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Effects of maternal edible THC consumption on offspring lung growth and function in a rhesus macaque model

Lyndsey E. Shorey-Kendrick^{1,**}, B. Adam Crosland^{2,**}, Matthias C. Schabel³, Ilhem Messaoudi⁴, Minzhe Guo⁵, Matthew G. Drake⁶, Zhenying Nie⁶, R. Clayton Edenfield⁷, Issac Cinco⁴, Michael Davies¹, Jason A. Graham⁷, Olivia L. Hagen⁷, Owen J.T. McCarty⁸, Cindy T. McEvoy⁹, Eliot R. Spindel^{1,*}, Jamie O. Lo^{2,7,*}

¹Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR, USA

²Division of Maternal Fetal Medicine, Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, OR, USA

³Advanced imaging Center, Oregon Health & Science University, Portland, OR, USA

⁴Department of Microbiology, Immunology, and Molecular Genetics, College of Medicine, University of Kentucky, Lexington, KY, USA

⁵Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

⁶Division of Pulmonary and Critical Care Medicine, Oregon Health & Science University, Portland, OR, USA

⁷Division of Reproductive and Developmental Science, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR, USA

⁸Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR, USA

⁹Division of Neonatology, Department of Pediatrics, Oregon Health & Science University, Portland, OR, USA

Correspondence: Jamie Lo (loj@ohsu.edu).

* Authors contributed equally

** Authors contributed equally

AUTHOR CONTRIBUTIONS

Lyndsey Shorey-Kendrick: Conceptualization, Investigation, Data curation, Formal Analysis, Writing – original draft. Adam Crosland: Conceptualization, Investigation, Data curation, Writing – original draft. Matthias Schabel: Investigation, Data curation, Formal Analysis, Writing – original draft. Ilhem Messaoudi: Data curation, Writing – review and editing. Minzhe Guo: Resources, Writing – review and editing. Robert Edenfield: Writing – review and editing. Mathew Drake: Writing – review and editing. Jane Nie: Writing – review and editing. Michael Davies: Data curation, and Resources. Issac Cinco: Data curation, Formal Analysis, Writing – review and editing. Jason Graham: Investigation, Data curation, and Resources. Olivia Hagen: Data curation, and Resources. Owen McCarty: Writing – review and editing. Cindy McEvoy: Writing – review and editing. Elliot Spindel: Study design, Supervision, Writing – review and editing, and Funding acquisition. Jamie Lo: Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Formal Analysis, Writing – original draft, Visualization, and Funding acquisition.

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DISCLAIMERS

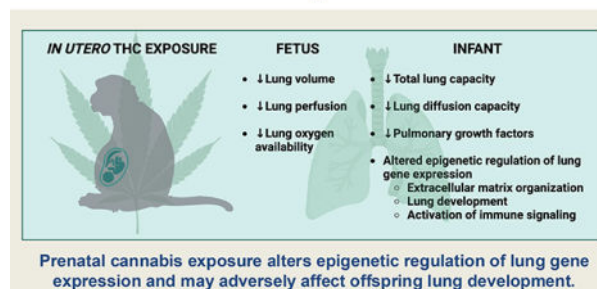
None.

Abstract

Prenatal cannabis use is rising, in part due to legalization and perceptions of safety. The impact of prenatal cannabis exposure on offspring development, especially respiratory health, remains largely unknown. The objective of this study was to determine whether *in utero* exposure to delta-9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis, is deleterious to offspring lung development and function using a rhesus macaque model. Female rhesus macaques received a daily edible containing either THC (2.5mg/7kg/day, equivalent to a heavy medical cannabis dose) or placebo during gestation and postnatally. Serial *in utero* magnetic resonance imaging (MRI) was performed during pregnancy at approximately G110 and G150. At 6 months of age infants underwent pulmonary function testing, followed by tissue collection for molecular analysis (bulk-RNAseq, whole genome bisulfite sequencing, and spatial-RNAseq). THC exposed infants displayed significantly reduced forced residual capacity (FRC) which correlated with non-significant decreases in total lung capacity (TLC), lung diffusion capacity (DLCO) and lower fetal lung perfusion, oxygen availability, and lung volume measured by MRI. Consistent with these decreases in volume indices, levels of pulmonary growth factors were decreased in bronchial alveolar lavage at 6 months. Molecular analysis of infant lungs revealed altered epigenetic regulation of gene expression, including at genes involved in extracellular matrix organization and lung development, and activation of immune signaling. Our study suggests that exposure to edible prenatal THC alters epigenetic regulation of lung gene expression and may negatively affect offspring lung development and function. Data from this study will help guide healthcare provider counseling on cannabis use in pregnancy.

Graphical Abstract

Effects of *in utero* THC on offspring lung development



NEW & NOTEWORTHY

In a translational rhesus macaque model, chronic prenatal delta-9-tetrahydrocannabinol (THC) exposure resulted in decreased lung volumes in offspring measured at 6 months of age. These decreases correlated with altered DNA methylation in lung, including at genes involved in extracellular matrix organization, lung development, and activation of immune signaling, and changes in lung cell composition as measured by spatial transcriptomics. These findings add to the growing evidence that prenatal cannabis exposure may adversely affect offspring development.

Keywords

Cannabis; lung; pregnancy; respiratory health; THC

INTRODUCTION

Cannabis is the most common federally illegal drug used in pregnancy(1) and the prevalence continues to rise, partly due to changing legal restrictions and a perception of safety (2, 3). Despite guidance from the American College of Obstetricians and Gynecologists advising pregnant individuals against cannabis use(4), many individuals continue to use due to the lack of awareness regarding potential risks. The lack of evidence-based data regarding the potential effects of prenatal cannabis use has also hindered healthcare providers from counseling patients effectively(5, 6).

There is concern for adverse birth outcomes given that delta-9-tetrahydrocannabinol (THC, the main psychoactive component of cannabis) can readily cross the placenta and bind to cannabinoid receptors expressed in the developing fetus(7). The current literature suggests that prenatal cannabis exposure is associated with an increased risk of preterm birth, stillbirth, and small for gestational age infants, but is limited by weak mechanistic understanding, reliance on patient self-report and failure to control for important confounders such as polysubstance use(7). We have previously shown that prenatal edible THC consumption in rhesus macaques is associated with DNA methylation alterations in the placenta and multiple fetal tissues, including brain, heart, and lung, at genes involved in anatomical structure morphogenesis and development that may affect long-term offspring outcomes(8). Overall, there is a paucity of data on the specific effects of prenatal cannabis use on offspring lung development, but what is known is concerning(9–11).

In the lung, cannabinoid receptor 1 (CB1) expression has been reported in airway epithelial cells, alveolar type II cells, parenchymal cells, and vascular cells(9, 11, 12). THC has also been reported to inhibit lung angiogenesis, which could affect alveolar and capillary development, and gas exchange (12, 13). In a large population-based birth cohort (n=3,551,167), our group found that individuals with a cannabis-related diagnosis (n=23,007) had a higher proportion of births with neonatal respiratory distress syndrome (5.9% vs. 3.4%, $p<0.001$) and bronchopulmonary dysplasia (0.2% vs. 0.1%, $p<0.001$)(14).

The lack of adequate safety data is partly due to the scarcity of relevant preclinical models with strong translation to human health. These studies cannot be performed in humans due to the inability to obtain tissue, and the high prevalence of polysubstance use and other relevant confounding variables. In addition, rodent models are not suitable because of the immaturity of rodent lungs at birth, the absence of respiratory bronchioles in rodents and the lack of critical lung progenitor cell types (15, 16).

To address this critical knowledge gap, our study expands upon our initial work on the reproductive effects of cannabis to determine if maternal cannabis use impacts offspring pulmonary health, a considerably understudied area with substantial public health concern.

This study leverages our established rhesus macaque model of chronic prenatal edible THC use(8, 17, 18). This model is directly relevant to human pregnancies and avoids the limitations encountered by prior human studies including confounding from different modes of cannabis delivery and toxins from smoking. Edibles are the second most common mode of cannabis delivery amongst pregnant individuals(19, 20). Rhesus macaques and humans also exhibit similar plasma THC metabolism, and physiologic, genetic, anatomic (including lung), reproductive, and endocrine properties(21).

As the prevalence of prenatal cannabis use continues to increase and emerging data suggests it may adversely impact offspring outcomes, it is critical and timely to study the potential risks to offspring pulmonary biology and underlying mechanisms. Thus, the objective of this study is to determine the impact of chronic prenatal THC use on offspring lung development and function, and to explore the role of the lung epigenome and transcriptome linking cannabis exposure to offspring respiratory health.

MATERIALS AND METHODS

Experimental Design

This study used indoor-housed sexually mature, female rhesus macaques (n=8) maintained on a standard chow diet (TestDiet, St. Louis, Missouri) with an additional cookie with either THC (n=4) or placebo (n=4) daily. Although there are two primary cannabinoids in cannabis, THC and cannabidiol (CBD), we initially focused on the direct effects of THC alone to increase rigor and reproducibility and minimize potential confounders. CBD exhibits low binding affinity for CB1 and CB2 receptors(22), and there is variability in CBD product composition (e.g., can also contain THC, pesticides) and poor labeling accuracy (i.e., half of CBD product labels are inaccurate)(23).

Cookies containing THC (THC edible) were made using research-grade THC obtained directly from the National Institute on Drug Abuse (NIDA) Drug Supply Program (24, 25). Edibles were administered prior to the animal's daily chow to ensure consumption on an empty stomach and to confirm complete ingestion. Animals were slowly titrated up to 2.5mg/7kg/day of THC, equivalent to a heavy human medical cannabis dose, using published weight-based medical cannabis acclimation recommendations (26) approximately 4 months prior to undergoing time-mated breeding as previously published (24). THC-exposed pregnant animals continued to consume a daily THC edible (2.5mg/7kg/day) throughout pregnancy and postnatally. We previously established that the proposed THC dosing in the rhesus macaque recapitulates the same THC pharmacokinetics reported in humans following a similar edible weight-based THC dose. In addition, in medical cannabis dispensaries in California and Washington states, the median THC dose of edible products is approximately 54mg, thus the dose proposed in this study (25mg for a 70kg adult) reflects contemporary dosing (27, 28). Lung Blood-oxygen level dependent (BOLD)-MRI consisting of T_2^* measurements was performed near gestational days 110 and 150. All animals underwent natural delivery at term (~168 days). At 6 months, infants underwent pulmonary function testing prior to necropsy (**Figure S1**).

All protocols were approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee and conformed to all guidelines for humane animal care (IP0001389). Methods are reported in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>)(29). All methods of euthanasia utilized at the ONPRC are consistent with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals(30). Sodium pentobarbital (Vortech Pharmaceuticals) was delivered intravenously followed by exsanguination under a deep surgical plane of anesthesia.

Fetal Lung Magnetic Resonance Imaging (MRI)

Lung Blood-oxygen level dependent (BOLD)-MRI consisting of T2* measurements was performed at approximately 110 and 150 days of gestation. Animals were maintained on 1–2% isoflurane and intravenously administered a neuromuscular blocker (cis-atracurium at 0.1mg/kg) prior to fetal magnetic resonance imaging, for temporary arrest of maternal motion to improve image quality. Maternal respirations were controlled by mechanical ventilation and images captured during expiratory breath-holding. MRI scans were conducted using an NHP-dedicated Siemens Tim Trio (3T) MRI system (Erlangen, Germany) using a circularly-polarized transmit, 15-channel receive radiofrequency “extremity” coil (QED, Cleveland, OH). Following localization of the uterus and acquisition of single-shot turbo spin-echo (HASTE) anatomic images in the coronal and axial planes, axial 2D multislice spoiled gradient echo (SPGR) images spanning the entire uterus were acquired at six in-phase echo times with monopolar readout gradients(31). Fetal oxygen perfusion was quantified using BOLD-MRI as previously published(31). Fetal lung volumes were estimated separately for left and right lung by voxel volume weighted numerical integration over lung regions of interest (ROIs) drawn on maps of T2*. Right and left lung volumes were summed for each gestational age measured.

Pulmonary Function Testing (PFTs)

PFTs were performed in intubated, anesthetized infant monkeys as previously published (32, 33). Measurements included total lung capacity (TLC), vital capacity (VC), functional residual capacity (FRC), inspiratory capacity (IC), residual volume (RV), and expiratory reserve volume (ERV). In addition, spirometry measurements included forced vital capacity (FVC), forced expiratory flows at 50% FVC (FEF50), and forced expiratory volume in 200 msec (FEV200). After spirometry, carbon monoxide lung diffusion capacity (DLCO) and alveolar volume (VA) were measured. The lung was inflated with the test gas (0.5% Ne; 0.5% CO; 21% O₂; balance nitrogen) using a volume that was two-thirds of vital capacity. Following 4-seconds for neon equilibration and CO uptake, the same volume was withdrawn, and the final concentrations of Ne and CO were measured with a gas chromatograph (3000 Micro GC, INFICON, East Syracuse, NY). Measurements were performed 2–3 times with at least 3 minutes between replicates. VA was calculated using the lung inflation volume of test gas and the concentrations of neon in the test gas and the volume withdrawn following the 4-second equilibration; there were no corrections for dead space volume. DLCO was calculated from VA and the CO concentrations in test gas and the volume withdrawn following 4-seconds of CO uptake. Values within 10% were averaged and expressed under standard temperature and pressure conditions.

Bronchial Alveolar Lavage (BAL) Collection and Processing

At necropsy, lungs were removed en bloc with as much trachea as possible. Using a 60cc syringe with attached sterile tubing, 50mL of PBS was used to inflate the lungs through the trachea, with the tube inserted approximately 2cm above the carina. The PBS was immediately pulled back into the same 60cc syringe, with a return volume typically between 35 and 45mL. Three 1mL aliquots of BAL were aliquoted and saved for additional analysis. BAL sample was centrifuged at 2000rpm for 10 minutes to pellet cells. Supernatant was collected for Luminex analysis and assessed for concentrations of immune mediators using the R&D 36-plex NHP XL Cytokine Premixed Kit (Cat# FCSTM21–36, Bio-Techne, Minneapolis, MN, USA) following manufacturer's instructions. Samples were acquired using the MAGPIX xMAP instrument (Luminex Corporation, Austin, TX, USA). Data was analyzed using the Luminex Xponent software.

Nucleic Acid Extraction

Following BAL, infant lung samples were collected, wrapped in foil, and snap frozen in liquid nitrogen. Frozen lung tissue was later broken into small pieces on a stainless-steel plate on dry ice. For each infant, approximately 30 mg of tissue was transferred to a tube containing 750 μ L of RLT buffer with 10% β -Mercaptoethanol and a 5mm stainless steel bead. Samples were sonicated in a Tissue Lyser at 25hz in 1 minute bursts until fully homogenized. The homogenate was centrifuged at max RCF for 30 seconds and supernatant was then extracted using the Qiagen All Prep DNA/RNA/miRNA universal kit and the QIAcube for automated sample processing.

Whole Genome Bisulfite Sequencing (WGBS)

DNA extracted from frozen lung samples was sent to Novogene for DNA fragmentation, methylation library preparation, bisulfite conversion, and sequencing on an Illumina NovaSeq 6000 with 150bp paired-end reads at $\geq 60\times$. Raw FASTQ sequence files were processed with our standard in-house WGBS alignment protocol. Briefly, paired readsets were preprocessed with Trimmomatic(34) and aligned to the Mmul_10 reference genome using Bismark(35) to map bisulfite converted reads and determine cytosine methylation status.

DNA Methylation Analysis

Differentially methylated regions between THC and control samples were identified using the *DMRICH* package in R and are defined as having a 10% difference or greater between groups (36, 37). Default parameters were used with the following exceptions: "Percent of samples per a group for CpG coverage cutoff", which was set to 0.5 (50%); number of permutations for block and DMR analyses was set to the total number of samples (n=8); and cutoff value for single CpG coefficient (effect size) used to discover testable background regions was set at 0.1 (10%). We used Ensembl transcript annotations instead of the default Bioconductor annotations as recommended for non-model organism genomes. The *GOfuncR* option was selected for gene ontology analysis, which performs permutation based enrichment testing for the DMRs relative to the background regions and

accommodates for gene length bias. By default, DMRs are only mapped to genes if they are between 5 kb upstream and 1 kb downstream.

Bulk RNAseq Library Construction and Data Processing

RNA extracted from frozen lung samples was sent to Novogene for RNA QC, library preparation, and sequencing. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis, A-tailing, adapter ligation, size selection, amplification, and purification. Libraries were quantified with Qubit and real-time PCR and assessed for size distribution with a bioanalyzer. Quantified libraries were pooled and sequenced on an Illumina NovaSeq 6000 with ≥ 30 M (150bp paired-end) reads per sample. FASTQ sequence data was preprocessed with Trimmomatic(34) and aligned to Mmul_10 reference genome using STAR(38) stranded alignment to generate a table of counts. After trimming and alignment the raw RNA-seq count table consisted of 35,432 Ensembl annotated genes. Count data was transformed to counts per million (cpm) and we filtered out genes with low coverage (≤ 0.1 cpm in ≥ 5 samples, based on the minimum number of samples per group). We next performed principal component analysis (PCA) and sample clustering on the remaining genes ($n = 17,464$) to check for potential outliers. We estimated fractions of 10 immune cell types within the bulk lung RNAseq using the *immunedecconv* package and the quanTISeq method (39).

Bulk RNAseq Analysis

The *edgeR* package(40) was used for differential expression analysis between THC-exposed and control groups and results visualized using heatmaps with *ComplexHeatmap* (41), volcano and boxplots with *ggplot2*(42). We used the *STRINGdb*, *enrichplot*, and *clusterProfiler* packages(43, 44) to visualize interaction networks among differentially expressed genes (DEGs) and to examine enrichment of Gene Ontology (GO) terms, KEGG Pathways, Keywords, PMIDs, Tissues, etc. We also used Weighted Gene Co-expression Network Analysis (*WGCNA*) (45) to identify correlated modules of expression and to relate expression to phenotype data, visualized using functions from the *comethyl* package(46).

Methylation-Expression Correlation (eQTM)

To examine the correlation between log normalized expression of nominal DEGs with methylation, we first filtered the WGBS Bismark object to only include CpGs with 10X coverage in 50% or more of samples and performed Pearson correlation between expression and individual filtered CpGs within 25kb. We next examined the correlation between DMRs and expression using the smoothed methylation value per DMR per sample and expression of transcripts within 25kb.

Spatial RNAseq Library Construction and Data Processing

The 10x Genomics CytAssist-enabled Visium assay uses probe sets representing 18,085 human genes to spatially profile transcription across tissues. Hybridized probes were captured on a 5,000 spot array over a 6.5×6.5mm area of tissue. Briefly, 5 μ m sections of FFPE tissue were mounted on microscope slides, deparaffinized, stained with hematoxylin

and eosin and imaged on a Nikon Ni-E microscope with motorized stage as per kit guidelines (10x Genomics, Demonstrated Protocol CG000520). Decrosslinked tissue was hybridized with the Visium Human Transcriptome Probe Kit v2 and ligated probes captured on Visium slides using the CytAssist instrument. Libraries were generated from captured probes as per manufacturer's instructions (10x Genomics, User Guide CG000495). Paired end sequencing was performed by Novogene at a depth of >25,000 read pairs per spot. Space Ranger (v2.0.1) was used on the University of Kentucky HPC to align and count reads. Data was assessed for quality based on web summary files from Space Ranger and based on distribution of features.

Spatial Gene Expression Analysis

Output files from Space Ranger were loaded into *Seurat* (v4.4.0) for data pre-processing and analysis (47) in R (v4.2.2). We first filtered each dataset to remove spots with less than 500 features detected. We then normalized count data using the SCTransform function (vst.flavor=v2) (48) prior to integration after selecting a list of informative features (49). We next performed joint dimension reduction on the integrated Seurat object (RunPCA, RunUMAP, FindNeighbors, and FindClusters) to identify clusters of spots with co-expression that are representative of distinct structures and cell mixtures, with approximately 1–10 cells per 55-micron spot. Next, we overlaid cluster annotation on spatial images, extracted average expression (i.e. per cluster, gene, and treatment), and performed differential expression analysis between treatments using the non-parametric Wilcoxon rank sum test for each cluster. Additionally, we used a human reference (LungMAP_HumanLung_CellRef_Seed.v1.1.2), composed of 48 normal lung cell types with cell identities derived from the LungMAP CellCards(50) to identify enriched cell populations within clusters. We used these estimated cell proportions and cluster gene markers to manually annotate clusters along with information regarding underlying structural features.

Pseudobulk Analysis of Spatial RNA seq

To confirm the results from differential expression analysis between treatment groups using bulk RNAseq, we performed a pseudobulk analysis of differential expression using the spatial RNAseq dataset. To do so, we aggregate the counts per gene per sample across all spatial clusters and then perform filtering and differential expression analysis between groups using *edgeR* and the same process used for bulk RNAseq. We directly compared the log counts per million between bulk and pseudobulk datasets from the same animals using scatterplots and correlation analysis. We also compared the logFC between the two platforms to validate the direction and magnitude of change in the top DEGs and performed pathway enrichment analysis on all candidate DEGs.

Statistical Analysis and Data Integration

Statistical comparison between treatment groups for individual outcome measurements (i.e. measurements at MRI, PFT, and necropsy) was performed as follows: means and standard errors for each measurement were calculated using the *emmeans* package. P-values are from the ANOVA between treatment groups using unadjusted models and the *car* package, and models adjusted for crown-rump length at PFT when applicable. Additional integration

of datasets was performed using functions from comethyl and WGCNA to reduce 'omic data into co-regulated modules, which are then correlated with sample traits and outcome measurements.

RESULTS

Study sample characteristics

As described in the methods, pregnant dams with chronic THC consumption (THC, n=4) or placebo (CON, n=4) were allowed to deliver naturally at term. There was an equal ratio of male to female offspring per group. Offspring were kept with their mothers for normal rearing after delivery. There were no significant differences in baseline characteristics between THC- and CON-exposed dams, or offspring characteristics at birth or PFT as shown in Table 1.

Prenatal THC exposure is associated with reduced lung volume

Blood-oxygen level dependent (BOLD)-MRI consisting of T_2^* measurements was performed near gestational days 110 (G110) and 150 (G150). In THC exposed fetuses there were non-significant decreases in lung volume ($p=0.108$; $p=0.268$), lung perfusion, and oxygen availability ($p=0.089$; $p=0.328$) at ~G110 and G150, respectively (Figure 1). All animals underwent natural delivery at term and at 6 months of age, offspring were sedated, intubated, and PFTs were performed. The lung measures of functional residual capacity (FRC) and total lung volume (TLC) were significantly reduced in the THC exposed animals ($p = 0.015$ and 0.047 , respectively; Figure 2A). After adjusting for crown-rump length at PFT, the difference in FRC remained significant ($p=0.029$) and TLC association was slightly attenuated ($p=0.076$). Static lung compliance and gas exchange, as measured by diffusing capacity of the lungs for carbon monoxide (DLCO), also tended to decrease ($p = 0.11$ and 0.13 respectively). Forced expiratory flows (FEFs) and volumes (FEVs) were not significantly different between groups (Table S1).

Prenatal THC exposure is associated with reduced growth factors in bronchoalveolar lavage

Quantification of cytokines in the bronchoalveolar lavage (BAL) collected at necropsy was performed to explore potential differences between THC versus control animals and correlation with respiratory outcomes. We observed significant decreases in the lung growth factors brain-derived neurotrophic factor (BDNF), fibroblast growth factor-basic (FGFb), and vascular endothelial growth factor (VEGF) and in the pleiotropic cytokine interleukin-15 (IL-15) in THC animals (Figure 2B). The full list of cytokines quantified in BAL is provided in Table S2. Additionally, lung volume measured at different stages of development and by different methods were significantly correlated with each other and with BAL cytokines (Figure 2C).

Prenatal THC exposure is associated with differential gene expression in infant lung

We next performed differential expression analysis between THC-exposed and control lungs using bulk-RNAseq. There were 730 genes nominally different between treatment groups (0 FDR significant; Figure 3A and Table S3) and pathway analysis revealed

enrichment of gene ontology (GO) terms related to immune signaling (Figure 3A and **Table S4**) and activation of KEGG pathways involved in cytokine-cytokine receptor interaction (NES= 1.853; p=0.0017). Given this finding, we estimated fractions of 10 immune cell types within the bulk lung RNAseq and found significantly decreased proportion of natural killer (NK) cells in the THC exposed animals (**Figure S2**). Adjusting for cell fractions in differential expression analysis resulted in 1,453 nominally significant DEGs (Figure 3B and **Table S5**). Cell fraction adjusted DEGs were enriched for GO terms related to extracellular matrix organization and development including “lung development”, “respiratory tube development”, and “kidney development” (Figure 3B and **Table S4**) and gene set enrichment analysis revealed suppression of the “retrograde endocannabinoid signaling” KEGG pathway (NES= -2.015; p=0.0007).

RNA expression is correlated with lung measurements

We used WGCNA to cluster all genes into correlated modules and identify gene networks associated with treatment and biological measurements (e.g., FRC, TLC, etc.). Using this approach, we identified a total of 74 modules, two of which were significantly correlated with THC treatment (MEdarkorange and MEorangered3; (Figure 3C). The MEdarkorange module eigenvalues were also correlated with BDNF and FGFbasic protein concentration in BAL, DLCO, and FRC (Figure 3C). Pathway analysis of the MEdarkorange genes (n=124; **Table S3**) identified enrichment for immune tissues and cell types, which is similar to results of pathway and KEGG enrichment of all DEGs without cell type adjustment. The MEorangered3 module eigenvalues were also correlated with BDNF and (BOLD)-MRI T₂* measures at ~G110, however this module only 19 contained genes and therefore we did not perform pathway analysis.

Prenatal THC exposure is associated with differential methylation in infant lung

DNA was isolated simultaneously from the same samples used for RNAseq. We identified 3,558 nominally significant differentially methylated regions (DMRs) with 10% or greater difference in methylation between THC-exposed and control lungs (70% hypermethylated, 30% hypomethylated) (Figure 4; **Table S5**). The top 3 DMRs were annotated to phosphatidylinositol glycan anchor biosynthesis class Z (PIGZ; difference= -54%), transient receptor potential cation channel subfamily M member 8 (TRPM8; difference= -28%), and PX domain containing serine/threonine kinase like (PXX; difference= -34%). GO enrichment testing suggests that DMRs are enriched for genes located in cellular junction and involved in cell junction organization (Figure 4C). Enrichment testing for gene region (genic) annotations identified positive enrichment of DMRs in promoter, exon, intron, and 3'UTR genomic features and depletion in intergenic regions. DMR annotations were also enriched for CpG Islands, Shores, and Shelves, and depleted in Open Sea regions (**Figure S3**).

RNA expression is correlated with DNA methylation (eQTM analysis)

For each candidate differentially expressed gene we performed correlation with DNA methylation (DNAm) using CpGs within 25kb to examine epigenetic dysregulation of gene expression following maternal THC use during pregnancy. Out of 730 DEGs in our

unadjusted model, 673 (over 92%; **Table S3**) were correlated with DNAm at one or more CpG located within 25kb of one or more measurable transcripts, and 97 unique DMRs were correlated with gene expression (included in **Table S6**). Similarly, over 95% of DEGs following adjustment for cell types were correlated with DNA methylation at one or more CpG (**Table S5**).

Spatial transcriptomics identifies differential expression within specific cell clusters

Bulk-RNAseq represents the average of gene expression across all cell types within a sample and can mask differences between and within cell populations. Therefore, we next determined spatial niches of differential expression and underlying lung structures using the 10x Genomics Visium Spatial Gene Expression platform which provides an average resolution of 1 to 10 cells per 55-micron spot. We measured expression of 17,318 genes (>95% of human capture probes), demonstrating that the Visium platform is highly compatible with rhesus macaque DNA. Data integration and dimension reduction revealed 13 distinct clusters of spots (Figure 5A) and the top marker genes for each cluster are visualized in **Figure S4**. Clusters were manually annotated based on 1) cell type mapping per spot to the Human LungMap reference (Figure 5B), 2) underlying tissue morphology (Figure 5C), and 3) functional enrichment analysis of marker genes for each cluster (Figure 5D) (51). The only significant difference in the percentage of spots between treatments was in the “0 cluster” ($p=0.008$), although there were trends for decreased percentage of cluster 4, and increased percentage of spots in clusters 3, 5, and 11, in the THC-exposed lungs (**Figure S5**).

Differential expression analysis within each cluster identified 186 unique spatial cluster DEGs (sDEGs) with Bonferroni $p<0.05$ between THC and control lungs, although several sDEGs were identified in more than one cluster resulting in 484 total instances of differential expression (**Table S7**). The majority of sDEGs were from the clusters enriched for vascular smooth muscle cells (cluster 10), ciliated cells lining airways (cluster 6), and immune cells (cluster 11) (Figure 6A). Functional analysis of all sDEGs identified enrichment of genes involved in extracellular matrix organization, regulation of peptidase activity, muscle contraction, and wound healing (**Table S8**). Results from enrichment analysis of sDEGs in each cluster separately were similar to results for all sDEGs, with response to transforming growth factor beta, and response to hyperoxia enriched in clusters 6 and 11, and sDEGs in cluster 11 uniquely enriched for terms related to immune system processes and regulation of tube size (Figure 6B; **Figure S6**). Pseudobulk analysis of spatial transcriptomics, which aggregates expression per gene across spatial clusters, support results from bulk-RNAseq, as shown by significant positive correlation of effect sizes (logFC) with bulk DEG candidates ($r=0.68$; $p<2.2e-16$).

DISCUSSION

This is the first study to examine the effects of maternal THC edible consumption on offspring respiratory health in a rhesus macaque model. Although non-human primate studies are small in sample size, it is a strong translational model that overcomes limitations of human studies and has a demonstrated track record of predicting clinical respiratory

outcomes (33, 52, 53). In this study, there are several key findings that highlight the potential adverse impact of THC exposure on offspring lung function and development. Most importantly, maternal THC edible consumption resulted in significantly decreased lung volume measured by pulmonary function testing at 6 months of age, though only FRC remained significant after adjustment for infant length. However, measurement of total fetal lung volume by *in utero* MRI and repeated measurements over time in the same animals suggests these changes began early in development (before G110, equivalent to the late second trimester) and are persistent to at least 6 months of age. These results suggest the potential for altered developmental programming of the lung and potential long term deleterious effects of *in utero* THC on offspring lung. This is reminiscent of the long term effects of maternal tobacco smoking on offspring lung function, which are known to persist into childhood and adulthood, leading to an increased risk of respiratory morbidity(54), and which were only discovered decades after the common use of tobacco products(54, 55). Because edibles and smoking are the two most common forms of cannabis use in pregnant individuals, it is possible that smoked cannabis will result in even worse offspring respiratory health outcomes due to the combined effect of THC binding to cannabinoid receptors in the fetal lung and the toxins from cannabis smoke.

Additionally, there is significant co-use of tobacco and cannabis products during pregnancy and approximately 50% of individuals who use one product during pregnancy will use both (14, 56, 57). Given that prenatal tobacco exposure also effects offspring lung development and function(54), there are likely to be additive effects of co-use of tobacco and cannabis during pregnancy. This is supported by our clinical analysis of pregnant women who used cannabis and or tobacco during pregnancy showing that use of either product alone increased risks of infant death, NICU admission, respiratory distress syndrome and bronchopulmonary dysplasia and that these risks were increased with combined use (14). In addition, *in utero* tobacco exposure appears to primarily affect airway and alveolar structure (54) while our results shown here suggest prenatal cannabis may have greater effects on lung volumes, blood flow and gas exchange, thus increasing the possibility of additive negative effects on offspring lung development and function. It is also important to note that the effects of tobacco use during pregnancy on lung development appear driven primarily by nicotine rather than the other toxins in tobacco smoke suggesting that smoking cannabis or consuming THC edibles during pregnancy will have similar effects on offspring lung development irrespective of the toxins in cannabis smoke (58).

Potential underlying mechanisms for the observed effects of prenatal THC on lung volume, correlated decreases in perfusion and oxygen availability of the fetal lung, and in pulmonary gas exchange in THC-exposed offspring at 6 months include decreased levels of critical lung factors measured in bronchoalveolar lavage fluid, including VEGF and BDNF, and FGFbasic. VEGF is highly expressed in lung and endothelial cells and is essential for vascular bed formation during embryogenesis(59). Previous studies have demonstrated that insufficient VEGF in newborn mice leads to alveolar simplification(60), while lung-specific repression of VEGF was associated with reduced lung volume, alveolar simplification, and increased respiratory rate(61). Therefore, altered VEGF following *in utero* THC exposure may partially explain observed decreases in offspring lung volumes and gas exchange (DLCO) in our study, as both alveolar surface area and the pulmonary capillary bed are

crucial determinants of gas exchange. Moreover, recent single-cell sequencing experiments have identified a BDNF⁺ stromal subtype which appears to contribute to branching morphogenesis in developing lungs(62). In a separate study, BDNF was found to be mainly expressed by alveolar type I cells, and greater expression of BDNF was observed in mice exposed to lipopolysaccharide (LPS), widely used to elicit inflammation and acute lung injury (63). Therefore, lower BDNF measured in BAL of THC exposed animals may be related to an altered regulation of inflammatory and immune responses.

Although cannabinoids administered to adults have been shown to suppress the immune system(64–66), there is limited information on the effects of THC exposure on the developing immune system(67). One study in mice noted decreased thymic cellularity and alterations in T cell populations and function following acute THC administration during pregnancy, however the doses used are not reflective of “real-world” exposure (68). Our study suggests that prenatal THC exposure is associated with cytokine activation and inflammation in the infant lung, which may impact susceptibility to future respiratory infection and have direct consequences on lung function. These findings support our observation of increased rates of bronchopulmonary dysplasia in THC-exposed neonates seen in our large population-based cohort study(14).

Our study is strengthened by our integration across datasets which included correlation of biological outcomes with each other, with bulk transcriptional signatures (e.g. WGCNA modules), and with DNA methylation signatures of epigenetic regulation. Moreover, we performed pseudobulk analysis of spatial expression in a different piece of lung tissue per animal to validate bulk-RNAseq measures. Of note, spatial transcriptomic analysis additionally revealed differential expression within specific cellular niches that were not identified by bulk-RNAseq and we observed enrichment of biological processes in spatial DEGs extremely relevant to lung development and function including extracellular matrix organization, response to hyperoxia and wound healing in the ciliated cells, and growth factor binding and regulation of tube size in airway smooth muscle cells, which agree with bulk-RNAseq results adjusted for immune cell fractions. Notably, several genes in the collagen, lysyl oxidase (LOXL), matrix metalloproteinase (MMP), and “a disintegrin and metalloproteinase with thrombospondin motifs” (ADAMTS) family genes were decreased in lungs from THC exposed infants. Prenatal exposure to nicotine also alters immune signaling, collagen deposition, and expression of extracellular matrix proteins, suggesting that combined exposure to cannabis and tobacco products may have interactive effects on these overlapping pathways(69–72). However, the specificity of THC effects and underlying molecular mechanisms compared to smoked marijuana or other prenatal insults (e.g. tobacco exposure) remains undetermined.

Additional strengths of our study include utilization of a translational rhesus macaque model to overcome the limitations of existing studies, such as the inability to accurately measure cannabis exposure (e.g., timing, duration, frequency, dose, type of product, and administration route). Our study used weight-based THC dosing that parallels human medical cannabis dosing for rigor and reproducibility. Daily THC edibles were selected as the mode and frequency of administration to recapitulate typical human THC use in pregnancy(19, 73) and to examine the direct effects of THC-only. Additionally, our study

overcomes the paucity of human studies on prenatal cannabis exposure due to lack of feasibility in performing repeated *in utero* imaging and obtaining tissue for molecular analysis, and ethical challenges related to research on maternal use of federally illegal drugs, such as cannabis.

The study has several limitations, including a small sample size due to the limited resource and high cost of NHP studies that may limit the generalizability of our study's results. Our study was underpowered to detect molecular differences after multiple testing correction and we rely on integration across datasets and pathway level results to identify candidates for further study. Additionally, this study was initially designed to study effects of prenatal THC on infant brain development and behavior(18), therefore we were unable to perform stereologic analyses of lung tissue as they were not uniformly inflated and fixed. Thus, although the correlated findings of decreased lung volumes, changes on BOLD-MRI, and altered DNA methylation and gene expression strongly support the biologic relevance of this study, follow up studies with larger sample sizes are needed. Future studies should examine the impact of prenatal THC exposure on airway structure and alveolar development using advanced imaging and stereology, which was not possible in this study.

Overall, although the results presented herein are not definitive, they raise clear concerns for potential effects of maternal THC edible consumption on offspring respiratory health. Children born with decreased lung function at birth are more likely to follow a lower trajectory of lung function as they age, increasing their risk to develop respiratory diseases such as chronic obstructive pulmonary disease (COPD) as adults(74). Therefore, this study suggests that prenatal THC exposure may have important influence on future respiratory health and further studies in human populations are needed. The findings of this study add to the current, limited safety data on prenatal cannabis exposure, in particular the potential impact of chronic prenatal and postnatal THC exposure on infant lung development. As the prevalence of prenatal cannabis use is rising, along with significant increases in potency, there is an urgent need for evidence-driven recommendations on the safety of use both prenatally and postnatally. The data from this study will help guide patient counseling on cannabis use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

Raw datasets for this study are publicly accessible through the NIH Sequence Read Archive (SRA) via *BioProject* accessions: PRJNA1195032 and PRJNA1208011, and at: <https://doi.org/10.6084/m9.figshare.28144076>.

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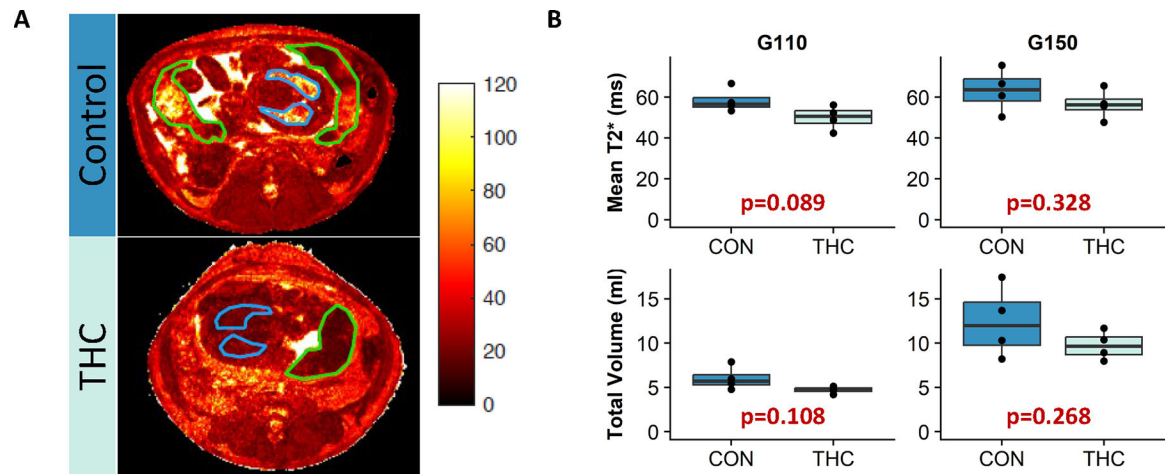


Figure 1.

Prenatal THC-associated is associated with decreased oxygenation and lung volume by Blood-Oxygen-Level Dependent (BOLD) MRI. (A) Representative axial BOLD MRI images from control (top) and THC-exposed (bottom) fetuses at approximately gestational day 150 (G150; late-third trimester). Lungs are outlined in blue and placental lobes in green. Brighter intensity (longer T2*) indicates greater oxygen availability in the control animals and darker areas (shorter T2*) demonstrate decreased oxygen availability in THC-exposed animals. (B) Boxplots of Mean T2* and calculated total lung volume (sum of right and left lung volumes) at G110 and G150 assessed by BOLD-MRI. Means and standard errors were calculated using the *emmeans* package. P-values are from unadjusted ANOVA between treatment groups (n=4 animals per group).

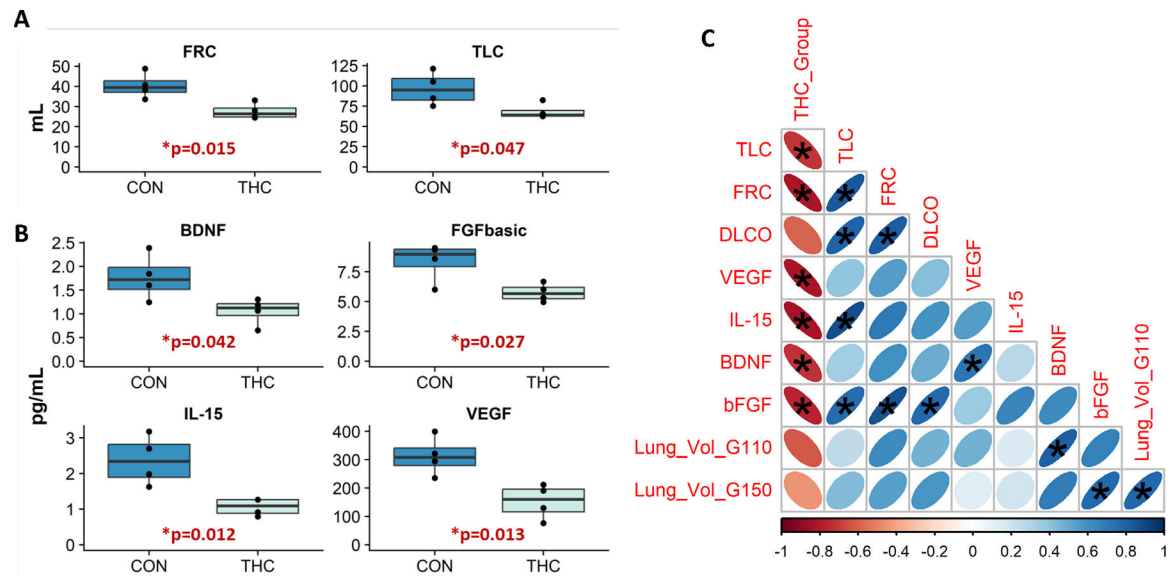
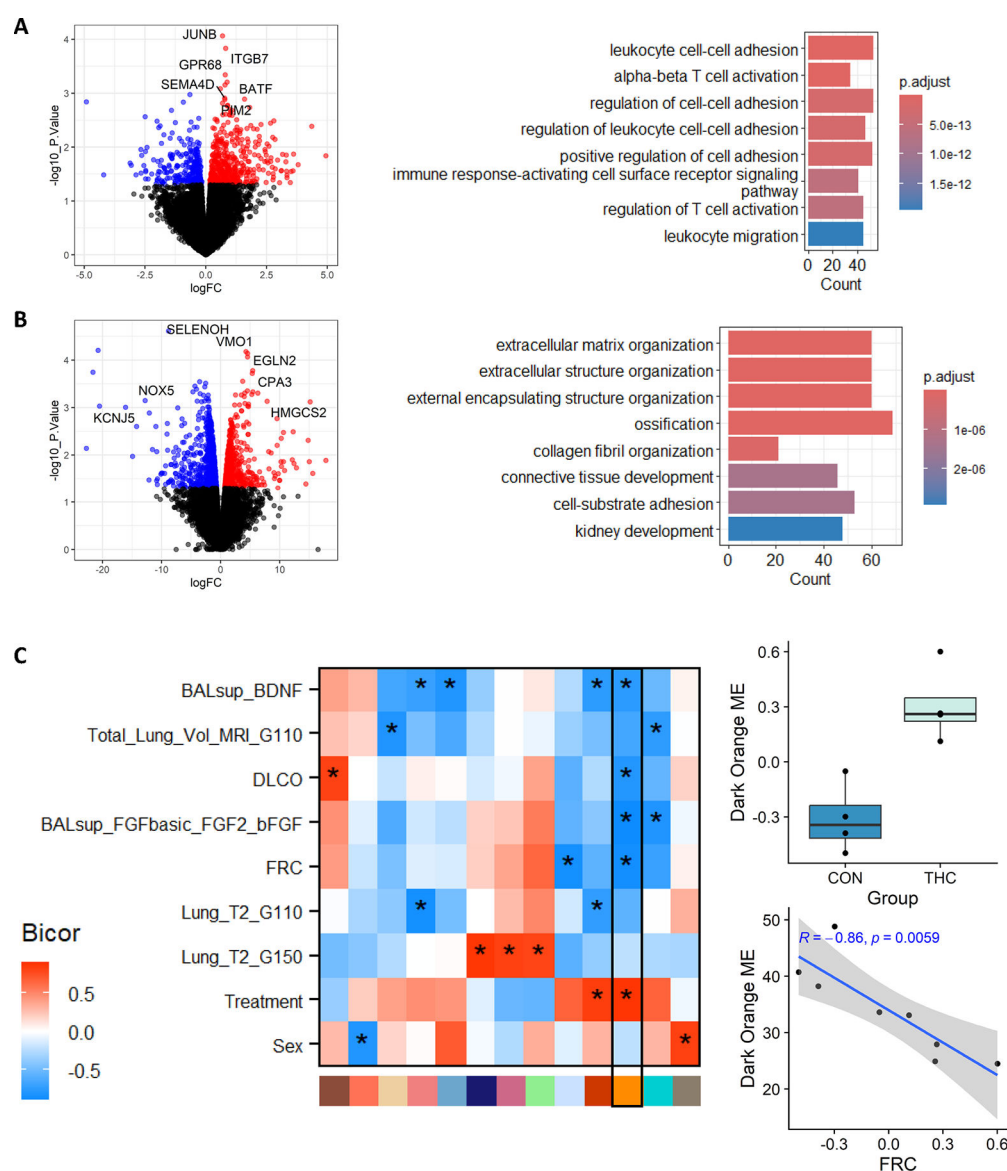


Figure 2.

Prenatal THC-associated is associated with lung measures at pulmonary function testing (PFTs) performed at 6 months of age and cytokines in bronchoalveolar lavage (BAL) fluid. (A) Boxplots represent the median and interquartile range of functional residual capacity (FRC) and total lung volume (TLC) as well as (B) select cytokines measured in BAL following PFTs. P-values are from unadjusted ANOVA between treatment groups (n=4 animals per group). (C) We use a correlation chart to examine within animal correlation between MRI measures, PFT measures, and cytokine measures in BAL. Ellipses are scaled and colored according to Pearson correlation values between measurements with blue indicating positive correlation and coral indicating negative correlation. Asterisks (*) denote $p < 0.05$.

**Figure 3.**

Prenatal THC exposure is associated with differential gene expression in infant lung and RNA expression is correlated with lung measurements. (A) Results are presented from differential expression analysis between THC and CON samples using edgeR unadjusted for covariates and (B) after adjustment for immune cell fractions. In the volcano plots on the left, the x-axes represent logFC and the y-axes represent $-\log_{10}P$ value. The color of points indicates nominal significance for upregulated (red) and downregulated (blue) expression. Barplots on the right represent top enriched Gene Ontology (GO) terms enriched among DEGs. (C) From WGCNA analysis, the top significant module-trait correlations are visualized in the heatmap with red indicating positive correlation values and blue indicating negative. The asterisks indicate $p < 0.05$. The boxplot (top right) shows the separation of MEDarkorange by treatment and the scatterplot (bottom right) shows the correlation between MEDarkorange and FRC.

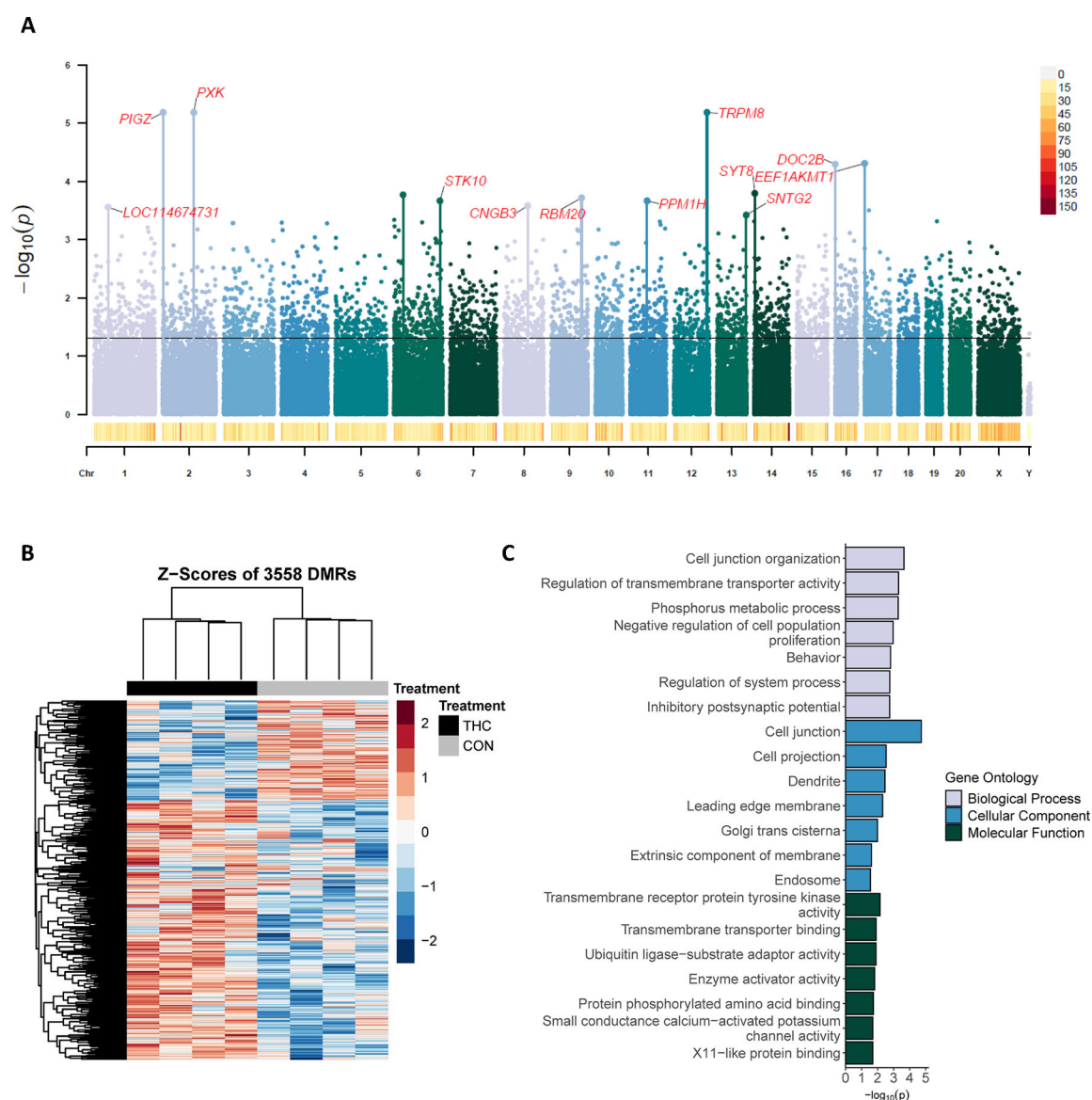
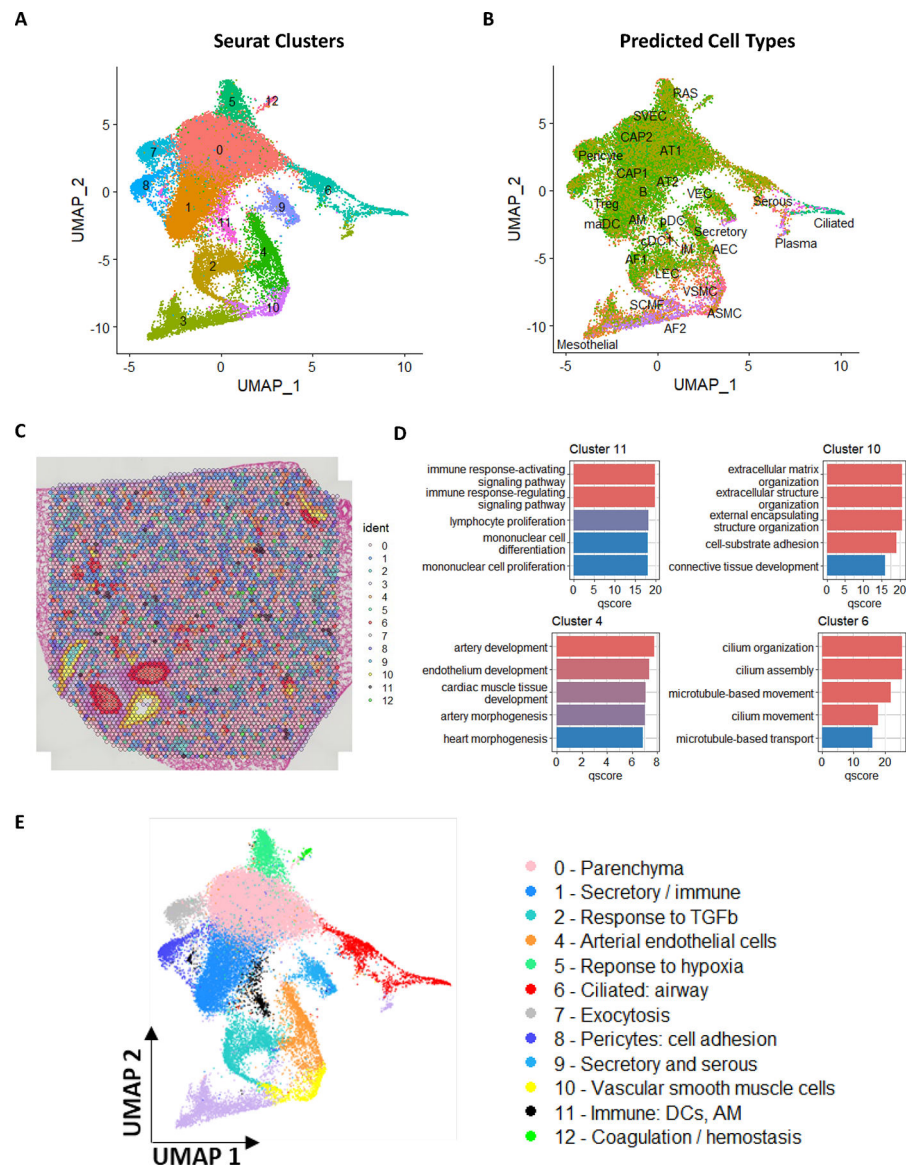


Figure 4.

Prenatal THC exposure is associated with differential methylation in infant lung. The DMRichR pipeline was used to identify genomic regions with 10% or greater difference in methylation between THC and control samples. (A) Manhattan plot summarizes THC-DMRs with chromosome position on the x-axis and the $-\log_{10}(P\text{-value})$ on the y-axis; the black line indicates $p < 0.05$ and red labels highlight genes annotated to top DMRs. (B) Heatmap showing individual smoothed methylation value z scores for each DMR. (C) Barplots show the top 7 Gene Ontology (GO) terms for each category identified using GOfuncR, with the $-\log_{10}(P\text{-value})$ on the x-axis and GO terms labeled on the y-axis.

**Figure 5.**

Spatial transcriptomic cluster annotation. (A) UMAP of 13 clusters identified based on co-expression patterns after quality control, data integration, and dimension reduction. (B) Dominant predicted cell populations of each spot were determined using Human LungMap single-cell reference expression. (C) Cluster identity was overlaid on H&E stained tissue in order to add tissue morphology annotation. (D) Functional enrichment analysis of marker genes in each cluster was performed to support annotation. (E) UMAP clusters were recolored and manually annotated based on the combined evidence from (B-D).

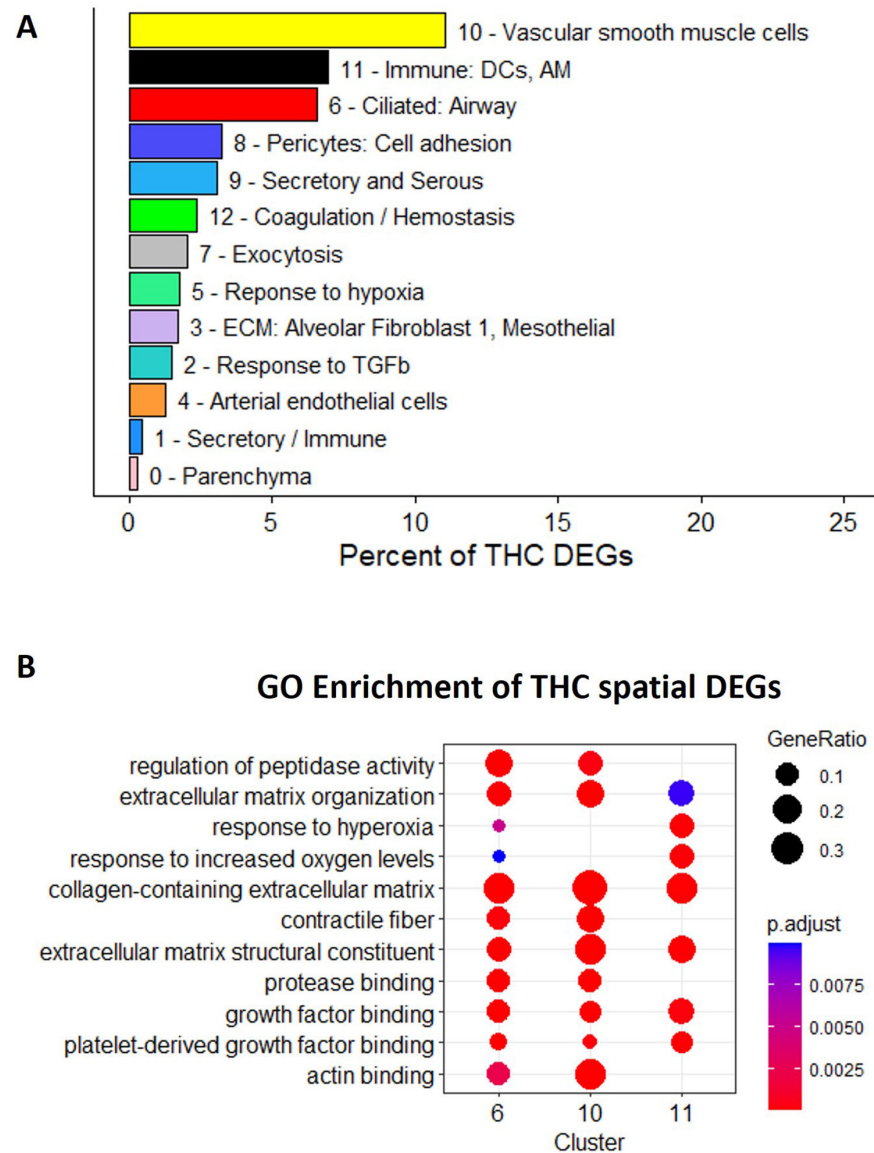


Figure 6.

Prenatal THC-associated is associated with differential expression within spatial clusters. (A) Within each spatial cluster, we identified differential expression of genes (DEGs) between THC and control lungs and summarize the percentage in each cluster out of the total number of DEGs in all clusters. (B) Dotplots summarize GO enrichment analysis for the 3 clusters with greatest number of DEGs; the size of the circles indicates the ratio of the number of observed DEGs out of the total number of genes in each pathway and the color indicates relative significance, with more red indicating a lower p-value.

Table 1.

Subject demographics

Characteristic	Control (n=4)	THC (n=4)	p-value
Maternal age (years)	8.704 ± 0.729	10.706 ± 2.12	0.154
Parity	3	3.25 ± 1.5	0.761
Maternal Weight at G60 (kg)	7.200 ± 0.877	7.788 ± 1.369	0.501
Maternal Weight at ~G150 (kg)	8.725 ± 0.754	9.175 ± 1.239	0.562
Fetal sex (male:female)	1:3	1:3	1
P180 Infant Weight (kg)	1.57 ± 0.11	1.38 ± 0.11	0.110
P180 Infant Crown-Rump Length (cm)	31.9 ± 0.6	31.32 ± 0.6	0.522

Mean and standard error were calculated using the *emmeans* package. P-values are from the unadjusted ANOVA between treatment groups using the *car* package.