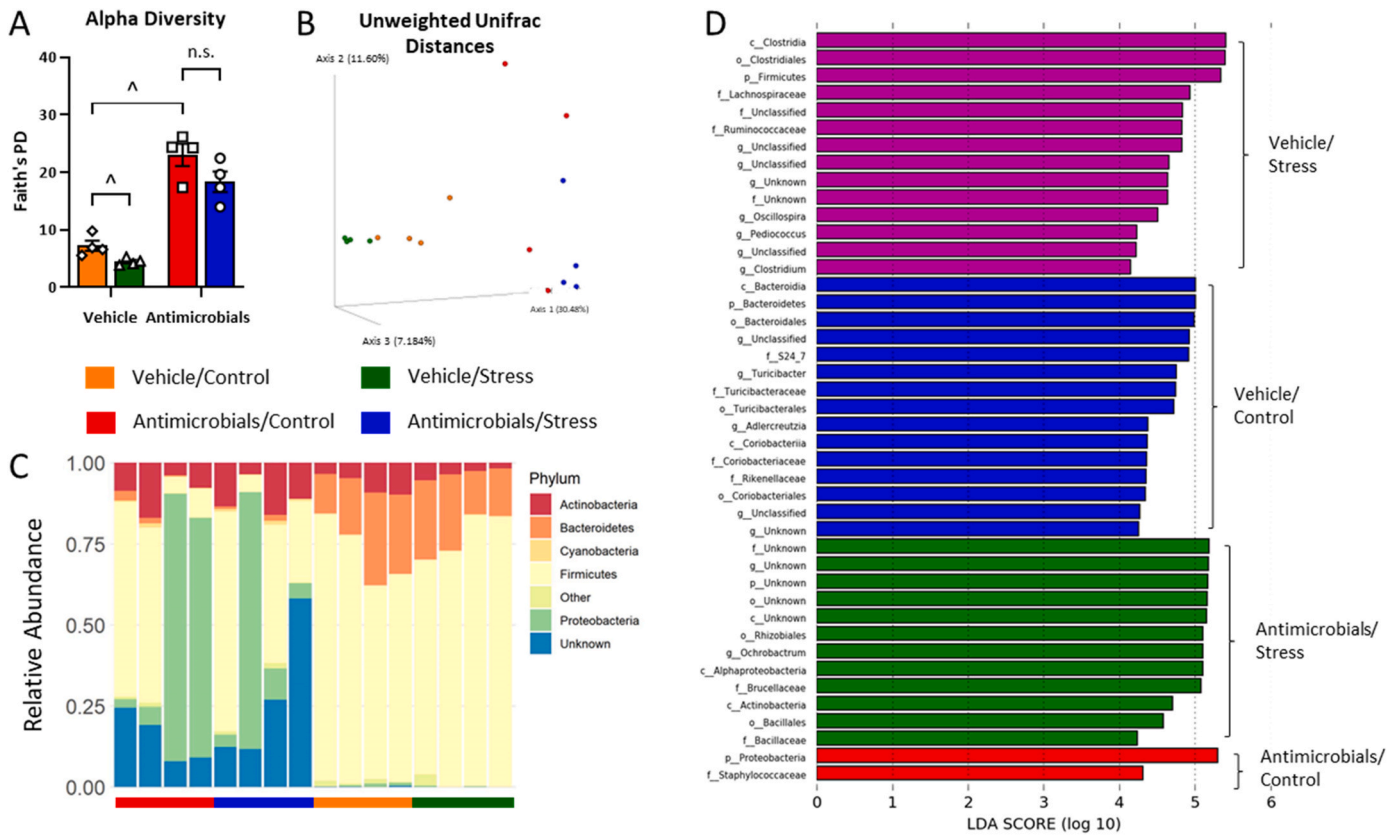

Fig. 2. Prenatal stress and antimicrobials disrupt the maternal gut microbiome. A. Alpha diversity, as measured by Faith's phylogenetic diversity (PD), is decreased by stress and increased with antimicrobial treatment. B. Stress and antimicrobials also shift beta diversity, as measured by unweighted unifrac distances and plotted using principal coordinate analysis. C. LEfSe analysis reveals taxa that are differentially abundant in the four groups. Bars represent mean ± SEM. A. n = 4 dams/group. Kruskal-Wallis test: ^ indicates significant pairwise comparison (^ $p < 0.05$).

to quantify bacterial copy number. Standard amplification was performed using universal 16S primers (Forward Primer: CGGTGAATACGTTCYCGG; Reverse Primer: GGWTACCTTGTTACGACTT (Galley et al., 2021)) with the following thermoprofile: 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s; 1 cycle of 72 °C for 10 min. The standard was then purified using the QiaQuick PCR Purification Kit (Hilden, Germany) and quantified using a Qubit 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and the Qubit dsDNA Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, MA). Copy number of the standard was calculated using the following formula: (concentration x 6.022×10^{23})/(123×10^{9} x 660). A standard curve was then prepared by diluting the amplified standard 10-fold 10 times. The copy number of total bacteria in each sample was then determined by running qPCR using the universal 16S primers (1 μM) and the PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The following thermoprofile was run using the QuantStudio 3 Real-Time PCR Systems machine (Applied Biosystems, Foster City, CA): 1 cycle of 50 °C for 2 min and 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 56 °C for 75 s. Copy number of each sample was calculated using the standard curve.

For sequencing, double stranded (ds)DNA was quantified with the Qubit 2.0 Fluorimeter (Life Technologies, Carlsbad, CA) using the dsDNA Broad Range Assay Kit. The dsDNA was sent to the Genomic Services Core at the Institute for Genomic Medicine at Nationwide Children's Hospital, Columbus, OH, for library preparation and high-throughput sequencing. Paired-end (250 nt forward and reverse) sequences for the V4 hypervariable region of the 16S rRNA gene (515F-806R) were generated on the Illumina MiSeq. Paired end raw reads were merged using Quantitative Insights into Microbial Ecology (QIIIME) version 2 Release 2021.4. The sequences were denoised – trimmed and filtered – using DADA2 (Callahan et al., 2016) and a phylogenetic tree was generated using the MAFFT and FastTree programs. Alpha diversity was measured using Faith's Phylogenetic Diversity (PD), and beta diversity was measured using unweighted and weighted UniFrac distances, as determined with QIIME2. Differences in Faith's PD was determined using the Kruskal-Wallis one-way analysis of variance (ANOVA) followed by pairwise comparisons. Differences in unweighted and weighted UniFrac distances were determined using a permutational multivariate analysis of variance (PERMANOVA) followed by pairwise comparisons. The Benjamini-Hochberg method was used to correct for false discovery rate.

2.7. Statistical analyses

With the exception of the 16S rRNA sequencing data, all statistical tests were performed using Graphpad Prism 8. For all data, multifactorial analysis of variance (ANOVA) tests followed by Tukey's post hoc tests were performed to determine effects of sex. For data in which the main effect of sex or interaction effects of stress were not significant, data were averaged by litter to account for litter effects. Two-way ANOVAs were then performed to determine main effects of stress and antimicrobials and stress × antimicrobials interaction effects, followed by Tukey's post hoc tests. The Grubbs method was used to identify outliers (alpha = 0.05), which were excluded from analyses. Significance was defined as $p < 0.05$.

3. Results

3.1. Antimicrobial treatment during pregnancy reduces maternal fecal bacterial load

To determine the interaction between maternal gut microbes and prenatal stress in influencing immune outcomes, we administered a cocktail of broad-spectrum antibiotics and antifungals (AM) to pregnant mice to reduce the bacterial and fungal load in the maternal intestine. Of note, since the use of antibiotics has been shown to allow for fungal species to increase in abundance and colonize the gut (Dollive et al., 2013), antifungals were added to the cocktail to prevent the confounding effects of fungal overgrowth. The specific antibiotics and antifungals that comprise the cocktail – neomycin, vancomycin, meropenem, and nystatin – were chosen to act primarily on the gut microbiome when administered orally as they are poorly absorbed in the intestinal tract (Lyu et al., 2016; Rao et al., 2011; Raza et al., 2021; Taylor, 2005), and are therefore expected to have limited direct effects on the host. Furthermore, AM were administered to the mice through the drinking water to limit additional stress exposure by oral gavage. We ensured that the mice received the treatment by measuring water consumption throughout the duration of the treatment from E7.5 to E17.5. Although there was a main effect of stress on water consumption, with stressed dams drinking more water per day compared to control (Fig 1b; $f(1,55) = 11.99$, $p = 0.001$), AM did not impact water consumed ($f(1, 55) = 0.1515$, $p = 0.699$), indicating that the AM group received the treatment.

We then aimed to validate that AM reduced bacterial load in the gut of the pregnant dams. To do so, we extracted DNA from fecal samples collected prior to initiation of treatment on E7.5 and immediately following restraint on E16.5. DNA was quantified using the Nanodrop One spectrophotometer and normalized to the mass of the sample. AM drastically reduced microbial contents in the gut (Fig 1c; $f(4.0,6.1) = 29.59$, $p = 0.0004$), with a decrease in fecal DNA concentration from the E7.5 pre-treatment timepoint to the E16.5 post-treatment timepoint for the AM-control ($p = 0.015$) and AM-stress ($p = 0.012$) conditions. Furthermore, fecal DNA concentration was lower in the AM-control group compared to the Vehicle (VEH)-control group ($p = 0.036$) and in the AM-stress group compared to the VEH-control group ($p = 0.031$). To further confirm that bacterial DNA specifically was reduced by AM, we performed qPCR for 16S using a sample-derived standard curve to quantify 16S copy numbers in our fecal samples. Indeed, there was a significant difference in 16S copy numbers in our samples (Fig 1d; $f(4,15) = 9.74$, $p = 0.0004$), with lower 16S copy numbers in the AM-control group compared to the VEH-control group ($p = 0.024$) and the VEH-stress group ($p = 0.012$) and lower 16S copy numbers in the AM-stress group compared to the E7.5 pre-treatment group ($p = 0.010$), the VEH-control group ($p = 0.003$), and the VEH-stress group ($p = 0.002$). Altogether, these data demonstrate that our model of AM treatment reduces maternal gut bacterial load.

3.2. Prenatal stress restricts maternal body mass gain, without impacting pregnancy outcomes

We have previously shown that prenatal restraint stress restricts maternal body mass gained during gestation (Antonson et al., 2020). Here, we replicate that finding. Although all groups started at the same body mass pre-gestation (Supp Fig 1a; stress: $f(1,55) = 2.097$; $p = 0.153$; antimicrobials: $f(1,55) = 0.008$; $p = 0.928$; interaction: $f(1,55) = 1.336$, $p = 0.253$), pre-antimicrobials (Supp Fig 1b; stress: $f(1,55) = 1.371$; $p = 0.247$; antimicrobials: $f(1,55) = 0.133$; $p = 0.717$; interaction: $f(1,55) = 0.895$, $p = 0.348$), and pre-stress (Supp Fig 1c; stress: $f(1,55) = 2.959$; $p = 0.091$; antimicrobials: $f(1,55) = 0.116$; $p = 0.735$; interaction: $f(1,55) = 0.906$, $p = 0.345$), there was a main effect of stress on maternal body mass gained from E10.5-E16.5 normalized to litter size (Fig 1e; $f(1,55) = 36.83$, $p < 0.0001$). However, AM treatment did not impact maternal gestational body mass gain ($f(1,55) = 0.026$; $p = 0.872$). Additionally, neither stress nor AM altered gestational length (Supp Fig 1d; stress: $f(1, 23) = 2.152$; $p = 0.156$; antimicrobials: $f(1,23) = 2.152$; $p = 0.156$; interaction: $f(1,23) = 2.152$; $p = 0.156$), litter size (Supp Fig 1e; stress: $f(1,5) = 0.954$; $p = 0.333$; antimicrobials: $f(1,55) = 0.00001$; $p = 0.997$; interaction: $f(1,55) = 1.973$; $p = 0.166$), or pup mortality postnatally (Supp Fig 1f; stress: $f(1,25) = 0.004$; $p = 0.948$; antimicrobials: $f(1,25) = 0.231$; $p = 0.635$; interaction: $f(1,25) = 0.077$; $p = 0.784$). Altogether, these data indicate that restraint stress, but not AM, restricts maternal body mass gained during pregnancy. Furthermore, neither stress nor AM induce adverse gestational outcomes, including preterm birth or pup loss.

3.3. Prenatal stress and antimicrobials disrupt the maternal gut microbiome

To determine the effect of prenatal stress and antimicrobial treatment on the maternal gut microbiome, we performed 16S rRNA sequencing of maternal fecal samples collected immediately following the last round of restraint stress on E16.5. Alpha diversity, as measured by Faith's phylogenetic diversity, was altered by both AM and stress (Fig. 2a; Kruskal-Wallis, $p = 0.004$). Pairwise comparisons revealed a difference between the VEH-control group and the VEH-stress group ($q = 0.025$), demonstrating that restraint stress reduces alpha diversity of the maternal gut microbiome. Additionally, there was a reduction in alpha diversity as measured by Faith's phylogenetic diversity in the E16.5 stress group compared with the E7.5 pre-treatment (data not shown). Furthermore, there was a difference between the AM-control group and the VEH-control group ($q = 0.025$) and the VEH-stress group ($q = 0.025$). Alpha diversity also differed between the AM-stress group and the VEH-control group ($q = 0.025$) and the VEH-stress group ($q = 0.025$). Of note, the alpha diversity in the AM-treated groups was increased compared to the VEH groups, indicating increased within-sample diversity. This could be explained by the reduction in the typical microbes present in the gut, which allows for the increased relative abundance of atypical, pathogenic microbes.

In terms of beta diversity, the four groups clustered separately when plotting unweighted UniFrac distances using a principal coordinate analysis (Fig. 2b; PERMANOVA, $p = 0.001$). Pairwise comparisons then revealed significant differences between the VEH-control group and the VEH-stress group ($q = 0.042$), the AM-control group ($q = 0.042$), and the AM-stress group ($q = 0.042$). Additionally, the VEH-stress differed from the AM-control group ($q = 0.042$) and the AM-stress group ($q = 0.042$). Similar results were observed for weighted UniFrac distances (PERMANOVA, $p = 0.002$, data not shown). Altogether, this suggests that stress and antimicrobial treatment disrupt the microbial community composition of the dams.

To identify specific microbes impacted by stress and AM, we used the linear discriminant analysis (LDA) effect size (LEfSe) algorithm to identify differentially abundant microbes. In total, LEfSe analysis (LDA>2.0, $p < 0.05$) yielded 43 taxa that were differentially abundant amongst the four groups (Fig. 2d). Of note, the AM-control group was enriched in *Proteobacteria*, which was also reflected in the barplot (Fig. 2c). The AM-control group also had higher relative abundance of *Staphylococcaceae*, while the AM-stress group had a higher relative abundance of *Actinobacteria* and *Alphaproteobacteria*, specifically *Rhizobiales, Brucellaceae*, and *Rhizobiales* within that class. Taxa enriched in the VEH-stress group include the *Lachnospiraceae* and *Ruminococcaceae* families, along with the *Oscillospira*, *Clostridium*, and *Pediococcus* genera. Finally, the VEH-control group had a higher relative abundance of *Turicibacter* and *Aldercreutzia* species and the *Coriobacteriaceae* and *Rickenellaceae* families.

3.4. Prenatal stress and antimicrobials independently reduce inflammation in the dam

There is mounting evidence that the gut microbiome regulates host immune function and response to stress. Thus, we aimed to characterize maternal immune function following exposure to AM treatment and stress. As a general measure of immune activation, spleen mass was measured on E17.5. There was a main effect of stress and of AM in reducing maternal spleen mass (Fig. 3a; main effect of stress: $f(1,27) = 5.964$, $p = 0.021$; main effect of antimicrobials: $f(1,27) = 6.653$, $p = 0.016$; no interaction effect: $f(1,27) = 2.443$, $p = 0.130$). There was also a concomitant increase in maternal serum corticosterone, with stress and AM independently impacting circulating glucocorticoids (Fig. 3b; main effect of stress $f(1,22) = 6.760$, $p = 0.016$; main effect of antimicrobials: $f(1,22) = 18.25$; no interaction effect: $f(1,22) = 0.573$, $p = 0.457$).

As we expected the antimicrobials to primarily act on the microbes in the intestine, we interrogated host immune function in the distal colon and ileum to determine the impact of stress and AM on inflammation within the gastrointestinal tract. To assess intestinal inflammation, a representative selection of proinflammatory cytokines and chemokines, including *Il1b*, *Il6*, *Tnf*, and *Ccl2* were probed by gene expression analysis, as these genes have previously been shown to be dysregulated by stress (Bailey et al., 2011; Chen et al., 2020; Gur et al., 2017, 2019; Wohleb et al., 2015). In the distal colon, there was a main effect of stress and of AM on expression of the proinflammatory cytokine *Il1b* (Fig. 3c; main effect of stress: $f(1,26) = 15.07$, $p = 0.0006$; main effect of antimicrobials: $f(1,26) = 14.65$, $p = 0.0007$; no interaction: $f(1,26) = 3.011$, $p = 0.095$) and a main effect of AM on expression of the proinflammatory chemokine *Ccl2* (Fig. 3c; main effect of antimicrobials: $f(1,27) = 8.98$, $p = 0.0058$; no effect of stress: $f(1,27) = 3.180$, $p = 0.086$; no interaction: $f(1,27) = 0.172$, $p = 0.682$). Expression of the proinflammatory cytokines *Il6* and *Tnf* were not impacted by stress or AM (Fig. 3c; *Il6:* no effect of stress: $f(1,27) = 1.90$, $p = 0.179$; no effect of antimicrobials: $f(1,27) = 0.003$, $p = 0.957$; no interaction: $f(1,27) = 0.156$, $p = 0.696$; *Tnf*: no effect of stress: $f(1,27) = 3.083$, $p = 0.090$; no effect of antimicrobials: $f(1,27) = 3.135$, $p = 0.088$; no interaction: $f(1,27) = 1.153$, $p = 0.293$). In the distal ileum, there was a main effect of AM in reducing expression of *Il1b*, *Il6*, *Tnf*, and *Ccl2*, with a main effect of stress only on decreasing expression of *Il6* and *Ccl2* (Fig. 3d; *Il1b*: main effect of antimicrobials: $f(1,29) = 19.12$, $p = 0.0001$; no effect of stress: $f(1,29) = 2.117$, $p = 0.156$; no interaction: $f(1,29) = 0.322$; *Il6*: main effect of antimicrobials: $f(1,29) = 11.16$, $p = 0.002$; main effect of stress: $f(1,29) = 5.203$, $p = 0.030$; no interaction: $f(1,29) = 0.212$, $p = 0.649$; *Tnf*: main effect of antimicrobials: $f(1,29) = 19.83$, $p = 0.0001$; no effect of stress: $f(1,29) = 3.472$, $p = 0.073$; no interaction: $f(1,29) = 1.522$, $p = 0.227$; *Ccl2*: main effect of antimicrobials: $f(1,29) = 6.699$, $p = 0.013$; main effect of stress: $f(1,29) = 6.604$, $p = 0.0156$; no interaction: $f(1,29) = 0.240$, $p = 0.628$). These data suggest that prenatal stress and AM both reduce proinflammatory cytokine expression in the maternal distal colon and distal ileum.

3.5. Prenatal stress induces neutrophilia in the dam, regardless of antimicrobial treatment

Given that stress and antimicrobials increased levels of circulating corticosterone and reduced inflammation in the gastrointestinal tract, we next aimed to characterize circulating leukocyte populations in the dam on E17.5 by flow cytometry.

To investigate circulating myeloid populations, $CD11b^{+}CD45^{+}$ cells were gated from total cells, and then further gated based on Ly6G expression and SSC properties to identify $Ly6G^{+}SSC^{Hi}$ neutrophils and $Ly6G^{-}SSC^{Lo}$ monocytes (Fig. 4a). Within the $Ly6G^{-}SSC^{Lo}$ gate, cells were further categorized as classical, intermediate, and alternative monocytes based on Ly6C expression (high, intermediate, and low, respectively), as previously described (Antonson et al., 2020). Exposure to stress during gestation increased the population of circulating neutrophils in the dam, with no effect of AM or interaction effect (Fig. 4b; main effect of stress: $f(1,21) = 5.760$, $p = 0.026$; no effect of antimicrobials: $f(1,21) = 2.152$, $p = 0.157$; no interaction: $f(1,21) = 1.000$, $p = 0.329$). However, neither stress nor AM impacted the classical ($Ly6C^{Hi}$), intermediate ($Ly6C^{Int}$), or alternative ($Ly6C^{Lo}$) populations (Fig. 4c–e; $Ly6C^{Hi}$: stress: $f(1,21) = 2.854$, $p = 0.106$; antimicrobials: $f(1,21) = 0.383$, $p = 0.543$; interaction: $f(1,21) = 2.026$, $p = 0.169$; $Ly6C^{Int}$: stress: $f(1,21) = 0.012$, $p = 0.916$; antimicrobials: $f(1,21) = 0.079$, $p = 0.781$; interaction: $f(1,21) = 0.401$, $p = 0.533$; $Ly6C^{Lo}$: stress: $f(1,21) = 1.933$, $p = 0.179$; antimicrobials: $f(1,21) = 0.027$, $p = 0.872$; interaction: $f(1,21) = 0.353$, $p = 0.559$).

To investigate circulating lymphoid populations, $CD45^{+}$ cells were gated from viable cells and then further gated based on $CD3^{+}$ and $CD19^{+}$ expression to identify T cells and B cells, respectively (Supp Fig 2a). Within the $CD3^{+}$ gate, cells were further classified into $CD4^{+}$ and $CD8^{+}$ T cells. Neither $CD19^{+}CD3^{-}$ B cells nor $CD3^{+}CD19^{-}$ T cells were altered by stress or AM (Supp Fig. 2b and c; B cells: stress: $f(1,25) = 0.858$, $p = 0.363$; antimicrobials: $f(1,25) = 2.059$, $p = 0.164$; interaction: $f(1,25) = 0.679$, $p = 0.418$; T cells: stress: $f(1,25) = 0.836$, $p = 0.369$; antimicrobials: $f(1,26) = 1.600$, $p = 0.218$; interaction: $f(1,25) = 0.661$, $p = 0.424$). Furthermore, neither stress nor AM affected circulating $CD4^{+}CD8^{-}$ T cells or $CD8^{+}CD4^{-}$ T cells (Supp Fig. 2d and e; CD4 T Cells: stress: $f(1,17) = 1.876$, $p = 0.189$; antimicrobials: $f(1,17) = 0.226$, $p = 0.641$; interaction: $f(1,17) = 0.029$, $p = 0.866$; CD8 T Cells: stress: $f(1,17) = 2.908$, $p = 0.106$; antimicrobials: $f(1,17) = 0.200$, $p = 0.661$; interaction: $f(1,17) = 0.877$, $p = 0.362$).

Altogether, these data provide evidence that restraint stress, but not antimicrobial treatment, during pregnancy increases circulating neutrophils, without impacting other myeloid or lymphoid populations.

3.6. Antimicrobial treatment modulates expression of inflammatory markers in the intrauterine environment

Since we observed reductions in gastrointestinal expression of inflammatory cytokines and chemokines due to stress and antimicrobial treatment, we next hypothesized that placental immune function would be impacted as well, as the interface between the dam and the fetus. We specifically examined *Ccl2* and *Il6* as these were the proinflammatory factors in the intrauterine environment that were altered by stress in our previous study (Chen et al., 2020). For all of the intrauterine tissues examined, one female and one male sample were analyzed per dam first for sex differences. However, as no main effects of sex or interaction effects were detected for any of the following parameters, the female and male values were averaged per dam and are the reported values.

AM reduced *Ccl2* expression in the placenta, with no effect of stress (Fig. 5a; main effect of antimicrobials: $f(1,28) = 12.29$, $p = 0.002$; no effect of stress: $f(1,28) = 0.093$, $p = 0.763$; no interaction: $f(1,28) = 1.191$, $p = 0.284$). However, expression of *Il6* was not altered by either AM or stress (Fig. 5b; antimicrobials: $f(1,28) = 1.343$, $p = 0.256$; stress: $f(1,28) = 0.329$, $p = 0.571$; interaction: $f(1,28) = 1.426$, $p = 0.242$). We then examined expression of *Tlr4* (toll-like receptor 4), which is a pattern recognition receptor that is activated by microbial components, and found that AM reduced *Tlr4* expression in the placenta (Fig. 5c; main effect of antimicrobials: $f(1,27) = 8.700$, $p = 0.007$; no effect of stress: $f(1,27) = 0.0007$, $p = 0.979$; no interaction: $f(1,27) = 2.666$, $p = 0.114$). Due to the increase in circulating neutrophils, we also investigated expression of the neutrophil chemoattractant *Cxcl1* in the placenta, which was not altered by either stress or AM (Fig. 5d; antimicrobials: $f(1,28) = 2.355$, $p = 0.136$; stress: $f(1,28) = 1.903$, $p = 0.179$; interaction: $f(1,28) = 1.570$, $p = 0.221$).

To assess fetal immune function, we examined expression of inflammatory markers in the fetal liver, which is the primary site of hematopoiesis during mid-late gestation in the mouse (Lewis et al., 2021). Interestingly, there was a stress × AM interaction effect on expression of *Ccl2* in the fetal liver, with stress reducing expression only in the AM

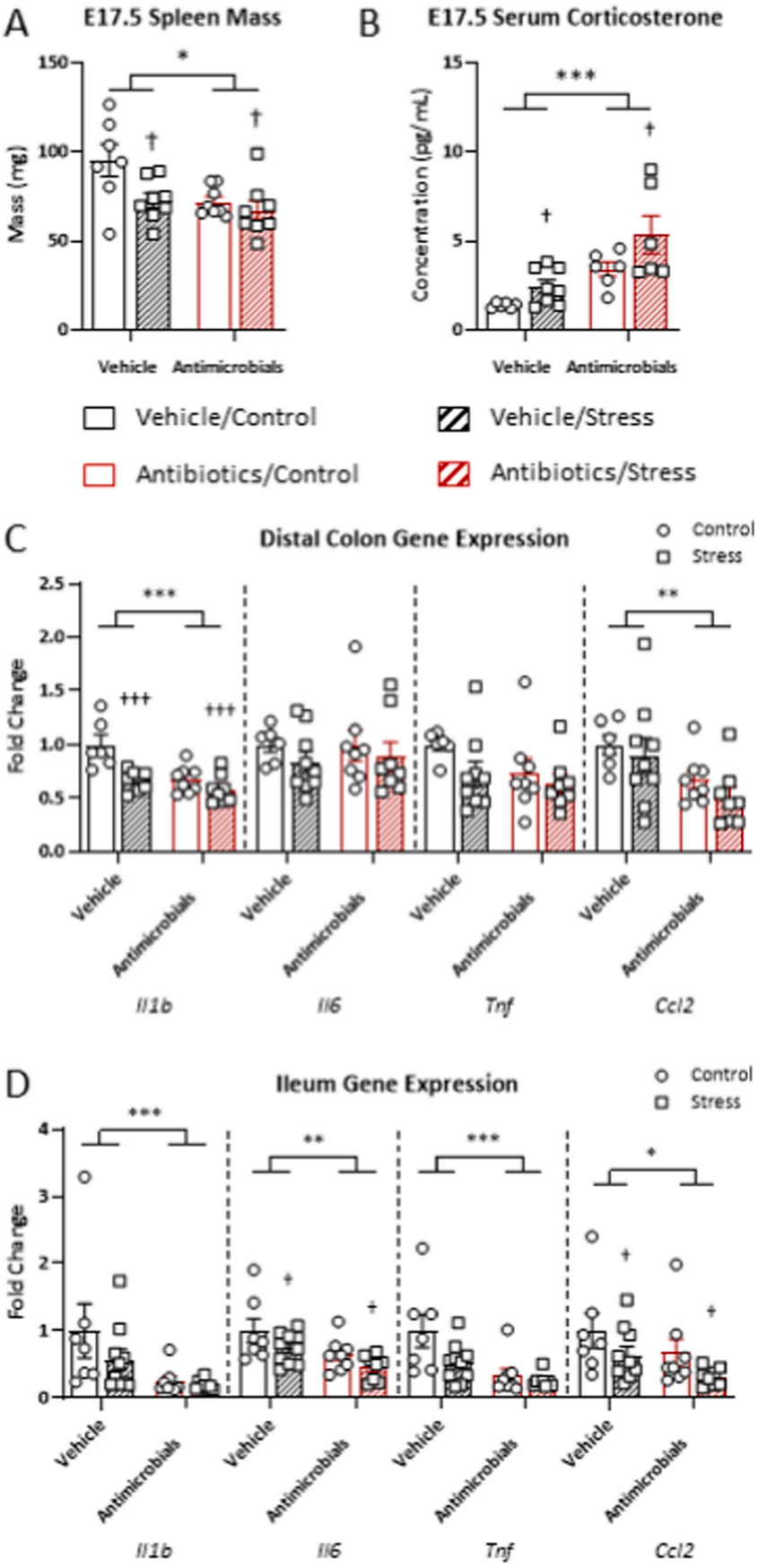

Fig. 3. Prenatal stress and antimicrobials independently reduce inflammation in the dam. Prenatal stress and antimicrobials A. reduce maternal spleen mass and B. increase circulating corticosterone, both general signs of systemic immune suppression. C. Prenatal stress reduces expression of colonic *Il1b*, while antibiotics treatment reduces expression of colonic *Il1b* and *Ccl2*. D. in the ileum, prenatal stress decreases expression of *Il6* and *Ccl2*, while antimicrobial treatment decreases expression of *Il1b*, *Il6*, *Tnf*, and *Ccl2*. Bars represent mean ± SEM. n = 6–10 dams/group. Two-Way ANOVA: † indicates main effect of stress (†$p < 0.05$; †††$p < 0.001$); * indicates main effect of antimicrobials (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

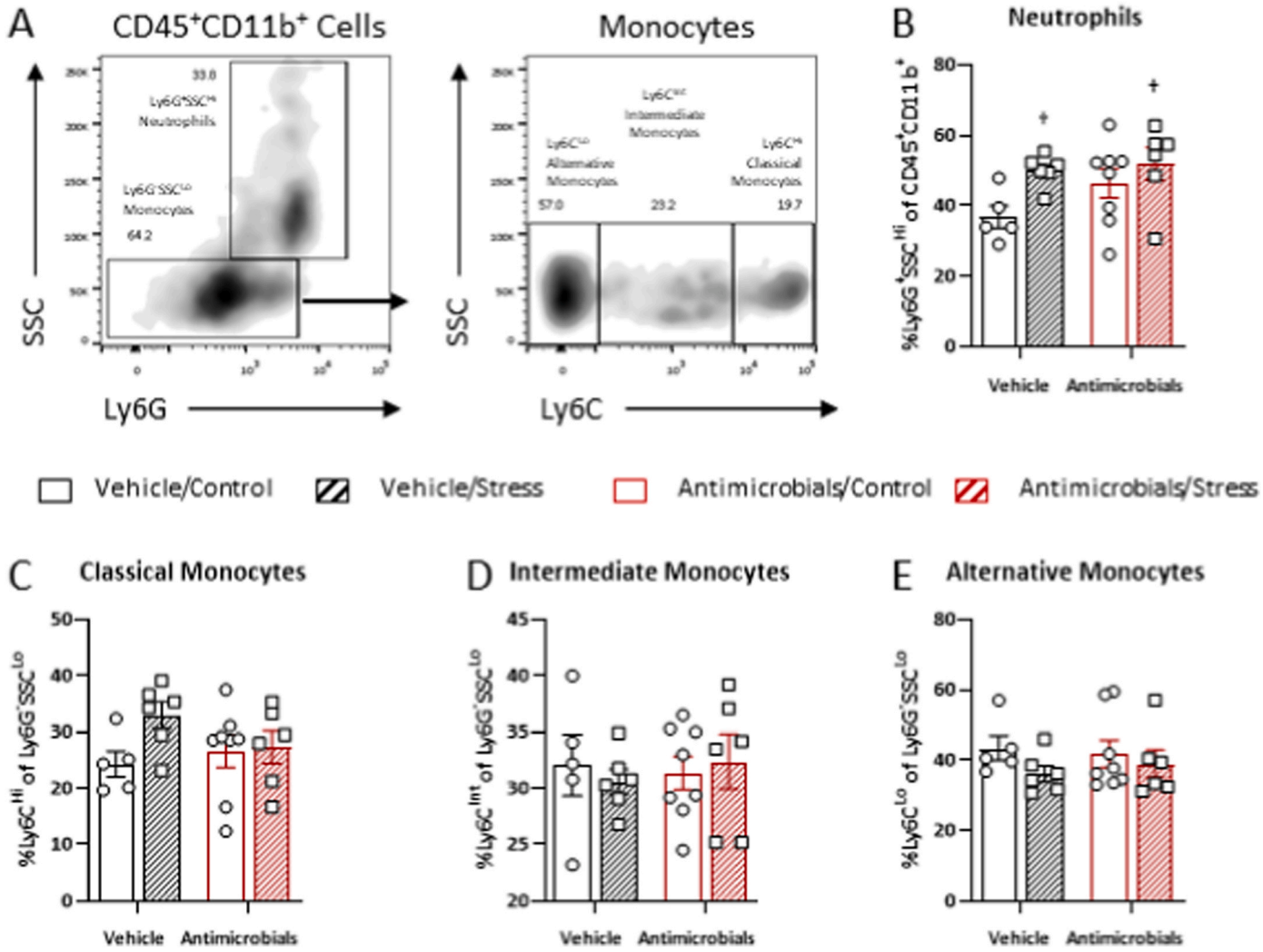

Fig. 4. Prenatal stress induces neutrophilia in the dam, regardless of antimicrobial treatment. A. Gating strategy for identifying circulating myeloid cells. $CD45^{+}CD11b^{+}$ cells were gated based on Ly6G expression and SSC properties to identify $Ly6G^{+}SSC^{Hi}$ neutrophils and $Ly6G^{-}SSC^{Lo}$ monocytes. Within the monocyte gate, cells were further categorized as $Ly6C^{Hi}$ classical monocytes, $Ly6C^{Int}$ intermediate monocytes, and $Ly6C^{Lo}$ alternative monocytes. B. Restraint stress increases the percent of neutrophils in circulation, with no overt changes in C. classical, D. intermediate, or E. alternative monocytes. Bars represent mean ± SEM. n = 5–8 dams/group. Two-Way ANOVA: † indicates main effect of stress (†$p < 0.05$).

condition (Fig. 5e; interaction: $f(1,8) = 5.620$, $p = 0.045$; Tukey's post hoc test for AM-control vs AM-stress: $p = 0.044$). This indicates a potential additive effect of AM and stress in reducing fetal inflammation, though this was not observed for *Il6* expression in the fetal liver (Fig. 5f; stress: $f(1,8) = 2.528$, $p = 0.151$; antimicrobials: $f(1,8) = 1.532$, $p = 0.251$; interaction: $f(1,8) = 1.535$, $p = 0.251$). We also examined inflammatory markers in the developing brain, as we have previously observed inflammation following exposure to stress (Chen et al., 2020; Gur et al., 2017). However, here, there is no overt effect of stress or AM on expression of *Ccl2* or *Il6* (Fig. 5g and h; *Ccl2*: stress: $f(1,28) = 0.023$, $p = 0.882$; antimicrobials: $f(1,28) = 0.056$, $p = 0.815$; interaction: $f(1,28) = 8.169$, $p = 0.008$, with no significant comparisons by Tukey's post hoc test; *Il6*: stress: $f(1,28) = 2.161$, $p = 0.153$; antimicrobials: $f(1,28) = 0.088$, $p = 0.769$; interaction: $f(1,28) = 0.692$, $p = 0.412$). This suggests that while AM reduces placental expression of *Ccl2* and *Tlr4*, the fetal liver and brain are largely spared.

3.7. Prenatal stress and antimicrobials reduce offspring body mass and modulate circulating leukocyte populations

Although the fetus seemed largely spared at the prenatal timepoint, there is evidence that prenatal stress and gestational antimicrobial treatment can impact offspring health outcomes later in life (Merlot et al., 2008; Miller et al., 2018; Nielsen et al., 2011). Thus, we first assessed pup body mass at postnatal day (P)7, P14, and P21 as general measures of offspring health. Body mass was measured for each pup and averaged per dam, without dividing the pups by sex. There was a main effect of AM in reducing pup body mass at P7 (Fig. 6a; main effect of antimicrobials: $f(1,24) = 7.058$, $p = 0.014$; no effect of stress: $f(1,24) = 1.004$, $p = 0.326$; no interaction: $f(1,24) = 3.537$, $p = 0.072$). At P14, there was a stress × AM interaction (Fig. 6b; interaction effect: $f(1,22) = 7.629$, $p = 0.011$), with decreased body mass of the VEH-stress pups compared to the VEH-control pups (Tukey's post hoc test: $p = 0.035$) and decreased body mass of the AM-control pups compared to the VEH-control pups (Tukey's post hoc test: $p = 0.002$). However, these effects are resolved by P21, at which point neither stress nor AM impact pup body mass (Fig. 6c; stress: $f(1,24) = 0.114$, $p = 0.738$; antimicrobials: $f(1,24) = 1.041$, $p = 0.318$; interaction: $f(1,24) = 3.727$, $p = 0.065$). Together, these data indicate that stress and antimicrobials influence offspring body mass in early life.

As we identified altered populations of circulating leukocytes in the dam following exposure to stress, we also interrogated circulating leukocyte populations in the offspring in adulthood to assess long-term effects of gestational stress and AM on the immune system. For all of the

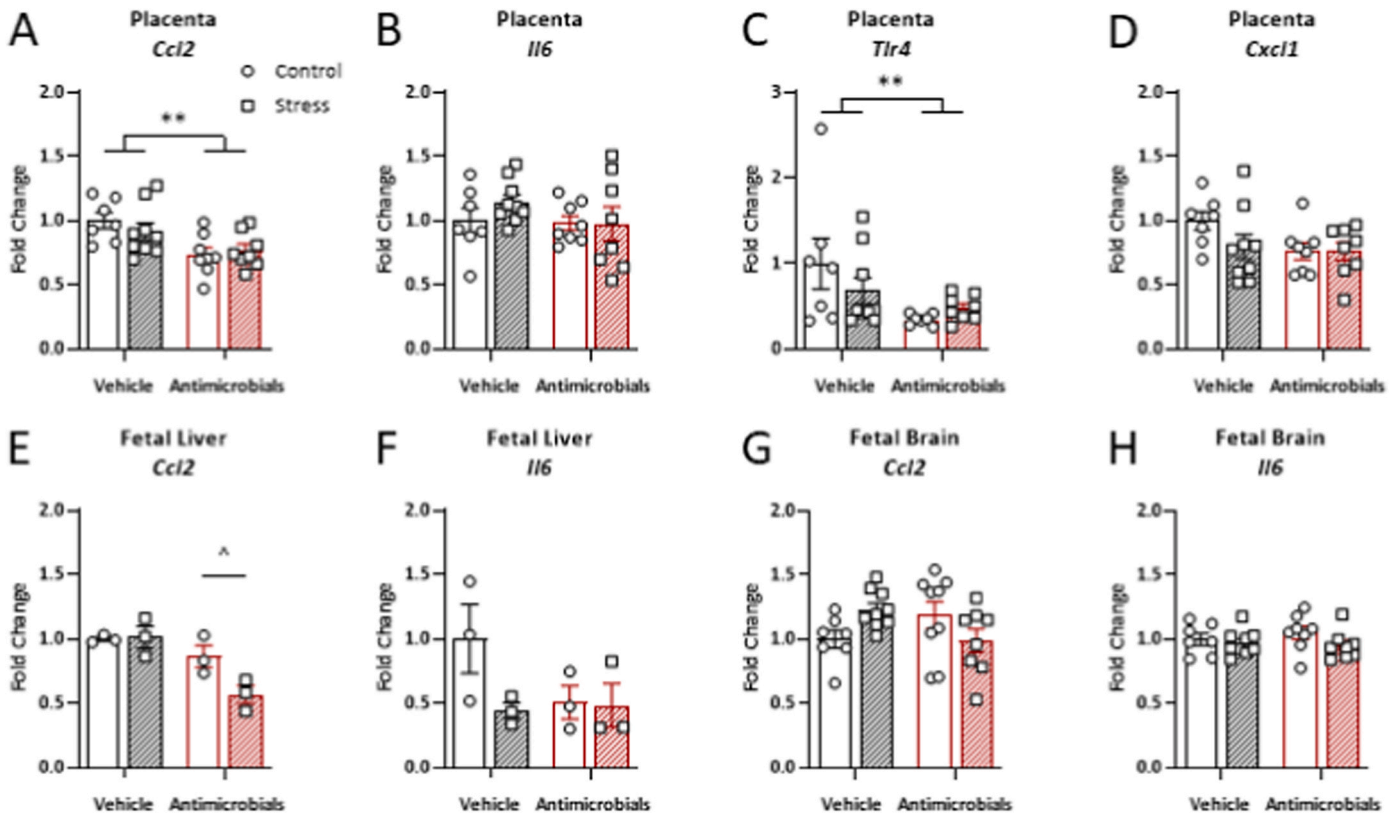

Fig. 5. Antimicrobial treatment modulates expression of inflammatory markers in the intrauterine environment. Antimicrobial treatment reduces expression of A. proinflammatory chemokine *Ccl2* and C. pattern recognition receptor *Tlr4* in the placenta, but does not impact expression of B. *Il6* or D. the chemokine *Cxcl1*. E. Exposure to stress reduced expression of *Ccl2* in the fetal liver only in the antimicrobial-treated mice, while F. *Il6* expression in the fetal liver was not affected by stress or antimicrobial treatment. Expression of G. *Ccl2* and H. *Il6* in the fetal brain was not altered by exposure to stress or antibiotics. Bars represent mean ± SEM. Placentas and fetal brains: n = 7–9 litters/group. Fetal livers: n = 3 litters/group. Two-Way ANOVA: * indicates main effect of antimicrobials (**p < 0.01); ^ indicates significant interaction followed by Tukey's post hoc test (^ p < 0.05).

following postnatal experiments, one female and one male offspring was analyzed per dam first for sex differences. However, as no main effects of sex or interaction effects were detected for any of the following parameters, the female and male values were averaged per dam and are the reported values.

To examine myeloid populations, CD11b$^+$ cells were gated from total whole blood cells, within which NK cells were identified based on expression of NK1.1 and SSC properties. Neither AM nor stress impacted circulating NK1.1$^+$SSC^{Lo} cells (Fig. 6d; stress: $f(1,22) = 1.126$, $p = 0.300$; antimicrobials: $f(1,22) = 2.329$, $p = 0.141$; interaction: $f(1,22) = 0.487$, $p = 0.493$). However, within the NK1.1$^-$ population, the % of cells that were Ly6G$^+$, and thus identified as neutrophils, was increased by both AM and stress (Fig. 6e; main effect of stress: $f(1,21) = 4.740$, $p = 0.041$; main effect of antimicrobials: $f(1,21) = 7.033$, $p = 0.015$; no interaction: $f(1,21) = 1.886$, $p = 0.184$), in part resembling the finding of stress-induced neutrophilia in the dams during gestation. Monocytes were then identified as Ly6G$^-$SSC^{lo} cells, and then further classified based on Ly6C expression into classical (Hi), intermediate (Int), and alternative (Lo) monocytes. None of the monocytes populations were impacted by stress or AM (Supp Fig. 3a–c; Ly6C^{Hi}: stress: $f(1,21) = 0.0097$, $p = 0.922$; antimicrobials: $f(1,21) = 0.157$, $p = 0.696$; interaction: $f(1,21) = 1.750$, $p = 0.200$; Ly6C^{Int}: stress: $f(1,22) = 0.0024$, $p = 0.961$; antimicrobials: $f(1,22) = 0.583$, $p = 0.453$; interaction: $f(1,22) = 0.023$, $p = 0.881$; Ly6C^{Lo}: stress: $f(1,22) = 0.201$, $p = 0.659$; antimicrobials: $f(1,22) = 0.139$, $p = 0.713$; interaction: $f(1,22) = 1.500$, $p = 0.234$).

In terms of lymphoid populations, B cells were first identified based on CD19 expression within the viable cells gate, though the percentage of cells that were CD19$^+$ was not altered by prenatal exposure to stress or AM (Fig. 6f; stress: $f(1,21) = 0.325$, $p = 0.575$; antimicrobials: $f(1,21) = 0.293$, $p = 0.594$; interaction: $f(1,21) = 1.015$, $p = 0.325$). However, T cells, gated based on CD3 expression from CD19$^-$ cells, were reduced by AM (Fig. 6g; main effect of antimicrobials: $f(1,21) = 5.107$, $p = 0.035$; no effect of stress: $f(1,21) = 0.629$, $p = 0.437$; no interaction: $f(1,21) = 0.428$, $p = 0.520$). Of the CD3$^+$ cells, CD4$^+$ T cells were not altered by stress or antimicrobials (Fig. 6h; stress: $f(1,22) = 0.823$, $p = 0.374$; antimicrobials: $f(1,22) = 3.554$, $p = 0.073$; interaction: $f(1,22) = 0.207$, $p = 0.653$), while there was a main effect of stress in increasing the CD8$^+$ T cell population (Fig. 6i; main effect of stress: $f(1,21) = 9.373$, $p = 0.006$; no effect of antimicrobials: $f(1,21) = 0.017$, $p = 0.898$; no interaction: $f(1,21) = 0.072$, $p = 0.791$). Altogether, these data indicate that prenatal stress and antimicrobial treatment differentially and broadly alter circulating leukocyte populations in the adult offspring.

4. Discussion

Prenatal stress can adversely impact both maternal and offspring health outcomes, including increased risk of developing neuropsychiatric disorders (Bale et al., 2010; Lancaster et al., 2010; O'Donnell et al., 2009; O'Hara and Wisner, 2014) and immune dysfunction (Merlot et al., 2008) for both mother and child. There is emerging evidence that the maternal gut microbiome interacts with the immune system to mediates the sequelae of stress during pregnancy; thus, we examined interactions between the maternal gut microbiome and maternal and offspring immune function in the context of prenatal stress. Through the use of a mouse model of prenatal restraint stress and antimicrobials-induced microbiota-reduction, we provide evidence that stress and antimicrobials treatment independently disrupt the maternal gut microbiome and

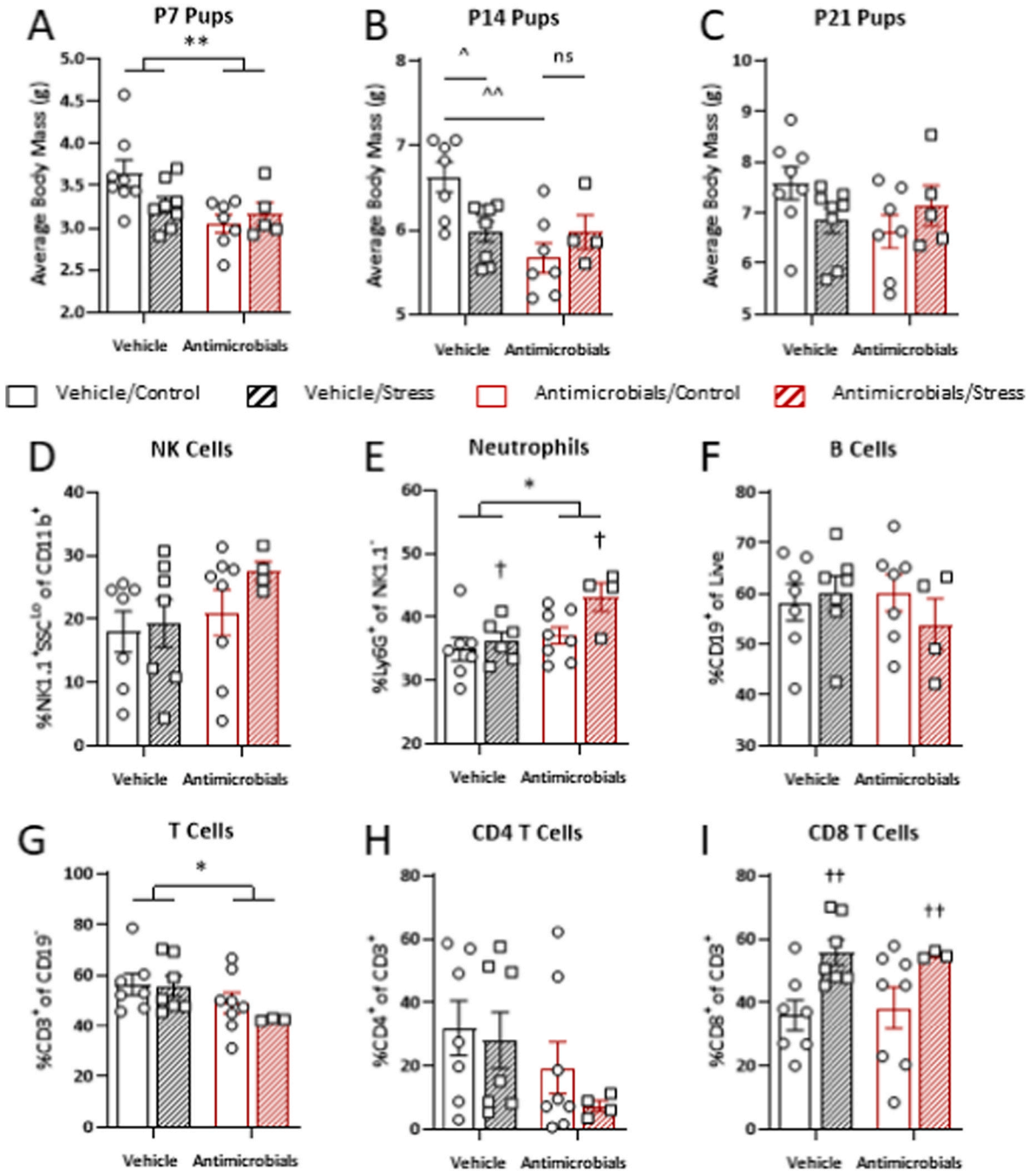

Fig. 6. Prenatal stress and antimicrobials reduce offspring body mass and modulate circulating leukocyte populations. A. Antimicrobial treatment reduced pup body mass at P7. B. At P14, there was a stress × antimicrobial interaction, with decreased pup body mass in the vehicle-stress group and the antimicrobial-control group compared to the vehicle-control group. C. These results were resolved by P21, at which point pup body mass did not differ between groups. In adult offspring, D. circulating NK cells were not impacted by stress or antimicrobials, while E. neutrophils were increased by both stress and antimicrobials. In terms of lymphoid populations, F. B cells were not altered by prenatal exposure to stress or antimicrobial treatment, though G. antimicrobials reduced circulating T cells. Within the circulating T cells, H. the percentage of CD4 T cells were not altered, while I. stress increased the CD8 T cell population. Bars represent mean ± SEM. n = 3–9 litters/group. Two-Way ANOVA: † indicates main effect of stress (†$p < 0.05$; ††$p < 0.01$)); * indicates main effect of antimicrobials (*$p < 0.05$; **$p < 0.01$); ^ indicates stress × antimicrobial interaction with significant Tukey's post hoc test (^ $p < 0.05$; ^^ $p < 0.01$).

reduce inflammatory gene expression in the distal ileum and distal colon, while largely sparing the fetus *in utero*. However, both stress and antimicrobials have long term consequences on offspring immune compartments.

The gut microbiome has increasingly become implicated in influencing and shaping host physiology, ranging from immune function to metabolism to neurodevelopment and behavior (Cryan and Dinan, 2012; Lambring et al., 2019; Pronovost and Hsiao, 2019; Rivera-Amill, 2014). Furthermore, there is mounting evidence that stress can alter composition of the intestinal microbiome, both clinically and preclinically (Bailey et al., 2011; Galley et al., 2017; Karl et al., 2017), as well as during pregnancy (Antonson et al., 2020; Gur et al., 2017; Jašarević et al., 2017). Consistent with these findings, we demonstrate that alpha diversity, as measured by Faith's phylogenetic diversity, was reduced in maternal fecal samples collected from those exposed to stress compared to control dams. Furthermore, beta diversity, as measured using

unweighted Unifrac distances, was altered by stress. In terms of specific taxa that were altered by stress exposure, there was an enrichment of *Oscillospira* species (spp.) and taxa of the *Ruminococcaceae* family in the vehicle-stress group, similar to what has previously been observed in restraint stress models (Galley et al., 2015). Furthermore, *Ruminococcaceae* has been shown to be increased in fecal samples from humans with major depressive disorder (Zheng et al., 2016), suggesting a link between this family and stress and psychiatric disorders. Altogether, these measures indicate that restraint stress disrupts the commensal microbial communities in the gut during pregnancy.

Given the finding that stress alters the maternal gut microbiome, and previous findings by our group that stress induces immune dysfunction in both the maternal and fetal compartments (Antonson et al., 2020; Chen et al., 2020; Gur et al., 2017, 2019), we then aimed to manipulate the microbiome to thoroughly interrogate the role of maternal gut microbes in mediating stress-induced immune dysfunction. To reduce the microbial load in the gastrointestinal tract during pregnancy, we administered pregnant dams a cocktail of broad-spectrum antibiotics and antifungals. Of note, the use of antibiotics has been shown to allow for fungal species to increase in abundance and colonize the gut (Dollive et al., 2013); thus, we included antifungals in our cocktail to prevent the confounding effect of fungal overgrowth. Specifically, our cocktail contained neomycin, vancomycin, meropenem, and nystatin, all of which have been shown to have minimal intestinal absorption (Lyu et al., 2016; Rao et al., 2011; Raza et al., 2021; Taylor, 2005), and thus are more likely to stay within the intestinal lumen and primarily affect the microbes in the gastrointestinal tract. Although this specific cocktail has not been previously published, neomycin and vancomycin are commonly used in microbiome reduction studies (Allen et al., 2012; Gury-BenAri et al., 2016; Kennedy et al., 2018; Levy et al., 2015), as are other members of the carbapenem family that includes meropenem (Hoban et al., 2016; Möhle et al., 2016; O'Connor et al., 2021). Nystatin has also been previously used to reduce fungal load in a study of the mycobiome in rats (Botschuijver et al., 2017). To confirm that our cocktail reduced microbial load, we measured DNA extracted from fecal samples using a Nanodrop spectrophotometer, showing reduced DNA yield from samples collected post-treatment. Furthermore, our antimicrobial treatment reduced the number of 16S copies in the fecal samples.

Additionally, 16S rRNA sequencing of maternal fecal samples collected on E16.5 demonstrated that the antimicrobial treatment drastically disrupted the commensal microbial communities, with increased alpha diversity as measured by Faith's phylogenetic diversity and beta diversity that cluster separately from vehicle-treated samples on a principal coordinate analysis plot of unweighted Unifrac distances. Although the increase in alpha diversity is contrary to findings of other studies using antibiotics treatments, which have shown decreases in measures of alpha diversity (Candon et al., 2015; Sun et al., 2019; Wu et al., 2021), this may be explained by the profound shift in composition in the antimicrobial-treated samples. We observed a reduction in the typical commensal microbes present in the gut and an increase in the relative abundance of rare taxa. For example, the relative abundance of *Proteobacteria*, is increased within the antimicrobial-treated groups compare to the vehicle groups, with a corresponding decrease in the typically predominant *Bacteroidetes* and *Firmicutes* phyla (Nguyen et al., 2015). This suggests that our antimicrobial treatment regimen reduces and disrupts the healthy microbial communities within the gastrointestinal (GI) tract but leaves rare taxa behind. Alternatively, it is possible that the addition of an antifungal treatment to the regimen impacted the bacterial communities, as antifungal treatments have been previously shown to induce an increase in bacterial alpha diversity (Qiu et al., 2015). Although antimicrobial treatment is commonly used as an alternative to germ-free models, our results suggest the importance of examining and identifying the particular microbes that remain following treatment with antimicrobials. Furthermore, this may indicate the difficulty of interpreting and generalizing across studies using antimicrobials as the composition of the microbiome in the antimicrobial-treated mice may differ depending on the specific agents used even if the treatment decreases the overall abundance of microbes.

Given these limitations, we examined the interaction between maternal commensal microbes and prenatal stress in impacting maternal and offspring immune function. There is mounting evidence that stress can disrupt maternal immune homeostasis during pregnancy, although the outcomes depend on the timing and severity of the stressor due to the changing immune milieu during gestation (Mor et al., 2017). Due to the careful regulation of maternal immune function during pregnancy, disruption of the pro- and anti-inflammatory balance, in either direction, can lead to adverse obstetrical outcomes. Here, we build upon previous work from our group investigating the maternal immune system following stress during pregnancy (Antonson et al., 2020). Of note, we replicated our previous findings that stress reduces maternal spleen mass and increases circulating corticosterone levels (Antonson et al., 2020), and further show that antimicrobial treatment independently reduces spleen mass and increases corticosterone. These results are in line both with studies showing that stress activates the hypothalamic-pituitary-adrenal (HPA) axis in pregnant rats, increasing circulating glucocorticoids (Stefanski et al., 2005; Takahashi et al., 1998), and induces systemic immune suppression in non-pregnant female mice as measured by spleen mass (Voorhees et al., 2013), and with studies in male mice showing similar phenotypes in models of antibiotics-induced microbial disruption or reduction (Desbonnet et al., 2015; Reikvam et al., 2011; Yamamoto et al., 2001). Furthermore, male germ-free mice have been shown to have an exaggerated HPA axis response to stress (Sudo et al., 2004), which supports the data here that stress augments maternal serum corticosterone levels in the antimicrobial-treated animals.

In addition to the systemic immune suppression observed due to stress and antimicrobial treatment, we found that stress and antimicrobial treatment reduced expression of pro-inflammatory cytokines and chemokines in both the maternal distal colon and distal ileum. Interestingly, this was in contrast to findings that chronic restraint stress enhances inflammatory cytokine expression in the colon in male rats (Li et al., 2021), as does antibiotics treatment in non-pregnant female mice (Sun et al., 2019). However, acute restraint stress and social stress in male mice reduced inflammatory cytokine expression in the colon, suggesting the differences observed may be due to the duration and severity of the stressor, or due to sex differences (Vagnerová et al., 2019; Vodička et al., 2018). Furthermore, in the same studies, there was a decrease in GI inflammatory cytokine expression in germ-free male mice compared to specific pathogen free mice (Vagnerová et al., 2019; Vodička et al., 2018). These differences may be due to the use of single antibiotics in the study showing inflammation instead of a broad-spectrum cocktail, suggesting distinct responses of GI tissue in response to microbiome *disruption* and to microbiome *reduction*.

While both stress and antimicrobial treatment increased circulating glucocorticoids and reduced GI inflammation, only stress impacted maternal circulating leukocyte populations. In accordance with previous studies demonstrating stress-induced neutrophilia in male and female mice (Bowers et al., 2008; Jiang et al., 2017; Schwab et al., 2005), here we show that prenatal stress increases neutrophils in circulation. However, in contrast to our data, a study in pregnant rats found that chronic social stress did not impact circulating neutrophils, and instead decreased monocytes, B cells, and NK cells (Stefanski et al., 2005). This discrepancy could be explained by the use of multiparous females in the study as parity impacts immune function (Natri et al., 2019; Nilormee et al., 2016), or by the type of stress as different stressors have been shown to elicit different shifts in leukocyte populations (Bowers et al., 2008). Although the present study indicates that the presence of commensal gut microbes does not mediate gestational stress-induced increase in circulating neutrophils, as this occurred in both the vehicle- and antimicrobial-treated groups, additional work is necessary to assess functional changes in these immune cells in addition to relative composition.

As there is evidence that maternal glucocorticoids and immune dysfunction can impact the developing fetus (Bartho et al., 2019; Cuffe et al., 2012; Estes and McAllister, 2016), we also examined cytokine and chemokine expression in fetal tissues. Similar to maternal GI tissues, antimicrobial treatment reduced expression of *Ccl2* in the placenta, further supporting the anti-inflammatory effect of antimicrobials. Indeed, as whole placental tissues were examined in this study and placental tissues contain both maternal and fetal components (Chen and Gur, 2019), it is possible that this effect is driven by the immune suppression in the maternal compartment. This is supported by the lack of effect of antimicrobials on *Ccl2* expression in the fetal liver, the primary site of hematopoiesis during embryonic development (Mikkola and Orkin, 2006), or the fetal brain, suggesting the fetal compartment is relatively spared from maternal antimicrobial treatment. Notably, the only difference that was observed was an interaction between antimicrobial treatment and stress in influencing fetal liver *Ccl2* expression, with stress decreasing *Ccl2* only in the antimicrobial-treated dams. This finding is in accordance with our observation that stress has an additive effect in suppressing maternal immune function. However, the distinct findings in placenta, fetal liver, and fetal brain tissues also suggests local regulation of the immune milieu in the intrauterine environment.

Additionally, a reduction in placental *Tlr4* expression was observed. As TLR4 is a pattern recognition receptor that recognizes components of microbes and is expressed on both immune cells and trophoblast cells in the placenta (Abrahams and Mor, 2005), the decrease in placental expression of *Tlr4* could be a result of the reduction of the maternal gut microbes, and thus, in the decrease in microbial ligands in circulation. Alternatively, the decrease in *Tlr4* could also be explained by direct signaling of glucocorticoids in the placenta to suppress immune function. Although few, if any, studies have examined the effect of antibiotics treatment on placental function, antibiotics reduced TLR4 activation following high fat diet treatment in male mice in the liver, muscle, and adipose tissue by decreasing circulating levels of lipopolysaccharide (Carvalho et al., 2012), a component of the gram negative cell membrane. This raises the possibility that disrupting the maternal gut microbiome can impact signaling in the placenta by impacting the presence of microbial components and ligands in maternal circulation, though additional research is warranted to elucidate how maternal stress and antimicrobial treatment can impact intestinal permeability and to investigate the direct impact of maternal corticosterone.

There is mounting evidence that maternal stress and antibiotics treatment have long term effects on offspring immune function (Merlot et al., 2008; Pronovost and Hsiao, 2019). Indeed, population studies have shown that maternal stress and antibiotics use during pregnancy were associated with increased risk of hospitalization due to infection in childhood (Miller et al., 2018; Nielsen et al., 2011). These have been further supported by animal studies generally showing inhibitory effects of stress on offspring innate and adaptive immunity, including decreased cytotoxicity of circulating NK cells in adolescent male offspring (Klein and Rager, 1995) and decreased number of circulating lymphocytes in adult male offspring (Götz and Stefanski, 2007; Llorente et al., 2002). Furthermore, maternal antibiotics treatment has also similarly been found to reduce elements of innate and adaptive immunity in the neonatal offspring (Deshmukh et al., 2014; Gonzalez-Perez et al., 2016). Similar to these findings, we show here a main effect of maternal antimicrobial treatment in reducing circulating T cells in the offspring. However, in contrast, we observed that stress increased CD8 T cells regardless of antimicrobial treatment, suggesting enhanced rather than suppressed immunity. One study of prenatal restraint stress in rats demonstrated a similar increase in circulating CD8 T cells in the adult male offspring (Vanbesien-Mailliot et al., 2007), suggesting stress-induced alterations in offspring circulating leukocyte populations may depend on type of stressor as the decreased circulating leukocytes were found in adult male offspring exposed to maternal social stress (Götz and Stefanski, 2007). In addition to these findings in the lymphoid compartment, we also observed an increase in circulating neutrophils in offspring exposed to stress and antimicrobials *in utero*. This is in contrast with previous studies either demonstrating no differences in circulating neutrophils in adult male offspring (Götz and Stefanski, 2007) or a decrease in granulocyte counts in male and female piglets (Couret et al., 2009), though again this could be due to differences in the type of stressor, with a maternal social stressor used in the first study and housing stress in pigs was used for the second. It is also possible that the neutrophilia observed in the adult offspring is due to dysregulation of the HPA axis, as a similar phenotype was observed in the dams, and prenatal stress and maternal microbes have both been implicated in shaping the HPA axis in male offspring (Glover et al., 2010; Jašarević et al., 2015); however, this was not directly assessed in this study and additional work is warranted to interrogate this mechanism. One additional important limitation to our postnatal studies is that the number of pups per litter were not standardized. As the number of pups in a litter can impact the amount of milk available per pup, and in turn impact pup weight gain (Kumaresan et al., 1967), it is possible that this is a contributing factor to our findings, though the number of pups did not differ between the four groups in our study.

In summary, through this study, we show that prenatal stress and maternal microbes have discrete roles in regulating the maternal immune system and in shaping the offspring immune system. Using a model of antimicrobial-mediated maternal gut microbiome reduction in combination with our established model of gestational restraint stress, we demonstrate that prenatal stress and antimicrobials both induce systemic and GI immune suppression in the dam. Furthermore, prenatal stress induces neutrophilia in both the dam and the offspring, regardless of antimicrobial treatment. Although additional research is necessary to fully elucidate the mechanisms by which maternal stress and gut microbes shape the offspring immune system during development, the data presented here provide evidence that maternal stress and the gut microbiome can impact both the maternal and offspring immune system.

CRediT authorship contribution statement

Helen J. Chen: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Allison Bischoff:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Jeffrey D. Galley:** Writing – review & editing. **Lauren Peck:** Investigation, Writing – review & editing. **Michael T. Bailey:** Supervision, Writing – review & editing. **Tamar L. Gur:** Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2022.100480.

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