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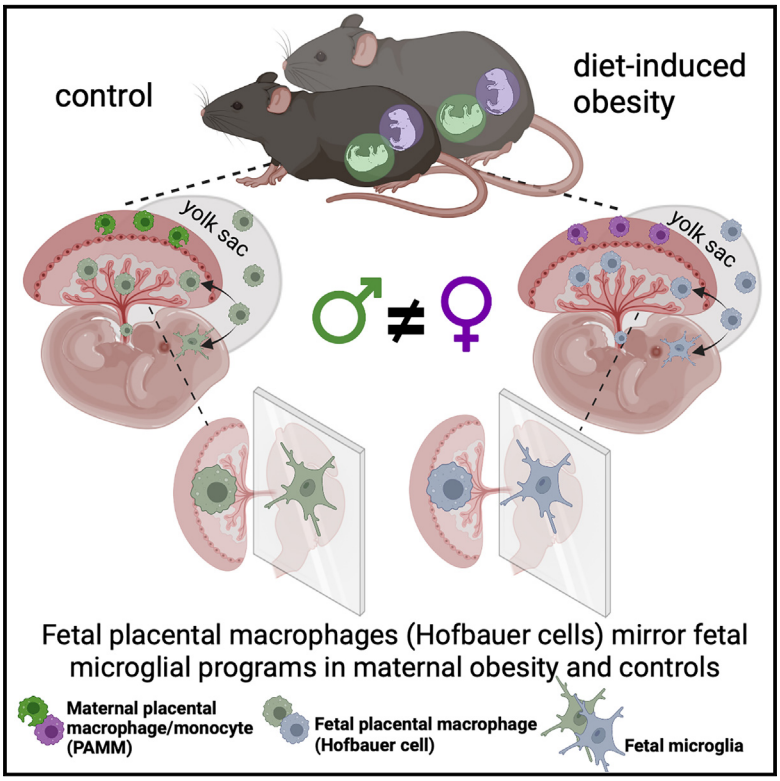
## Graphical abstract




## Highlights

- Microglia and fetal placental macrophages (Hofbauer cells) share a common yolk-sac origin
- scRNA-seq reveals shared transcriptional programs in these two cell types in maternal obesity
- There are sex differences in the impact of maternal obesity on Hofbauer cells and microglia
- Hofbauer cells may serve as biomarkers of the impact of maternal exposures on microglia

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## In brief

Batorsky et al. employ lineage tracing to demonstrate that fetal microglia and Hofbauer cells both originate in the yolk sac and exhibit shared transcriptional programs in the setting of maternal obesity. Hofbauer cells may serve as biomarkers for fetal microglia, providing insight into the impact of maternal exposures on neurodevelopment.

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# Hofbauer cells and fetal brain microglia share transcriptional profiles and responses to maternal diet-induced obesity

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## SUMMARY

Maternal immune activation is associated with adverse offspring neurodevelopmental outcomes, many mediated by *in utero* microglial programming. As microglia remain inaccessible throughout development, identification of noninvasive biomarkers reflecting fetal brain microglial programming could permit screening and intervention. We used lineage tracing to demonstrate the shared ontogeny between fetal brain macrophages (microglia) and fetal placental macrophages (Hofbauer cells) in a mouse model of maternal diet-induced obesity, and single-cell RNA-seq to demonstrate shared transcriptional programs. Comparison with human datasets demonstrated conservation of placental resident macrophage signatures between mice and humans. Single-cell RNA-seq identified common alterations in fetal microglial and Hofbauer cell gene expression induced by maternal obesity, as well as sex differences in these alterations. We propose that Hofbauer cells, which are easily accessible at birth, provide insights into fetal brain microglial programs and may facilitate the early identification of offspring vulnerable to neurodevelopmental disorders.

## INTRODUCTION

Microglia play a key role in neurodevelopment by modulating synaptic pruning, neurogenesis, phagocytosis of apoptotic cells, and synaptic plasticity.[1–4] Aberrant programming of fetal microglia in the setting of maternal immune activation has accordingly been identified as a key mechanism underlying abnormal fetal brain development[5–9] and likely contributes to the pathogenesis of neurodevelopmental and psychiatric disorders.[10–13] As microglia remain inaccessible in fetal life and postnatally, there is currently no way to identify which offspring may be most at risk for adverse neurodevelopmental and psychiatric morbidity after *in utero* exposures. Methods to determine whether or how *in utero* exposures may have primed fetal microglia could facilitate intervention during critical developmental windows when outcomes can potentially be modified.

Precursors of many tissue-resident macrophages, including microglia, originate in the fetal yolk sac.[14–16] As the yolk sac is a pre-placental structure, yolk-sac-derived macrophages likely also colonize the placenta, where they are called Hofbauer cells, although there has been recent debate regarding the potential ontogenies of these cells.[17–20] These cells share exposure to the same intrauterine environment as microglia, and their connection to the developing brain has been posited as a result of their role in the maternal-to-fetal transmission of neurotropic viruses such as Zika, cytomegalovirus, and HIV.[21–25] In support of commonality between these cell types, prior work by our groups demonstrated that maternal immune activation in the setting of high-fat diet primes both placental macrophages and fetal brain microglia toward a highly correlated, pro-inflammatory phenotype.[26,27] Still, whether Hofbauer cells manifest the same transcriptional programs as fetal microglia has not yet been investigated.

Maternal obesity is known to be associated with maternal immune activation[28–31] and with increased offspring risk for neurodevelopmental and neuropsychiatric disorders.[32–35] These

disorders demonstrate marked differences in prevalence by sex; as fetal sex is recognized to influence placental, fetal, and neonatal immune responses,[27,36–38] differences in microglial development and priming are an attractive candidate mechanism for mediating sex biases in these disorders.[39] Indeed, sex differences in the impact of maternal obesity on fetal brain development, microglial and placental function, and neurodevelopmental outcomes have been described,[26,31,40–45] with animal and human studies suggesting males have an increased incidence of structural brain changes, unfavorable brain metabolic profiles, autism spectrum disorder, and deficits in learning and memory. Yet, sex differences in single-cell microglial and placental macrophage programs have not yet been investigated.

To address these gaps in understanding, we sought to definitively trace Hofbauer cells from the fetal yolk sac to the placenta. Using an inducible macrophage reporter mouse model, we demonstrate that yolk sac-derived macrophages comprise the majority of tissue-resident macrophages in both placenta and brain in late embryonic development (embryonic day 17.5 [e17.5]). Further, we isolated placental resident macrophages and microglia from fetuses of diet-induced obese and control dams (wild-type C57BL/6J) at e17.5 and characterized cells using single-cell RNA sequencing (scRNA-seq). Using X and Y chromosome markers, we distinguished fetal placental macrophages, or Hofbauer cells, from placenta-associated maternal monocyte/macrophage (PAMM) populations, generating insights into transcriptional differences between Hofbauer cells and PAMMs both at baseline and in the setting of maternal diet-induced obesity. Specifically, we identified subpopulations of fetal placental macrophages that transcriptionally mirror fetal brain macrophages and found that maternal obesity affected gene expression similarly in both microglia and Hofbauer cells, both in terms of differentially expressed genes and biological processes. Functional analyses of differentially expressed genes provided insights into sex differences in immune function and the response to maternal obesity in both placental and fetal brain macrophages. By serving as a surrogate cell type that can reflect aberrant fetal microglial programs, Hofbauer cells may help identify offspring most vulnerable to neurodevelopmental morbidity in the context of maternal exposures such as obesity.

## RESULTS

### Tissue-resident placental macrophages are yolk sac derived

In both mice and humans, the maternal-fetal interface is composed of the maternally derived decidua and the fetally derived placenta. Fetal placental macrophages have been identified as early as 18 days post conception in humans, and 10 days post conception in mice (e10) before the full vascularization of the placenta.[46] This suggests that extra-embryonic placental macrophages (Hofbauer cells) likely derive from the yolk sac, as do many other tissue-resident macrophages, including microglia.[47]

To determine whether fetal placental macrophages are in fact yolk sac-derived, we crossed transgenic mice carrying a floxed Rosa-*tdTomato* allele[48] with tamoxifen inducible transgenic *Csf1R-Cre*$^{ER}$ mice to permanently label yolk sac progenitors. Csf1r is active in yolk sac progenitor cells at gestational day 8–9 (gd8-9), thus a 4-hydroxytamoxifen (4-OHT) pulse at gd8.5 will label yolk sac-derived macrophages prior to their migration out of the yolk sac.[49] Migration of macrophages out of the yolk sac to colonize the fetal brain and other tissues begins around e9,[14] and Csf1r+ placental macrophages are detectable beginning at e10.[46] Thus, we delivered 4-OHT to Cre$^{ER}$-negative dams (tdTomf/+ or tdTomf/f) crossed with Cre$^{ER}$-positive sires, so that 4-OHT only induced *Csf1R-Cre*$^{ER}$ activity in *fetal* macrophages within the embryos (Figure 1A). We then assessed tdTomato+ cells at e17.5 in placenta and brain from Cre$^{ER}$-negative and Cre$^{ER}$-positive embryos in conjunction with immunohistochemistry for ionized calcium binding adaptor molecule (Iba1), a marker for macrophages. In the placenta labyrinth, we saw robust colocalization of tdTomato and Iba1 at e17.5 (approximately 83% of Iba1+ cells were also tdTomato+; Figures 1B and 1C′). As expected, we also detected substantial colocalization in e17.5 brain (hippocampus shown in Figures 1B and 1D). We did not see any tdTomato signal in Cre-negative placenta or hippocampus (Figures 1C and 1D). Together, these data suggest that resident placental macrophages are primarily derived from yolk sac progenitor cells, similar to fetal microglia. Additional staining demonstrated that placental macrophages were found within the villous tissue and were not primarily located within the vasculature (Figure S1).

### Fetal placental and brain macrophages are heterogeneous populations with shared cluster-specific signatures

To identify subpopulations of fetal placental and brain macrophages, we performed scRNA-seq (10X Genomics) on macrophage-enriched single-cell suspensions from matched placenta and fetal forebrain tissue from both male and female embryos at e17.5 (Figure S2A). Matched placenta and fetal brains were collected from 18 mouse embryos, comprising nine embryos from obese dams (four male, five female) and nine embryos from control dams (four male, five female). A total of 197,000 cells were sequenced to an average depth of approximately 22,000 reads/cell. In order to identify cell types present in our data, we used graph-based clustering followed by identification of cluster-specific marker genes and comparison of marker gene expression and average expression profiles of all clusters with published placental[50–54] and fetal brain[55–57] datasets (see STAR Methods, Figures S2B and S2C, and Table S1). We selected macrophage and monocyte-like clusters for our main analysis in light of recent evidence of monocyte-to-macrophage transitional populations at the maternal-fetal interface,[58] which comprised >95% of the total myeloid cell population. The final clusters are visualized as uniform manifold approximation and projection (UMAP) plots for brain (Figure 2A) and placenta (Figure 2B) along with expression plots demonstrating the top three marker genes per cluster. Clusters have been named with a cell type prefix (Mg: microglia; HBC: Hofbauer cell; Mono_FBr: fetal brain monocytes; Mono_FPl: fetal placental monocytes; PAMM: placenta-associated maternal macrophages and monocytes) followed by the top marker gene in the cluster. Clusters primarily engaged in cell cycle functions (e.g., processes integral to DNA replication) end in _cellcycle.

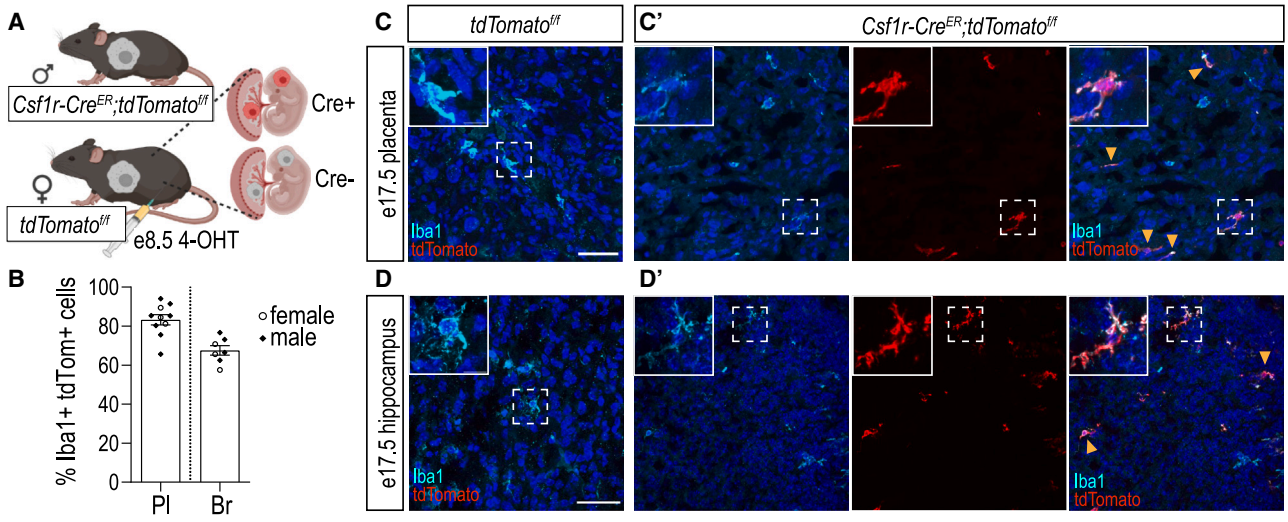



**Figure 1. Placental macrophages are yolk sac derived**

(A) Schematic of fetal yolk sac macrophage labeling. Male *Csf1R-Cre*$^{ER}$*;tdTomato*$^{f/f}$ mice were timed-mated to female *tdTomato*$^{f/f}$ mice. Pregnant females were injected with 4-hydroxytamoxifen (4-OHT) at gestational day 8.5. Embryos were collected at embryonic day 17.5.

(B) Percent of macrophages (Iba1+ cells) labeled with tdTomato in embryonic placenta and hippocampus following 4-OHT administration at e8.5. Open circles represent individual female embryos and closed diamonds represent individual male embryos ($n$ = 4 litters). Pl = placenta; Br = brain (hippocampus).

(C–C′) Representative images of Iba1 and tdTomato in control (C) and reporter (C′) placenta from e17.5 embryos.

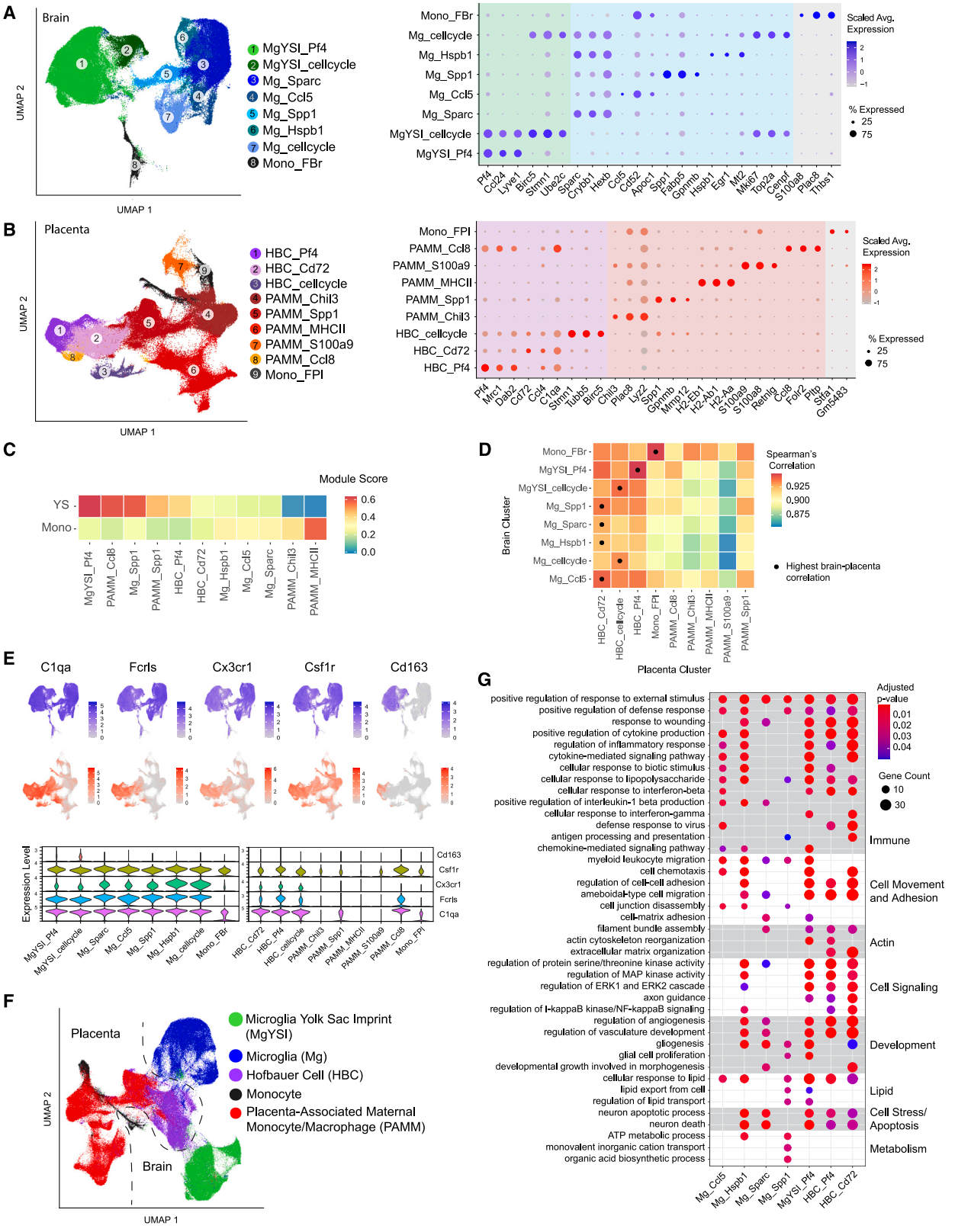
(D–D′) Representative images of Iba1 and tdTomato in control (D) and reporter (D′) hippocampus from e17.5 embryos. Arrowheads indicate double-positive (Iba1+ tdTomato+) macrophages/microglia in reporter tissue. Scale bar, 50 μm, inset scale 10 μm.

Interrogation of the transcriptional signatures of brain clusters revealed two distinct groupings of microglia. One grouping was defined by a more robust yolk sac signature, defined based on the signature of yolk sac-derived macrophages (Figure 2C).[58,59] We designated these clusters "yolk sac imprint microglia," MgYSI, which are further divided into two subpopulations, MgYSI_Pf4 and MgYSI_cell cycle.

Macrophages at the maternal-fetal interface are known to be a heterogeneous population consisting of both maternally and fetally derived cells. While maternally derived macrophages at the maternal-fetal interface have classically been referred to as decidual macrophages,[52,60,61] more granular characterization of heterogeneous maternally derived macrophage and monocyte populations has recently been performed,[58] with new nomenclature adopted: placenta-associated maternal monocyte/macrophages (PAMMs).[58,62] Specific subsets of PAMMs were identified in human placenta, including subtypes of maternally derived resident placental macrophages that are distinct from circulating maternal cells.[58] In this work, we sought to distinguish fetal placental macrophages or Hofbauer cells (HBCs) from PAMMs in murine placenta, understand the transcriptional programming of both cell types, and examine the similarity between murine and human HBC and PAMM signatures.

To distinguish fetal from maternal macrophages, we evaluated expression of male-specific markers DEAD-Box Helicase 3 Y-Linked (*Ddx3y*) and Eukaryotic translation initiation factor 2 subunit 3, and Y-linked (*Eif2s3y*) and female-specific marker X-inactive specific transcript (*Xist*) in brain and placenta clusters from male embryos. *Ddx3y*, *Eif2s3y*, and *Xist* were selected for their representative expression after interrogation of a broad X- and Y-chromosome-specific gene expression panel, including the antisense *Xist* transcript X (inactive)-specific transcript, opposite strand (*Tsix*), ubiquitously transcribed tetratricopeptide repeat containing Y-linked (*Uty),* and lysine demethylase 5D (*Kdm5d)* (Figures S2D and S2E). Maternally derived placental macrophage/monocyte clusters were confirmed via high expression of *Xist* and low/no expression of *Ddx3y* and *Eif2s3y* (Figures S2D and S2E), based on expression in cells isolated from placentas of a male fetus. The same approach applied to brain microglia demonstrated that all cells were fetally derived (Figures S2D and S2E), as expected. Using this approach, we identified three fetal placental macrophage clusters, HBC_Cd72, HBC_Pf4, and HBC_cellcycle (Figure 2B). These clusters have gene expression profiles similar to human Hofbauer cell clusters described in recent datasets.[51,52,58,63] We also identified one fetal placental monocyte cluster (Mono_FPl), and five PAMM (maternally derived macrophage) clusters, with expression profiles similar to those of recently identified PAMMs from human placenta.[58]

In order to identify the placental macrophage clusters with the most similar transcriptional profiles to brain macrophages, we analyzed the correlation of cluster-averaged gene expression (Spearman's correlation) between all pairs of brain and placental clusters (Figure 2D). These correlation analyses demonstrated that all microglial clusters were more similar to Hofbauer cell clusters than to maternally derived monocytes and macrophages (PAMMs) or to fetal placental monocytes (Mono_FPl). Multiple microglial clusters closely matched the transcriptional profile of HBC_Cd72, MgYSI_Pf4 highly correlated with the transcriptional profile of HBC_Pf4, and both microglial cell cycle signature clusters mapped closely to the HBC_cell cycle cluster. Of the PAMM clusters, PAMM_Spp1 was most closely related to

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microglial clusters and to Hofbauer cell clusters. Furthermore, combined clustering of both fetal brain and placental macrophages demonstrated that Hofbauer cells are the placental macrophage population with the most similar transcriptional signature to fetal brain microglia (Figure 2F). Both microglia and Hofbauer cells expressed high levels of the canonical tissue-resident macrophage markers *C1qa*, *Fcrls*, *Cx3cr1*, *Csf1r*, and *Cd163* among others (Figures 2E and S2F). In sum, these investigations demonstrated the closest relationships between Hofbauer cells and fetal brain microglia, particularly a subset of fetal brain microglia with a strong yolk sac-like signature.

We next sought to understand the conserved biological processes between Hofbauer cells and microglia via Gene Ontology (GO) biological process enrichment analyses of cluster marker genes (Figure 2G). Shared processes across populations of Hofbauer cells and microglia can be conceptually grouped into eight broad categories: immune signaling, cell movement and adhesion, actin processes (actin plays a critical role in microglial process elongation, and actin dynamics shape microglial effector functions, such as phagocytosis and response to inflammatory stimuli[64–66]), cell signaling, development (including vascular development/angiogenesis, gliogenesis, hemopoiesis), lipid transport, cellular stress response and apoptosis, and metabolism. The largest HBC cluster, HBC_Cd72, exhibits signatures of immune and inflammatory functions, processes mirrored by microglial clusters Mg_Ccl5, Mg_Hspb1, and Mg_YSI_Pf4. This cluster's involvement in regulating vascular development, angiogenesis, and gliogenesis is most similar to the functional signature of Mg_Hspb1, Mg_Sparc, and Mg_YSI_Pf4. The second largest HBC cluster, HBC_Pf4, is most closely related to Mg_YSI_Pf4 in its signature of actin and lipid-related processes and is engaged in fewer immune, lipid, and protein-related functions in comparison to HBC_Cd72. Both HBC population's cellular stress response and apoptotic functions are reflective of the functions of all microglial clusters apart from Mg_Ccl5, which is less engaged in these functions. Nearly all microglial clusters and both HBC clusters are engaged in lipid transport. The only microglial functions not closely mirrored by at least one cluster of Hofbauer cells were those related to ATP metabolism and biosynthesis. A complete list of enriched GO biological processes in cluster marker genes may be found in Table S2. Taken together, these analyses demonstrate that both fetal brain microglia and fetal placental macrophages are heterogeneous cell types, with Hofbauer cells mirroring microglia in both gene expression and inferred biological processes.

## Comparison of placental macrophage signatures within and across species

### *Within species comparison: Mouse HBCs vs. PAMM*

To employ HBCs to evaluate fetal neuroimmune development, it is important to distinguish them from PAMMs, which have distinct developmental trajectories. Marker genes that were more highly expressed in HBC compared with PAMM included *Complement 1q c chain* (*C1qc*), *Complement 1q a chain* (*C1qa*), *Platelet factor 4* (*Pf4*), *C-C motif chemokine ligand 4* (*Ccl4*), *DAB adaptor protein 2* (*Dab2*), and *Mannose receptor c type 1* (*Mrc1*), whereas *Placenta associated 8* (*Plac8*) as well as several MHC-II genes were more highly expressed in PAMMs (Figure 3A). Relative to PAMM markers, HBC marker genes were specifically enriched for GO biological processes related to chemokine signaling, ERK1/2 signaling, and regulation of neuron death.[67,68] PAMM markers were specifically enriched for MHC-II-mediated antigen presentation and processing, regulation of T cell activation, and cell-cell adhesion processes (Figure 3B). In contrast with recent observations in human first-trimester placenta,[58] *Folr2* was not as strong a marker for mouse HBCs (Figures 3C and 3D), but other key HBC (*Mrc1*) and PAMM (MHC-II gene *H2-Eb1*, *S100a9*) markers from the same study were well-represented in mouse HBC and PAMM subsets. These data suggest that while mouse and human placental macrophages have similar signatures, *Folr2* are not the strongest marker for HBC in mice, at least at the end of gestation. Furthermore, we calculated marker genes with validated, commercially available antibodies that would best distinguish HBC from PAMM (Figure 3E), including positive selection markers Mrc1 and Cd83, and negative selection markers Thbs1 and Cd74.

**Figure 2. Fetal placental and brain macrophages are heterogeneous populations with shared cluster-specific signatures**

(A) (Left) Uniform Manifold Approximation and Projection (UMAP) visualization of CD11b+ macrophage-enriched fetal brain microglia/monocyte cells reveals eight distinct clusters. Unless otherwise specified, clusters are named as "cell-type-prefix_top-marker-gene". Mg, microglia; Mono_FBr, fetal brain monocytes; YSI, yolk sac imprint. (Right) Cluster-average expression of the top three marker genes for each cluster (right), dot size indicates the percent of cells expressing the given gene, color intensity represents the scaled average gene expression.

(B) (Left) UMAP visualization of CD11b+ macrophage-enriched placenta macrophage/monocyte populations reveals four fetal and six maternal clusters. Fetal clusters were determined by significantly higher expression of Y chromosome markers *Eif2s3y* and *Ddx3y* relative to expression of X chromosome marker *Xist* (Figures S2D and S2E). Unless otherwise specified, clusters are named as "cell type prefix_top marker gene". HBC, Hofbauer cell; PAMM, placenta-associated maternal monocyte/macrophages; Mono_FPl, fetal placental monocytes. Color scheme indicates cell origin (purple = fetal macrophages; red = maternal; gray = fetal monocytes). (Right) Cluster-averaged gene expression of the top three marker genes for each cluster. Dot size indicates the percent of cells expressing the given gene.

(C) Module score for yolk sac-derived macrophages and embryonic liver monocytes.[58,59]

(D) Spearman correlation coefficients of cluster-averaged gene expression between brain and placenta clusters in (A) and (B). For each brain cluster, the placenta cluster with highest correlation is indicated with a dot.

(E) Expression levels of canonical microglia (top, blue) and Hofbauer cell marker genes (bottom, orange).

(F) UMAP visualization including both brain and placenta clusters shown in (A) and (B) shows similarity across brain-placenta compartments.

(G) Gene Ontology (GO) biological process enrichment analysis for select microglia and HBC cluster marker genes. The terms displayed were curated from among the top 25 most significant GO terms, selecting the processes most relevant to macrophage function, and reducing redundancy. Gene count gives the number of genes in the query set that are annotated by the relevant GO category. GO terms with adjusted $p$ value <0.05 were considered significant. Full results in Table S1.

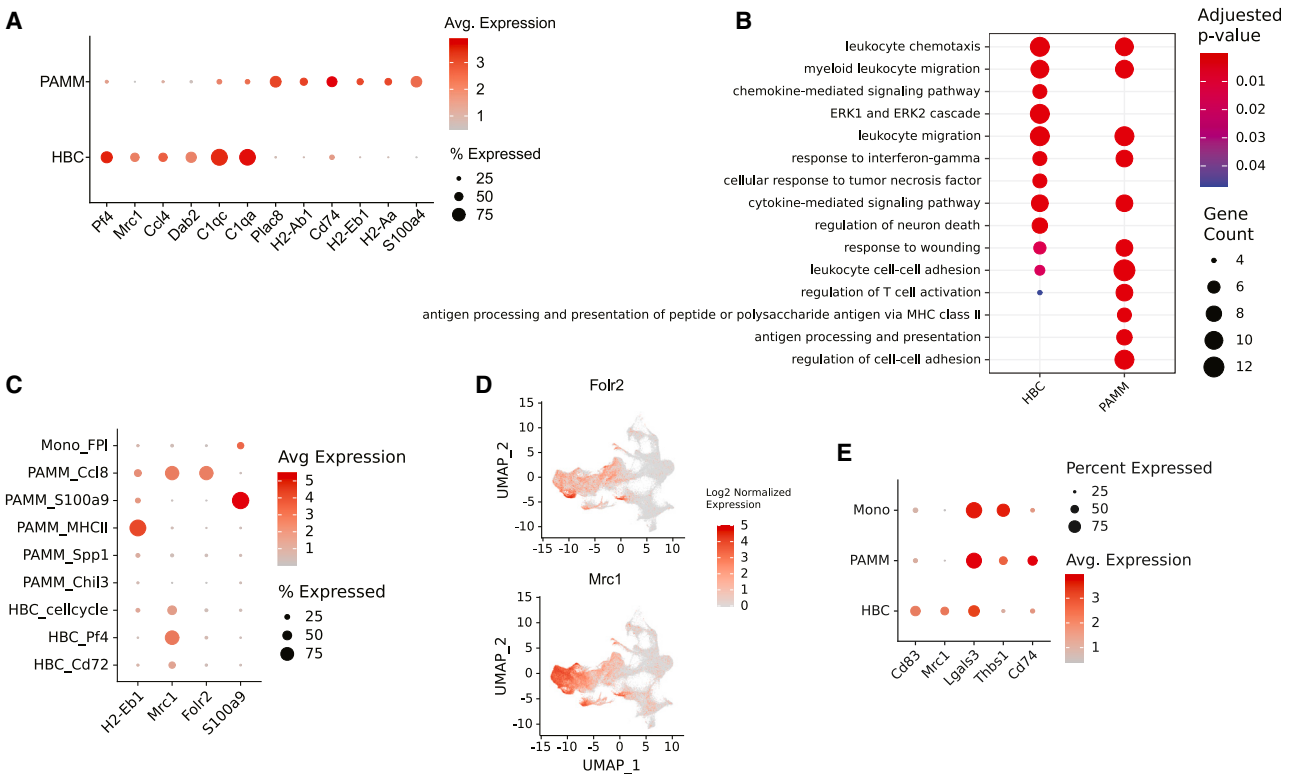



**Figure 3. Comparison of maternal and fetal placental macrophages**

(A) Cluster-average expression of top marker genes distinguishing PAMM from HBC. Dot size indicates percent of cells expressing the given gene.

(B) Gene Ontology (GO) biological process enrichment analysis for for HBC and PAMM marker genes. Gene count gives the number of genes in the query set that are annotated by the relevant GO category. GO terms with an adjusted *p* value <0.05 were considered significant.

(C) Cluster-average expression of genes that distinguish PAMM and HBC populations in human studies.

(D) Detailed expression of genes Folr2 and Mrc1 that distinguish PAMM and HBC populations.[58]

(E) Markers with validated antibodies that best separate HBC, placental monocyte, and PAMM populations.

## Across species comparison: Mouse vs. human HBCs and PAMM

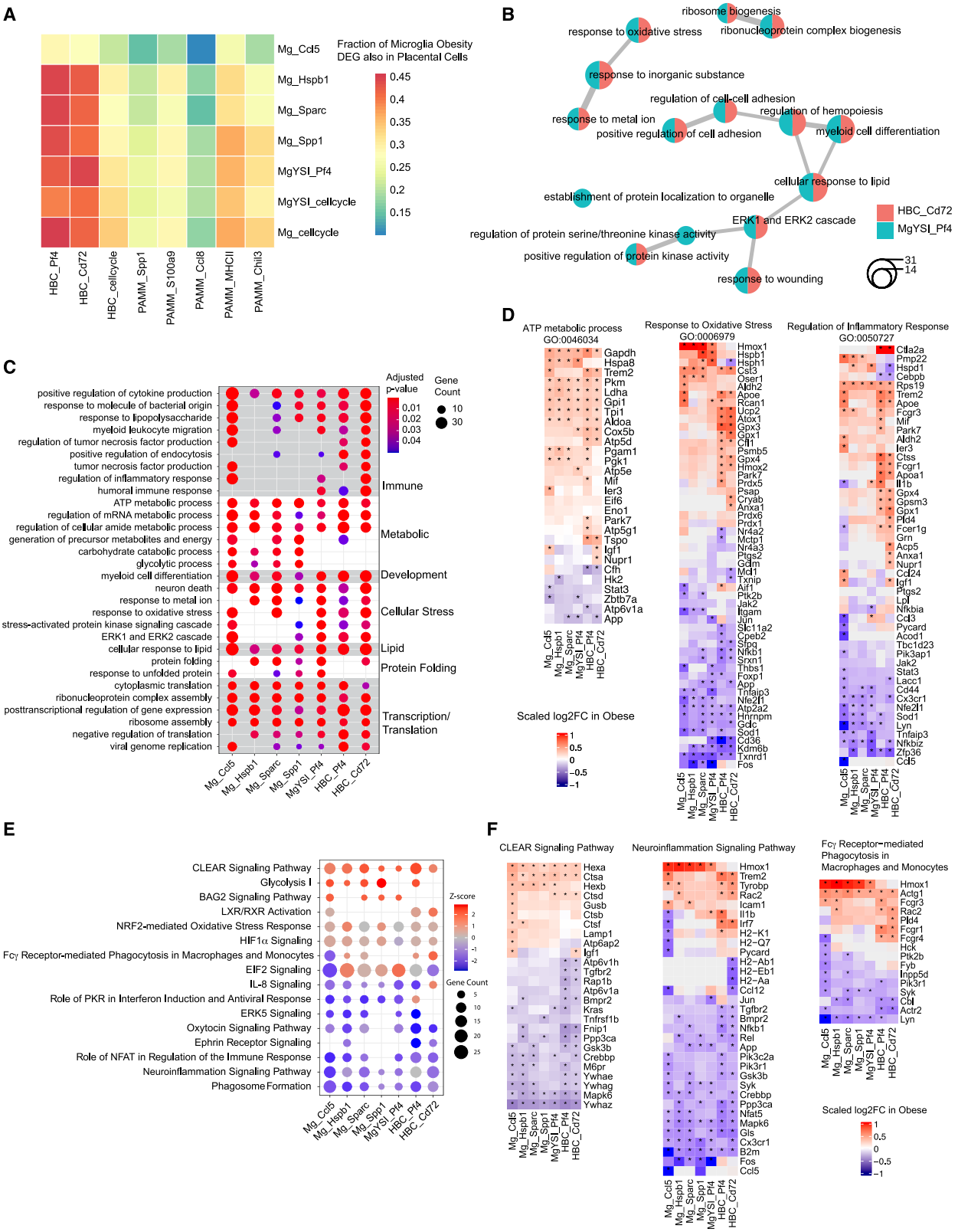
There are limited data on single-cell gene expression profiles of mouse placental macrophages, with recent debate regarding the potential ontogenies of HBCs.[17–20] In addition, mouse HBC profiles have not previously been compared with those from human placentas. We therefore next sought to understand similarities and differences between mouse and human placental macrophages. Mouse microglia are widely accepted as an excellent model for human microglia,[16,69–72] but substantially less is known about the ability of mouse placental macrophages to model their human equivalent. Mice and humans are known to share key similarities in immune system development, including the yolk sac as the initial site of hematopoiesis and origin for granulo-macrophage progenitors (e.g., the CX3CR1+ cells that give rise to microglia and Hofbauer cells)[73–77] and conserved cell surface antigen expression in yolk sac-derived macrophages.[16,78]

We compared our mouse scRNA-seq data to reference human datasets that contained high representation of HBC, representing human placentas obtained from 6 weeks through 40 weeks.[51,52,63] Comparison of our scRNA-seq data to that from human placenta datasets demonstrated strong correlation of cluster-averaged gene expression between mouse Hofbauer cells and human clusters identified as Hofbauer cells (notated as "villous Hofbauer cells, vil. HC, HB or vil.Hofb") at 6–11 weeks[51] (Figure S3A), 6–14 weeks[52] (Figure S3B), and full-term[53] (Figure S3C), with Spearman correlations 0.71–0.76. Taken together, these data demonstrate that placental resident macrophage and monocyte single-cell gene expression signatures are highly conserved across mice and humans.

## Fetal placental macrophages provide insights into fetal brain microglial programs in the setting of maternal diet-induced obesity

After establishing that Hofbauer cellular programs reflect those of microglia without perturbation, we next sought to examine whether Hofbauer cells could reflect microglial changes in a mouse model of high-fat diet-induced obesity. *C57BL/6J* females were placed on an obesogenic diet (Research Diets D12492, 60% kcal from fat) for 10 weeks to induce maternal obesity (Figure S4A) as described in prior publications by our group,[27,40,79] and we performed scRNA-seq as described above.

To determine the gene expression changes in fetal brain and placental macrophage populations in response to maternal

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obesity, we performed differential expression analysis within each cell cluster, including fetal brain microglia and placental macrophages from obese compared with control dams, considering male and female fetuses together (Figure 4). A total of 503 unique genes were dysregulated in fetal brain microglia by maternal obesity, and 660 genes in placental macrophages (including 444 in fetal placental macrophages and monocytes and 438 in maternal placental macrophage/monocyte clusters). A complete list of maternal obesity-associated differentially expressed genes (DEGs) by cluster within fetal microglia and placental macrophages is provided in Table S2. To characterize the similarity of the response to obesity in microglia and placental macrophages, we compared the DEGs in each pair of brain and placental clusters (Figure 4A). We found that genes differentially expressed in the setting of maternal obesity were more often shared between microglial clusters and Hofbauer cells than between microglia and PAMMs; there was an ~45% overlap between obesity-associated DEGs in all microglial clusters and the DEGs in HBC_Pf4 and HBC_Cd72, with the exception of microglia cluster Mg_Ccl5, which had only 33% overlap with the HBC DEGs. While the Mg_Ccl5 cluster shares with HBC clusters a functional signature related to immunity, cell movement and adhesion, and lipid regulation (Figure 2G), this cluster appears to differ from the other microglial clusters in its lack of marker genes related to actin cytoskeletal functions, cell-cell signaling, angiogenesis/gliogenesis, and functions related to apoptosis and cellular metabolism. Thus, the Mg_Ccl5 cluster may be engaged in more highly specialized functions than other Mg clusters and HBCs may not reflect this cluster as broadly as they do other subpopulations of Mg.

To explore the functional consequences of the gene expression changes induced by maternal obesity, we performed GO enrichment analyses of DEGs. Similar to the genes themselves, the GO categories enriched in the DEGs of microglial and placental clusters also showed substantial overlap and can be conceptually grouped into seven categories: immune signaling, metabolism, development, cellular stress, lipid response, protein folding, and transcription/translation (Figure 4C). These biological processes are enriched to varying extents in the DEGs of the other myeloid cell populations obtained in our full dataset (Figures S4B and S4C), with PAMM being more strongly impacted than fetal monocytes and granulocytes. In particular, 13 of the top 15 GO biological processes enriched in the DEGs of the clusters with the most similar DEGs, fetal MgYSI_Pf4 and placental HBC_Cd72, were common to both clusters (Figure 4B). The majority of the top GO terms were enriched in both clusters, and included processes relevant to microglial function, including cellular response to lipid, response to oxidative stress, and positive regulation of cell adhesion. Consistent with a recent study demonstrating that maternal high-fat diet induces inflammatory responses in the placenta and fetal brain,[26] we noted that immune-related processes were altered throughout Mg and HBC clusters in the setting of maternal obesity. Maternal obesity was associated with a dramatic alteration in metabolic processes in Mg and HBC clusters. The directionality of changes of the genes represented in these processes suggest a shift toward increased energy utilization or glycolysis in obesity-exposed macrophages (e.g., increased *Gapdh*), consistent with previous studies suggesting increased glucose metabolism in placentae from women with obesity and type 2 diabetes.[80,81] Overall, the direction of gene expression changes was consistent across Mg and HBC clusters for three key GO categories: ATP metabolic process, response to oxidative stress, and regulation of inflammatory response (Figure 4D), with a combination of both up- and downregulated genes.

To better understand the direction of effect of functional changes in macrophage populations in response to maternal obesity, we performed canonical pathway enrichment analysis using ingenuity pathways analysis (IPA, Qiagen) (Figure 4E, Table S3). These analyses confirmed a relative activation in glycolysis in both Mg and HBC clusters in the setting of maternal obesity and identified a relative activation of the coordinated lysosomal expression and regulation (CLEAR) signaling pathway in Mg and HBC clusters in the setting of maternal obesity. Activation of the CLEAR signaling pathway implies increased lysosomal activity in the setting of increased autophagy secondary to endoplasmic reticulum stress.[82–84] Evaluation of classic macrophage functions such as phagocytosis and inflammatory signaling revealed a complex picture, with both immune activation, but also a macrophage exhaustion phenotype. Specifically, the IPA analysis implicated activation of Fc$\gamma$-receptor-mediated phagocytosis in obesity-exposed microglia and HBC clusters, but suppression of the neuroinflammation signaling pathway (Figures 4E and 4F). This mixed picture of both macrophage activation and exhaustion in the setting of maternal immune activation is consistent with and extends published results, which

**Figure 4. Maternal obesity alters gene expression in fetal microglia and placental macrophages**

(A) Fraction of significant differentially expressed genes (DEGs) in obesity-exposed fetal microglia that are also significantly differentially expressed in obesity-exposed placental Hofbauer cells. DEGs between offspring of obese and control dams are shown. DEGs considered significant if adjusted $p$ value <0.05, abs(log2FC) > 0.25.

(B) Network plot of the top 15 GO biological processes enriched in the DEGs in fetal MgYSI_Pf4, shown in both MgYSI_Pf4 and placental HBC_Cd72. Nodes correspond to enriched GO categories, node size is proportional to number of genes, and edge thickness is proportional to the number of overlapping genes.

(C) Enriched GO biological processes in the DEGs of obesity-exposed microglia and Hofbauer cells. Select results are shown, full results are in Table S2. Shading corresponds to manual grouping of GO categories. Gene count gives the number of genes in the query set annotated by the relevant GO category.

(D) Up- and downregulation of DEGs implicated in ATP metabolism, response to oxidative stress, and regulation of inflammatory response in microglia and Hofbauer cells. DEG fold changes in obesity-exposed microglia and HBCs for three significantly enriched GO biological processes relevant to microglial function. *Indicates DEG with adjusted $p$ value <0.05.

(E) Select IPA canonical pathways enriched in obesity-exposed microglial and Hofbauer cell DEGs. Positive $Z$ score means pathway is activated in fetal macrophages of obese dams, negative $Z$ score means pathway is suppressed.

(F) Up- and downregulation of genes in fetal macrophages of obese dams for two significantly activated or inhibited IPA canonical pathways relevant to microglial function. DEG fold changes depicted. *DEGs with adjusted $p$ value <0.05.

describes not only fetal macrophage priming, activation, and increased phagocytosis[26,27] in response to maternal obesity and other maternal immune-activating exposures, but also macrophage and monocyte exhaustion phenotypes.[85,86]

We also observed GO processes that were not shared by the DEGs of microglia and HBC (Figure S4E), potentially reflecting how different tissue microenvironments alter cell responses to maternal obesity. Regulation of microtubule polymerization and neuron regulation were uniquely impacted in microglia, whereas mitochondrial metabolic processes and regulation of body fluid levels, a known function of macrophages in maintaining tissue homeostasis,[87] were more strongly altered in HBC. Differences in interaction with the cell microenvironment were further investigated using computational prediction of cell-cell interactions between brain and placental macrophages and other cell types present in our full dataset (Figure S5A), including characterization of how cell-cell interactions might be altered in the setting of maternal obesity (Figures S5B and S5C). As our experimental method enriched for macrophages, which may skew the frequency distribution of other cell types, these inferences must be interpreted with caution. We observed that maximal interaction strengths between both fetal brain and placental macrophages and their respective signaling partners are observed when the resident tissue macrophages are the *target* of the interaction—that is, when they express the receptor—rather than when they are the *source*—that is, when they express the ligand (see scale bar thickness in Figure S5A i vs. ii). This is consistent with these cells' known role in surveilling their microenvironment in order to respond to potential inflammation, injury, or other environmental changes. The major cell types that signal to Mg and MgYSI were, in decreasing order, fibroblast, endothelial cell, and monocyte. The interaction of these cells with microglia is stronger with yolk-sac imprint microglia (MgYSI) than other microglia (Mg). The major cell types that signaled to HBC and PAMM in our data were, in decreasing order, endothelial cell, fibroblast, and granulocyte. After exposure to maternal obesity, interactions where MgYSIs were the target were decreased and interactions where Mg were the target were increased (Figure S5B). In the placenta, interactions with both HBC and PAMM as the targets were decreased in maternal obesity (Figure S5B). Figure S5C demonstrates intercellular communication pathways predicted to have up- and downregulated signaling in obesity.

Taken together, these findings suggest shared gene expression and biological pathway changes in HBCs and microglia in the setting of maternal obesity and high-fat diet, despite occupying different spatial niches during development. Biological pathways that were uniquely altered by obesity in microglia vs. placental macrophages provide insight into how different tissue microenvironments may impact cell programs.

## Sex differences in fetal brain and placental macrophage responses to maternal obesity

We next investigated potential sex differences in the response of fetal placental and brain macrophages to maternal obesity, evaluating DEGs in obesity-exposed compared with control fetal brain and placental macrophages in a sex-stratified analysis (STAR Methods; Figure 5). Although cells were isolated on e17.5, prior to the hormonal surge commonly associated with brain masculinization,[39] we observed sex differences in gene expression changes induced by maternal obesity, suggesting that the local microenvironment or other non-hormonal factors are likely driving differences between male and female fetal brain and placental macrophages in late gestation. The number of DEGs caused by maternal obesity was significantly higher in all male HBC and PAMM placental clusters compared with female, as well in two microglial clusters, Mg_Spp1 and Mg_Hspb1 (Figure 5A). To facilitate the analysis of sex differences induced by maternal obesity, we combined clusters into four cell types: Mg, MgYSI, HBC, and PAMM. Overall, most DEGs changed in a similar direction (either upregulated or downregulated by obesity) in both males and females, especially in the two most similar populations MgYSI and HBC (Figure 5B); we called these genes "sex-consistent" ($N = 425$ DEGs in HBCs and 368 DEGs in MgYSI). Genes whose expression changed in opposite directions (e.g., upregulated in males but downregulated in females) in the setting of maternal obesity were designated "sex-dimorphic" ($N = 58$ DEG in HBCs and 92 DEG in MgYSI). GO enrichment analysis was used to determine biological processes that were altered in the maternal obesity-associated DEG in a sex-consistent manner in microglia and HBCs, and pathways that were altered in DEG in a sexually dimorphic manner (Figure 5B). The GO categories enriched in the sex-consistent genes were similar in microglia and HBC, including categories involving immune response, cell death and protein folding. GO enrichment analysis of the sex-dimorphic genes dysregulated by obesity in both HBC and microglia implicated pathways involved in the regulation of myeloid and leukocyte differentiation and hematopoiesis. Sex-dimorphic genes dysregulated in HBCs alone were concentrated in protein-folding categories. Sex-dimorphic genes dysregulated in microglia alone were implicated in neuronal cell death, response to interferon-alpha, negative regulation of cell-cell adhesion, and actin cytoskeleton-related genes.

To determine directionality of pathway activation or suppression, IPA analyses of DEGs in males and females were conducted. The activation or suppression of canonical pathways in male and female HBCs and MgYSIs were largely similar between sexes (Figure 5C). In both sexes, maternal obesity was associated with upregulation of pro-apoptotic and pro-autophagy signaling (EIF2 and CLEAR signaling) and downregulation of anti-apoptotic oxytocin and ERK 5 signaling in both HBCs and MgYSI. In contrast, maternal obesity had a sexually dimorphic effect on sirtuin signaling, with activation in female HBCs and MgYSI and suppression in male HBCs and MgYSI. Increased sirtuin signaling is linked to metabolic control, DNA repair, neuroprotection, and anti-aging effects,[88,89] suggesting a more favorable metabolic response in female HBCs and microglia in the setting of maternal obesity. The upregulation of the glycolysis I canonical pathway only in male HBCs and MgYSIs also suggests less efficient energy utilization by exposed male macrophages compared with female macrophages. In the neuroinflammatory response canonical pathway, males had a larger magnitude of gene expression fold changes compared with females, suggesting that male neuroinflammatory response may be more strongly dysregulated in the setting of obesity (Figure 5D). Fetal sex did not influence cluster identification or the proportion of cells

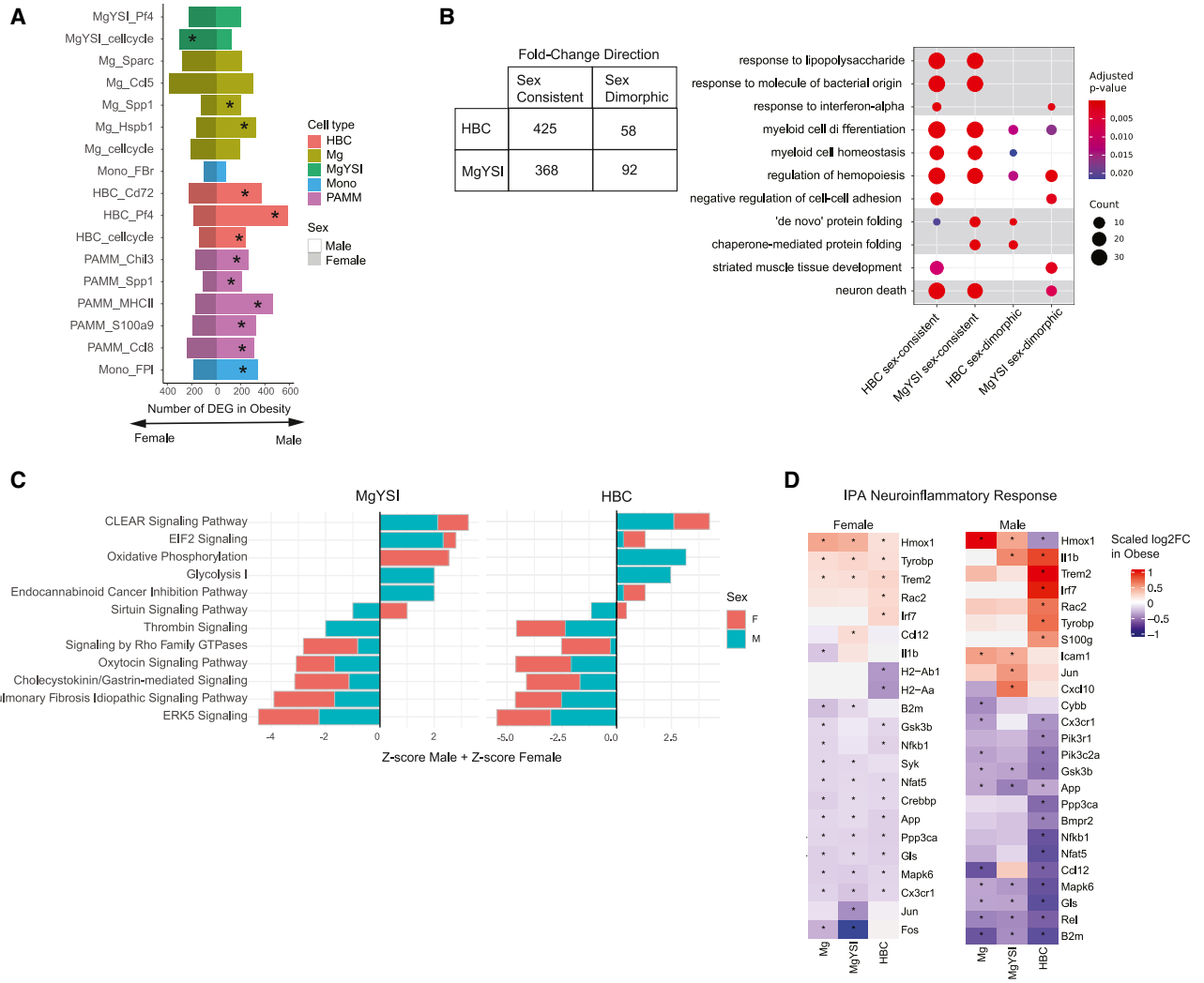


**Figure 5. Sex differences in the response of microglia and placental macrophages to maternal diet-induced obesity**

Differentially expressed genes (DEGs) between microglia and placental macrophages in fetuses of obese and control dams are shown, calculated separately for male and female offspring.

(A) The number of DEGs between obesity-exposed vs. control macrophages/monocytes is shown as a bar, colored by cell type, and shaded according to sex. *Indicates $p < 0.05$ difference between male and female.

(B) (Left) Table considers the total number of DEGs in yolk-sac imprint microglia (MgYSI) and HBC cell types that are DEGs in male and/or female cells. Genes are categorized as having a fold change in the same direction (sex-consistent) or different direction (sex-dimorphic) in males and females. (Right) GO biological process enrichment results for the set of sex-dimorphic and sex-consistent DEGs.

(C) Comparison of the top 12 IPA canonical pathways that are enriched in both Mg and HBC. The summed *Z* score from the female analysis and male analysis is shown, with positive *Z* score indicating activation and negative *Z* score indicating inhibition. (Full results are shown in Table S3.)

(D) Fold changes in fetal macrophages in offspring of obese versus control dams for the IPA canonical pathway neuroinflammatory response pathway for males and females. *DEGs with adjusted *p* value <0.05.

within clusters (Figure S4D) with the exception of Mg_Ccl5 and Mono_FBr in male brain, clusters that were slightly more represented in brains of fetuses from control dams (false discovery rate <0.05).

Taken together, these analyses demonstrated increased numbers of genes dysregulated by maternal obesity in male placental macrophages and two microglial clusters, and stronger dysregulation of neuroinflammatory pathway genes in obesity-exposed male microglia and fetal placental macrophages. These findings extend prior work demonstrating a greater impact of maternal obesity on male vs. female fetal brain gene dysregulation[40] and a greater impact of maternal obesity on pro-inflammatory priming of male fetal brain microglia and placental macrophages.[27] The increased dysregulation of the placental macrophage signature in exposed males relative to the brain macrophage signature is consistent with the known barrier function of the placenta, protecting the downstream fetal organs from maternal exposures.[90,91]

## DISCUSSION

Microglia, brain resident macrophages, are vulnerable to diverse maternal immune-activating exposures including bacterial and viral infection, metabolic inflammation such as that mediated by obesity and diabetes, environmental toxicants, and maternal stress, potentially contributing to adverse neurodevelopmental and psychiatric outcomes in offspring.[10,31,32,92–97] However, the mechanisms that dictate susceptibility to these outcomes are unclear, and there is a critical need to identify offspring at greatest risk. To identify more accessible readouts of aberrant microglial priming conferring risk for neurodevelopmental and psychiatric disorders,[10–13] we sought to evaluate whether extra-embryonic placental macrophages have similar transcriptional signatures as fetal brain microglia at baseline, and if placental macrophages can provide information about the impact of a maternal perturbation, diet-induced obesity, on fetal brain immune programs. Here, we demonstrate (1) a common yolk sac origin for microglia and Hofbauer cells, (2) similar transcriptional programs between subsets of microglia and Hofbauer cells both at baseline and (3) in response to maternal diet-induced obesity, providing evidence that term placental Hofbauer cells have the potential to uniquely inform on the programs of fetal microglia. We show (4) significant correlations between murine and human placental macrophage populations, suggesting the potential for Hofbauer cells to provide a readout of neurodevelopmental exposures in humans, as well. Finally, we demonstrate (5) sex differences in the impact of maternal obesity on fetal brain and placental macrophages, including more genes dysregulated in male placental macrophages and two male microglial clusters, and stronger dysregulation of neuroinflammatory genes in the male brain. The shared ontogeny and transcriptional programs between fetal placental macrophages and fetal brain microglia indicate that term Hofbauer cells, readily accessible at birth, may provide insight into microglial state and function after specific *in utero* exposures.

Consistent with prior studies, we observed significant microglial heterogeneity in fetal brains.[56,57,98] Not surprisingly, microglial clusters with the more robust yolk sac signature also highly express genes known to be associated with another yolk sac-derived tissue-resident brain macrophage population, CNS-associated macrophages (CAMs).[99–101] Our results also extend prior work in understanding fetal microglial responses to maternal obesity. We observed a mixture of activation and suppression of immune-related processes in fetal microglia and placental macrophages in response to maternal obesity. We previously reported that obesity primed Mg and HBCs toward a pro-inflammatory phenotype when treated with lipopolysaccharide (LPS).[27] More recent studies show that macrophage and monocyte exhaustion plays a role in obesity-associated cellular programming.[86]

Prior work has demonstrated sex-specific fetal brain gene expression signatures using whole fetal forebrain in a murine model of maternal diet-induced obesity, without subselection for any specific cell or immune cell subtype.[40] Given a strong sex bias in many microglial-mediated neurodevelopmental disorders, with autism spectrum disorder, attention-deficit hyperactivity disorder, and cognitive delay/learning disabilities all more common in males than females,[31,39,41,102,103] and understanding that male fetal macrophages (both brain and placental) may be more vulnerable to pro-inflammatory intrauterine priming compared with female macrophages,[27,104] we sought to investigate potential sex differences in fetal microglia and placental macrophage responses to maternal obesity. Our finding that significantly more genes were differentially regulated by obesity in male compared with female placental macrophages is consistent with prior work demonstrating a disproportionate impact of maternal obesity on male vs. female placental immune dysregulation.[26,27,105] The male fetus may be more vulnerable to maternal exposures, mediated in part through increased male placental reactivity.[38,106–108]

Beyond specific similarities between Hofbauer cells and microglia, we identified two elements that should facilitate the application of Hofbauer cells as proxies for microglia in future murine and human studies. First, despite their recognized importance in immune signaling at the maternal-fetal interface,[109] Hofbauer cells have been under-characterized due to challenges in cell isolation and maintenance of viability.[110] By enriching for macrophages and monocytes with a Percoll gradient followed by subselection for CD11b+ cells, our experiments were able to provide uniquely targeted single-cell sequencing and greater resolution on placental macrophage populations than has been previously achieved. This increased resolution yielded new transcriptional and functional insights about the under-characterized heterogeneity of Hofbauer cells and PAMMs. Consistent with other groups,[52,58,111] we found that maternal cells contribute significantly to the placental macrophage population.

The identification of markers more specific to Hofbauer cells should also enable their investigation as biomarkers by more readily distinguishing them from PAMMs. Our cell isolation protocol was selected due to its ability to permit isolation of fetal brain and placenta macrophages in parallel, in a timely fashion to optimize cell viability for sequencing. In our study with multiple male and female embryos, we could use Y chromosome-specific markers in male samples to resolve maternal from fetal macrophages in the placenta and extrapolate those insights to placental clustering in both sexes. While Folr2 demonstrated promise as such a marker for Hofbauer cells in first-trimester human placenta,[58] in late-gestation murine Hofbauer cells, we show that Mrc1 is a better candidate HBC marker, and specific MHC-II cell surface markers (H2-Eb1, H2-Aa, and H2-Ab1) may be used to distinguish PAMMs. Future studies can extend this work by validating the suggested markers, with the goal being to distinguish HBCs from PAMMs using fluorescence-activated cell sorting.

As a further step toward application of these cells to study human disease processes, we interrogated the transcriptional relationships between our Hofbauer cell and PAMM clusters and those of three independently published human datasets.[51,52,63] In all comparisons, the signatures of murine Hofbauer cells and PAMMs from our dataset were closely correlated with human Hofbauer cell and PAMM signatures, suggesting a highly conserved evolutionary relationship. Key similarities between mouse and human immune system development relevant to these experiments include the yolk sac as the initial site of hematopoiesis and origin for both microglia and Hofbauer cells.[73–77]

Although the mouse placenta does have some fundamental differences from human placenta, such as the degree of contact between fetal tissues and maternal blood, or variability in tissue structure between human placental villi and mouse fetal placental labyrinth, both species share important functions in nutrient transport, blood filtration, and immunocompetency.[112–114]

In summary, these data provide a precedent for using placental Hofbauer cells as a noninvasive biomarker of fetal brain microglial programming. These results extend previous work from our groups and others demonstrating that Hofbauer cells and fetal brain microglia respond similarly to bacterial infection,[104] and show similar exaggerated responses to the bacterial endotoxin LPS after priming by maternal obesity.[27] They lay the groundwork for longer-term translational studies in humans correlating Hofbauer cell inflammatory profiles with offspring neurological outcomes. Such studies will determine whether Hofbauer cells can serve as a clinical indicator of neurodevelopmental vulnerability, with the ultimate goal of identifying vulnerable offspring at birth and facilitating interventions during key developmental windows of plasticity. Defining molecular signatures of HBCs and microglia associated with maternal obesity is a necessary first step toward identifying candidate therapeutic approaches to ameliorate or rescue deleterious microglial programming.[115–119]

### Limitations of the study

One limitation of this work is that the relatively small number of replicates by fetal sex within the obese and lean subgroups may limit power to detect more modest sex differences than those reported here. While our single-cell data provide unique resolution on multiple clusters of maternal and fetal placental macrophages, translating these findings for clinical application will require identifying a more parsimonious group of surface antigens conserved across mouse and human placenta. Additionally, the potential gene expression panels identified here require further validation by characterizing their protein products and confirming their utility in human tissues. Ultimately, direct tests of the hypotheses we generated regarding dysregulated pathways will require parallel functional analysis of HBCs and microglia. We anticipate that the analyses described here will facilitate such functional experiments by allowing isolation of more precise subsets of Hofbauer cells and microglia, and providing guidance as to what functions should be interrogated.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2024.114326.

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## AUTHOR CONTRIBUTIONS

R.B. and A.M.C. contributed equally, and as co-first authors. A.G.E. conceived the study and, together with S.D.B., A.M.C., and R.B., designed the experiments. Acquisition of data: A.M.C., R.B., S.K., E.A.B., L.L.S., and A.G.E. Analysis and interpretation of data: R.B., A.M.C., B.A.D., D.K.S., S.D.B., and A.G.E. Drafting of the manuscript: R.B., A.G.E., and A.M.C. Revising the manuscript critically for important intellectual content: A.M.C., R.B., L.L.S., S.K., E.A.B., B.A.D., R.H.P., D.K.S., S.D.B., and A.G.E. All authors have given final approval for submission.

## DECLARATION OF INTERESTS

A.G.E. serves as a consultant for Mirvie, Inc. outside of this work. A.G.E. receives research funding from Merck Pharmaceuticals outside of this work. R.H.P. is a founder and member of the scientific advisory board of Psy Therapeutics; a member of scientific advisory boards for Swan AI Studio, Belle Artificial Intelligence, Genomind, and Circular Genomics; and consults to Alkermes, Burrage Capital, and Vault Health. He serves as an associate editor for JAMA Network Open. All of these roles are outside the present work.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Antibodies** | | |
| Chicken anti-Iba1 | Synaptic Systems | Cat# 234 006, RRID:AB_2619949 |
| Rabbit anti-RFP | Rockland | Cat# 600-401-379, RRID:AB_2209751 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 4-hydroxytamoxifen | Millipore-Sigma | Cat #H6278 |
| Percoll | Millipore-Sigma | Cat# GE17-0891-01 |
| Collagenase A | Millipore-Sigma | Cat# 11088793001 |
| **Critical commercial assays** | | |
| CD11b (Microglia) Microbeads, human and mouse | Miltenyi Biotec | 130-093-634 |
| MACS Separation Columns (LS) | Miltenyi Biotec | 130-042-401 |
| QuadroMACS Separator | Miltenyi Biotec | 130-090-976 |
| MACS MultiStand | Miltenyi Biotec | 130-042-303 |
| **Experimental models: Organisms/strains** | | |
| Mouse: FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J | The Jackson Laboratory | JAX:019098, RRID:IMSR_JAX:019098 |
| Mouse: B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J | The Jackson Laboratory | JAX:007914, RRID:IMSR_JAX:007914 |
| Mouse: C57BL/6J | The Jackson Laboratory | JAX:000664, RRID:IMSR_JAX:000664 |
| **Software and algorithms** | | |
| Fiji | Schindelin et al.[120] | https://fiji.sc/ |
| Cellranger 3.0 | 10x Genomics | https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger |
| R 4.0.0 | R Foundation | https://www.r-project.org/ |
| Seurat 4.0.3 | Hao et al.[121] Satija et al.[122] | https://satijalab.org/seurat/ |
| DoubletFinder 2.0.3 | McGinnis et al.[123] | https://github.com/chris-mcginnis-ucsf/DoubletFinder |
| SingleR 1.2.4 | Aran et al.[124] | https://bioconductor.org/packages/release/bioc/html/SingleR.html |
| celldex 0.99.1 | Aran et al.[124] | http://bioconductor.org/packages/release/data/experiment/html/celldex.html |
| clusterProfiler 4.0.5 | Wu et al.[125] | https://guangchuangyu.github.io/software/clusterProfiler |
| Tidyverse 1.3.1 | Wickham et al.[126] | https://tidyverse.tidyverse.org/ |
| Ingenuity Pathway Analysis 2021b | QIAGEN, Krämer et al.[127] | https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/ |
| Speckle 0.0.3 | Phipson et al.[128] | https://github.com/phipsonlab/speckle |
| sc2marker 1.0.3 | Li et al.[129] | https://github.com/CostaLab/sc2marker |
| CellChat 1.6.1 | Jin et al.[130] | http://www.cellchat.org/ |
| **Deposited data** | | |
| Raw and analyzed data | This paper | Single-cell RNAseq data (Raw Fastq and processed gene count matrices) are available as GEO series GSE252343 |
| All code necessary to reproduce these analyses | This paper | https://github.com/rbatorsky/fetal-mac-edlow |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources/reagents should be directed to and will be fulfilled by the lead contact, Andrea Edlow (aedlow@mgh.harvard.edu).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- Single-cell RNA-seq data are available as GEO series GSE252343.
- All code necessary to reproduce these analyses is available at https://github.com/rbatorsky/fetal-mac-edlow.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Strains and husbandry conditions

All procedures relating to animal care and treatment conformed to Massachusetts General Hospital Center for Comparative Medicine Program, Duke University Animal Care and Use Program, and NIH guidelines. Animals were group housed in a standard 12:12 light-dark cycle. The following mouse lines were used in this study: *FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J* (Jackson Laboratory, stock no. 019098, referred to as *Csf1r-Cre$^{ER}$* hereafter), *B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J* (Jackson Laboratory, stock no. 007914, referred to as *tdTomato$^{f/f}$* hereafter), and *C57BL/6J* (Jackson Laboratory, stock no. 000664). *Csf1r-Cre$^{ER}$* animals were backcrossed to *C57BL/6J* mice for one generation prior to breeding with *TdTomato$^{f/f}$* animals. *Csf1r-Cre$^{ER}$* was maintained in the males for all experimental studies, and genotyping was performed per Jackson Laboratory published protocols for each strain. For diet-induced obesity versus lean control experiments, *C57BL/6J* females were placed on either an obesogenic diet (Research Diets D12492, 60% kcal from fat) for 10 weeks to induce maternal obesity, or a control diet (D12450J, 10% fat) matched for protein, fiber and sucrose content for the same duration of time, prior to breeding with *C57BL/6J* males on the control diet, as described in prior publications by our group.[27,40,79] Pre-breeding dam weight curves and gestational weight curves are depicted in Figure S4A. Pregnant *C57BL/6J* dams were euthanized at gd17.5 and embryonic brains and matched placentas were retrieved as described below (Tissue collection for sequencing).

### Transgenic breeding/maintenance

Male *Csf1r-Cre$^{ER}$;TdTomato$^{f/f}$* mice were crossed with *TdTomato$^{f/f}$* or *Tdtomato$^{f/+}$* females to generate control *TdTomato$^{f/f}$* or *Tdtomato$^{f/+}$* and experimental *Csf1r-Cre$^{ER}$;TdTomato$^{f/f}$* or *Csf1r-Cre$^{ER}$;TdTomato$^{f/+}$* animals within the same litters. We did not see any spontaneous recombination (e.g., tdTomato fluorescence or RFP immunoreactivity) in control animals. Pregnancy was determined by the presence of a copulation plug (gestational day 0.5 (gd0.5)), and maternal weight was measured at gd0.5 and gd8.5 (to confirm pregnancy weight gain). Pregnant females were injected intraperitoneally (i.p.) with 10 mg/kg 4-hydroxytamoxifen (4-OHT, Millipore-Sigma cat #H6278) dissolved in corn oil (Sigma) at gd8.5 and euthanized at gd17.5 with $CO_2$ followed by rapid decapitation. Sex was recorded and is clearly noted throughout the manuscript.

## METHOD DETAILS

### Tissue collection for immunohistochemistry

Uterine horns containing embryos were rapidly dissected and placed on ice in sterile 1X PBS. Individual embryos were separated, and placenta, brain, and tail tissue (for genotyping) were collected. Placenta and brain tissue were fixed in 4% paraformaldehyde in PBS (PFA, Sigma) overnight at 4°C, cryoprotected in 30% sucrose +0.1% sodium azide in PBS (Sigma), and embedded in OCT (Sakura Finetek, Torrance, CA) before being cryo-sectioned. Sections were frozen at −80°C for storage. 40μm cryosections were collected directly onto Superfrost slides (Fisher), permeabilized in 1% Triton X-100 in PBS, and blocked for 1 h at room temperature using 5% goat serum (GS) in PBS +0.1% Tween 20. Sections were then incubated for 2 nights at 4°C with chicken anti-Iba1 (Synaptic Systems, 234 006) and rabbit anti-RFP (Rockland, 600-401-379). Following PBS washes, sections were then incubated with anti-rabbit Alexa 594 (placenta and brain), anti-chicken Alexa 647 (placenta) or anti-chicken Alexa 488 (brain) (1:200; ThermoFisher), and DAPI (100μg/mL). Ten z-stacks of 0.5μm optical thickness were taken using a Zeiss AxioImager.M2 (with ApoTome.2) from at least 5 sections (each 400μm apart) from the fetal compartment of each placenta. Ten z-stacks of 0.5μm optical thickness were also taken from at least 3 hippocampal sections (each section being 200μm apart).

### Tissue collection for sequencing

Uterine horns containing embryos were rapidly dissected and embryos were placed on ice in sterile PBS. Brains and placentas were isolated, and fetal forebrain and placenta were diced finely with sterile blade and placed into collagenase A (Millipore-Sigma; 11088793001) digestion solution on ice. Cd11b-positive cells were isolated from both placenta and brain tissue as previously

described.[27,131] Briefly, samples were serially dissociated into a single cell suspension using hand-flamed Pasteur pipettes, and the resultant suspension was enriched for macrophages and monocytes using a 70%/30% Percoll gradient (Millipore-Sigma; GE17-0891-01). This enriched macrophage/monocyte solution was incubated with human and mouse CD11b microbeads (Miltenyi Biotec; 130-093-634), and cells were further enriched for Cd11b-positive macrophages/monocytes using Miltenyi MACS Separation Columns (LS; 130-042-401) and a Miltenyi QuadroMACS Separator (130-090-976). Fresh CD11b+ cells from 8 to 9 biological replicates per diet group (4 male fetal brains and matched placentas per diet group, 5 female fetal brains and matched placentas per diet group) were then prepared for single-cell RNA sequencing (10X Genomics v3.0 chip). Paired fetal brain and placental samples were collected for 18 mice, giving 36 total samples. Approximately 5,900 cells/sample were sequenced, giving 197,000 sequenced cells with an average depth of approximately 22,000 reads/cell).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Immunohistochemistry quantification

Maximum intensity projections were generated with FIJI (FIJI Is Just ImageJ[120]), and the Cell Counter plugin was used to aid in manual counting of Iba1+ and tdTomato/RFP+ cells. Counts were done by an individual blinded to the age, sex, and genotype of the tissue. Sample sizes can be found in the legend for Figure 1.

### Single cell RNA-sequencing analysis

10x Data analysis and clustering: 10x scRNA-seq data was aligned with 10x Genomics Cell Ranger (v3.0)[132] against the mm10 mouse reference genome. Downstream analysis was performed with R (v4.0.0) package Seurat (v4.3.0).[121,122,133,134] Initial filtering was performed on each sample as follows: cells with UMI count <500, gene count <250, or fraction of reads aligning to mitochondrial genes >0.2 were removed; genes detected in <10 cells were also removed. All samples were normalized and putative doublet cells were removed using predictions from DoubletFinder (v2.0.3).[123] All samples were integrated to remove batch effects from individual animals using the Seurat Single Cell Transform workflow[135] using the top 3000 variable features, followed by clustering using the Louvain algorithm on the shared nearest neighbor graph, as implemented in the Seurat FindClusters function with resolution 0.4, and visualization by UMAP using the first 50 dimensions. Cluster marker genes were identified using Seurat function FindAllMarkers. Markers were considered significant with adjusted $p$-value <0.05 and average log fold-change between two groups >0.25.

### Identification of cell types

Annotation of clusters with cell types was done in three steps: 1) First, clusters were annotated using R package SingleR (v1.2.4) with celldex (v.0.99.1)[124] packages built-in MouseRNAseq reference; 2) annotation was confirmed using the top Spearman correlation coefficient between cluster-averaged gene expression in our clusters and cluster-averaged gene expression cell types from published datasets for mouse microglia[55–57] and human placenta[51,52,63] using the cluster correlation method recently described[53] (results shown in Figure SI4); 3) cell-type assignment was refined by manual examination of marker genes. Clusters identified as Monocyte- or Macrophage-like were selected and re-clustered (the full set of identified clusters is shown in Figures S2B and S2C). Single-cell gene signature enrichment scores were calculated using the AddModuleScore function in Seurat using gene signatures described by Thomas et al.[58] The test for differential cell-type proportions was conducted using a moderated t test on the logit-transformed proportions as implemented in the propellor function from the R package Speckle.[128] Marker genes with valid antibodies were selected using the Detect_single_marker function from the R package sc2marker.[129]

### Differential gene expression analysis

Differentially expressed genes (DEG) were calculated with each cluster or cell-type in fetal brain and placental cells between obese and control dams using the FindMarkers function in Seurat with arguments test.use = ''MAST'' and latent.vars = ''sample''. Genes were considered significantly differentially expressed with adjusted $p$-value <0.05 and absolute log2 fold change >0.25.

### Functional enrichment analyses

Gene Ontology biological process enrichment analysis was performed on both cluster marker genes and differentially expressed genes using R package clusterProfiler (v. 4.0.5)[125] and underlying database AnnotationDb org.Mm.e.g.,.db (v3.13.0). IPA Canonical Pathway and Diseases and Functions analysis performed with IPA (Content Version: 90348151). GO terms and IPA pathways with adjusted $p$-value <0.05 were considered significantly enriched.

### Cell-cell communication analysis

Putative ligand-receptor interactions between cell-types was performed using CellChat v.1.6.1 R package and underlying database.[130] CellChat identifies differentially over-expressed ligands and receptors for each cell-type and models the strength of interaction using mass action kinetics. Since our dataset was enriched for macrophages, the inferred interactions strengths are likely biased toward macrophages. Thus, we analyzed only interactions between macrophage populations and other cell types.