Human fetal microglia acquire homeostatic immune-sensing properties early in development

L. Kracht1,*, M. Borggrewel1, S. Eskandar2,*, N. Brouwer1, S. M. Chuva de Sousa Lopes3,†‡, J. D. Laman1, S. A. Scherjon2, J. R. Prins2, S. M. Kooistra1†‡, B. J. L. Eggen1†‡

Microglia, immune cells of the central nervous system (CNS), are important for tissue development and maintenance and are implicated in CNS disease, but we lack understanding of human fetal microglia development. Single-cell gene expression and bulk chromatin profiles of microglia at 9 to 18 gestational weeks (GWs) of human fetal development were generated. Microglia were heterogeneous at all studied GWs. Microglia start to mature during this developmental period and increasingly resemble adult microglia with CNS-surveilling properties. Chromatin accessibility increases during development with associated transcriptional networks reflective of adult microglia. Thus, during early fetal development, microglia progress toward a more mature, immune-sensing competent phenotype, and this might render the developing human CNS vulnerable to environmental perturbations during early pregnancy.

Microglia are the resident myeloid cells of the central nervous system (CNS) and contribute to tissue homeostasis and pathology. Under homeostasis, microglia survey the CNS parenchyma and express receptors involved in monitoring and immune-sensing functions, termed the sensome (1). Microglia colonize the brain prior to neurogenesis, myelination, and blood-brain barrier formation and are a largely self-maintaining and long-lived population in healthy CNS (2, 3). Environmental perturbations during pregnancy affect microglia functioning (4, 5) and can be associated with epigenetic reprogramming (6, 7). Microglia are important for CNS development, and in view of their longevity and epigenetic memory, early perturbations in microglia might have long-lasting effects that could affect CNS development and function (8, 9).

In mice, microglia emerge from early erythromyeloid progenitors in the extraembryonic yolk sac at embryonic day 7.5 (E7.5) and subsequently colonize the developing brain rudiment at E9.5 (10). Mouse microglia development is accompanied by changes in gene expression and epigenetic profiles (5). Microglia proliferate and differentiate in early embryonic stages and support neuronal development (synaptic pruning) in late embryonic and early postnatal stages. They acquire their homeostatic and immune surveillance profile at late postnatal and adult stages (4, 5). During embryonic development and early postnatal stages, mouse microglia are heterogeneous (11–13).

In human embryos, shortly after the closure of the neural tube, amoeboid ionized calcium-binding adaptor protein 1-positive (IBA1pos) microglia appear at gestational week (GW) 4.5 in the leptomeninges, the ventricular edge, and the choroid plexus of the brain (14). From these sites, microglia colonize the telencephalon and diencephalon. During this process, microglia transform to ramified cells, which are recognizable as early as GW12 (15, 16). To date, limited bulk RNA sequencing (RNA-seq) data of human mid-gestation fetal microglia indicate that mouse and human microglia share developmental gene expression signatures (4). However, an in-depth understanding of cellular development and heterogeneity of microglia during human fetal development is lacking. Here, 15,782 microglia were analyzed with single-cell RNA sequencing (scRNA-seq) data of human mid-gestation fetal microglia, confirming the presence of microglia (Fig. 1D). Microglia morphology was observed in most tissues, noreactivity was detected in fetal CNS tissue at CD11BposCD45int microglia, but not in other nonmicroglia CNS cells, with a median number of 22,330 unique molecular identifiers (UMIs) and 977 unique genes per cell after filtering. The number of UMIs and percentages of ribosomal and mitochondrial RNA were similar across all samples and ages (fig. S1, A and B). To verify that the CD11BposCD45int population was microglia, we compared the transcription profile to CNS cell type–specific gene sets from two independent human datasets (17, 18) (table S2). Expression of microglia genes was enriched in CD11BposCD45int microglia, but not in other (CD11BposCD45neg) CNS cells (Fig. 1C). IBA1 immunoreactivity was detected in fetal CNS tissue at all gestational ages, and a typical ramified microglia morphology was observed in most tissues, confirming the presence of microglia (Fig. 1D).

Microglia are heterogeneous during human fetal development and exhibit an activated, phagocytic gene expression profile

To determine transcriptional microglia heterogeneity during development, we performed an unsupervised clustering analysis, resulting in 16 distinct clusters (Fig. 2A) present at all GWs (Fig. 2B). All clusters exhibited similar amounts of mitochondrial and ribosomal RNA and UMIs per cell (fig. S1C). Thus, cluster formation was not caused by differences in cell quality. Variation between samples was minor, and sample bias did not affect clustering (fig. S2A). Although sex did not affect cluster distribution (fig. S2B), male/female gene expression differences were difficult to assess, owing to unequal male and female sample numbers per GW (table S1). Clusters were annotated on the basis of cluster-enriched genes (fig. S2, C and D, and table S3), which were determined by differential gene expression analysis of one cluster compared with all other clusters.

Cells in myeloid clusters 9 and 10 expressed the nonmicroglia myeloid cell markers LYVE1 and S100A4, respectively (fig. S2, C and D). These clusters also expressed genes of the MS4A family, which have been associated with a microglia–brain border macrophage intermediate during mouse development (13). Microglia clusters 11 to 16 were characterized on the basis of unique expression of cluster-enriched genes MRPL23, PARP4, MX1, HRA, HBG, ZP3, and NAMPT (fig. S2, C and D).

All microglia clusters expressed canonical microglia markers CSFIR and CX3CR1 (Fig. 2C). Expression of homeostatic microglia genes P2RY12 (Fig. 2C), P2RY13, and TMEM119 (fig. S3A) was less frequent but present and validated in situ for TMEM119 (fig. S3, B and C). Microglia of all clusters expressed multiple genes previously associated with an activated, phagocytic microglia phenotype identified in aging and neurodegenerative diseases [also called the DAMs/MGnD (disease-associated microglia and microglial neurodegenerative) phenotype] (19–21) (Fig. 2C and fig. S3A). AXL, APOE, and CD68, markers for activated,
phagocytic microglia, colocalized with IBA1 in situ, confirming their expression by microglia (Fig. 2, D and E, and fig. S3D).

**Microglia clusters are associated with GW and exhibit distinct functional profiles**

In view of the GW-dependent distribution of cells on the uniform manifold approximation and projection (UMAP) (Fig. 3A) and the differential contribution to clusters (Fig. 2B), we characterized GW-associated clusters.

The percentage of cells in cluster 5 increased from GW9 to -18 and showed distinct expression of immediate-early genes (IEGs) (Fig. 3B). cJUN protein was also detected in IBA1pos microglia in situ (Fig. 3C), making artifactual induction of IEGs exclusively by the microglia isolation procedure unlikely. Gene ontology (GO) terms associated with the cluster-enriched genes were mainly immune- and inflammation-related (Fig. 3D and table S4).

Microglia from all gestational ages contributed to cluster 6, but it was enriched for cells from GW10 to -13 (Fig. 3E). Cells of cluster 6 uniquely expressed the cell cycle genes MKI67 and SPC24 (Fig. 3E), were annotated to G2/M and S phase on the basis of conserved cell cycle genes (fig. S2B), and were associated with GO term cell division (Fig. 3D and table S4). Immunostaining confirmed expression of MKI67 in IBA1pos microglia across multiple GWs (Fig. 3F), indicating the presence of proliferating microglia.

Clusters 7 and 8 had a strong bias for GW9 to -10 microglia, whereas these clusters had little contribution of cells from GWs >15 (Fig. 3G). Cluster-enriched genes included neuronal genes and phagocytosis genes (Fig. 3, G and H). Phagocytosis of other cells or debris may explain the presence of these neuronal transcripts.

More than 40% of microglia in clusters 2 and 3 were derived from GW11 to -12 samples (Fig. 3H). Although microglia of all clusters expressed genes associated with microglia activation (Fig. 2, C, D, and E), some of these genes were more enriched in GW11 to -12 microglia (Fig. 3H). GO terms associated with GW11 to -12 microglia cluster–enriched genes were related to phagocytosis and brain development (Fig. 3D and table S4).

Clusters 1 and 4 exhibited higher expression of homeostatic microglia markers and primarily contained cells from GW15 to -17 (Fig. 3I). Homeostatic markers CX3CR1 and VISTA...
colocalized with IBA1 in situ (Fig. S3, E and F), demonstrating their expression by microglia (22). Enriched genes of GW15 to -17 microglia were annotated with GO terms for cytokine and immune system processes (Fig. 3D and table S4).

**Microglia undergo developmental transition toward adult, homeostatic microglia**

To further delineate the observed developmental progression (Fig. 3), we performed pseudotime analysis (23). Assigned pseudotimes (Fig. 4A) corresponded with the gestational ages at different GWs. (C) UMAPs depicting expression of microglia markers in log counts. (D and E) AXL and IBA1 (D) and APOE and IBA1 (E) coexpression in CNS tissue of GW10 to -18 fetuses. Lower images show single channels. White arrowheads indicate colocalization. Insets depict 2× magnifications of the indicated areas. MG, microglia.

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**Fig. 2. Human fetal microglia are heterogeneous and exhibit an activated, phagocytic profile.** (A) UMAP of 15,782 cells and 16 clusters depicted with size (percentage of total cells in a specific cluster) and annotation (20 fetal samples; n = 1 to 4 per GW). (B) Bar plots depicting the percentage of cells in each cluster.
Fig. 3. GW-associated microglia clusters have diverse functional profiles.
(A) UMAP depicting GWs (20 fetal samples; \( n = 1 \) to 4 per GW).
(B) UMAP highlighting specific cluster, bar plots indicating the percentage of cells in cluster across GWs, and violin plots depicting log expression of cluster-enriched genes for IEG MG.
(C) cJUN and IBA1 coexpression in CNS tissue of GW10 to -18 fetuses.
(D) Alluvial plot depicting the top five GO terms per cluster, plotted for all clusters. Ribbon thickness reflects the number of genes in GO terms.
(E) UMAP highlighting specific cluster, bar plots indicating the percentage of cells in cluster across GWs, and violin plots depicting log expression of cluster-enriched genes for cell cycle MG.
(F) MKI67 and IBA1 coexpression in CNS tissue of GW10 to -18 fetuses.
(G to I) UMAPs highlighting specific clusters, bar plots indicating the percentage of cells in respective clusters across GWs, and violin plots depicting log expression of cluster-enriched genes for GW9 to -10 MG (G), GW11 to -12 MG (H), and GW15 to -17 MG (I).
Significantly enriched genes per cluster, compared with all other clusters, are indicated. *\( P \) (adjusted) < 0.05, model-based analysis of single-cell transcriptomics (MAST). White arrowheads indicate colocalization. Insets depict 2× magnifications of the indicated areas. MG, microglia.
Mouse microglia development is orchestrated by distinct transcription factors (TFs) (5). To unravel putative gene regulatory mechanisms underlying the transcriptional changes in developing human fetal microglia, single-cell regulatory network inference and clustering (SCENIC) (28) was used. SCENIC analyzes co-expression and TF motifs in scRNA-seq data.

Sixty-six gene regulatory networks were identified that segregated into two main hubs after unsupervised clustering associated with older and younger GWs (Fig. 5A). Gene regulatory networks associated with younger GWs were enriched for diverse general cellular functions, including cell cycle (E2F2), morphogenesis (SOX4/11), and differentiation and chromatin remodeling (SPI) (29) (Fig. 5, A and B). Gene regulatory networks associated with older GWs were more microglia specific, including many ETS TF family members, such as ETS1-2, ELF1, ELK3, and SPI1 (PU.1) (Fig. 5, A and C), which are crucial for microglia development and function (30, 31).

Furthermore, gene regulatory networks associated with older GWs were more microglia specific, including many ETS TF family members, such as ETS1-2, ELF1, ELK3, and SPI1 (PU.1) (Fig. 5, A and C), which are crucial for microglia development and function (30, 31). In young GWs, microglia-regulating networks were enriched for diverse general cellular functions, including cell cycle (E2F2), morphogenesis (SOX4/11), and differentiation and chromatin remodeling (SPI) (29) (Fig. 5, A and B). Gene regulatory networks associated with older GWs were more microglia specific, including many ETS TF family members, such as ETS1-2, ELF1, ELK3, and SPI1 (PU.1) (Fig. 5, A and C), which are crucial for microglia development and function (30, 31). In young GWs, microglia-regulating networks were enriched for diverse general cellular functions, including cell cycle (E2F2), morphogenesis (SOX4/11), and differentiation and chromatin remodeling (SPI) (29) (Fig. 5, A and B). Gene regulatory networks associated with older GWs were more microglia specific, including many ETS TF family members, such as ETS1-2, ELF1, ELK3, and SPI1 (PU.1) (Fig. 5, A and C), which are crucial for microglia development and function (30, 31).

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with HOMER (32). In the combined peaks of all samples, putative binding motifs of essential microglia TFs were enriched (table S7). Differential peak analysis indicated that more peaks were associated with older (GW >13; 1338 peaks) than with younger developmental stages (GW <13; 33 peaks) (Fig. 5D and table S8). In peaks that were enriched in microglia at GW <13, the SPI TF motif was present, which was also detected by SCENIC (Fig. 5, A, B, and E). DNA sequences underlying peaks enriched in microglia at GW >13 contained many ETS TF family motifs, including PU.1 (Fig. 5E), which was validated at the protein level (Fig. 5F), in agreement with the SCENIC results (Fig. 5, A and C). These findings indicate that increased chromatin accessibility during microglia development is accompanied by the activation of gene networks driving microglia-specific functions.

Discussion

Microglia are the CNS-resident myeloid cells and are critical for brain development and later tissue homeostasis (4, 5). After seeding the developing CNS from the yolk sac, and also closure of the blood-brain barrier, microglia form a self-sustained population with highly variable turnover and with negligible contribution from peripheral immune cells (33). Studies in mice have revealed that during development, different gene regulatory networks drive microglia proliferation, differentiation, and maturation and that perturbances have long-lasting functional consequences (4, 5).

Here, the transcriptomic profile and chromatin organization of human fetal microglia during early to mid-gestation (GW9 to -18) development are presented. In contrast to microglia in the healthy adult CNS (34), we found that fetal microglia are highly heterogeneous. They progressively mature from GW13 onward, which is regulated by the activity of increasingly complex gene regulatory networks, and they already display functional properties characteristic of mature human microglia at mid-gestation.

Human fetal microglia share extensive transcriptional similarities with microglia during mouse development, with analogous proliferative, glycolytic, and activated, phagocytic capacities (4, 12, 13). The presence of glycolysis-related genes in microglia at GW9 to -10 underscores their undifferentiated and activated state, since immune-activated mouse and human microglia (25, 35) as well as undifferentiated cells such as stem cells (36) and embryonic cells (13) use glycolysis as an energy source.

Early to mid-gestation microglia share transcriptional features with a phagocytic microglia population that is transiently present during postnatal mouse development and associated with myelinating brain regions (12, 13). In mice, microglia support myelination, neurogenesis (37), and oligodendrogenesis (38). Human microglia may play similar roles, as the gestational period GW9 to -18 coincides with oligodendrocyte (39) and neuronal (40) development. These human fetal phagocytic microglia express genes detected in DAM/MGnD microglia that are observed in neurodegenerative mouse models (19–21). The similarities between developmental human microglia...
subtypes and DAM/MGnD microglia in mice suggest that developmental transcriptional programs are reactivated in neurodegenerative diseases (22, 13) in which microglia are increasingly implicated.

At later GWS, the frequency of IEG-expressing microglia increases. Expression of IEGs in microglia was previously attributed to ex vivo activation (6, 12). However, we detected cJun in fetal CNS tissue prior to microglia isolation, indicating that IEGs were not (exclusively) induced by the isolation procedure or the collection and experimental methods used here. IEG expression might reflect a necessary responsiveness of microglia to local environmental cues of the developing CNS.

In all investigated GWS, small microglia clusters were present (clusters 11 to 16) for which we could not assign specific functional properties because of a low number of cluster-enriched genes. In situ validation of these minor clusters was further hampered by the limited number of cells in these respective clusters and by the small size of early fetal brain tissues.

With increasing GWS, human microglia acquire a more homeostatic phenotype, reflected by an increasing overlap between genes expressed in fetal (especially beyond GW13) and juvenile and adult human microglia. This overlap was likely underestimated, because juvenile and adult human microglia were analyzed by bulk sequencing and may have included less abundantly expressed genes not detected in single-cell sequencing. Moreover, expression of microglia sensome genes (1), encoding receptors important in environmental sensing, increased at later gestational ages, pointing to the emergence of immune-sensing microglia during early fetal development.

The detected increase in chromatin accessibility and associated gene regulatory networks in fetal microglia from older GWS allows for the activation of more complex gene programs that are required for the immune sensing, synaptic pruning, phagocytic, and tissue-supportive functions of microglia.

The emergence of immune-sensing microglia is highly relevant in view of environmental perturbations during pregnancy that disturb mouse microglia development (4, 5) and impair CNS functions in adult mice (41). Perturbed microglial development has been linked to human neurodevelopmental and psychiatric disorders (8, 9). A higher risk for the development of autism is associated with feverish infections, particularly in the second trimester (42, 43). Because microglia express receptors involved in environmental sensing at this gestational period, they may contribute to fetal CNS sensitivity to the environment during early pregnancy.

Together, our findings demonstrate that microglia are highly heterogeneous during early human fetal development and mature by mid-gestation. This explains the vulnerability of the developing human CNS to environmental perturbations at this developmental period during pregnancy, with potentially long-lasting consequences.

REFERENCES AND NOTES

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Competing interests: The authors have no competing interests to disclose. Data and materials availability: All next-generation sequencing data can be viewed at NCBi GEO under accession number GSE141862. Code used for data analysis is deposited at Zenodo (44).

SUPPLEMENTARY MATERIALS
science.sciencemag.org/content/369/6503/530/suppl/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 to S10
References (45–57)
MDAR Reproducibility Checklist

View/request a protocol for this paper from Bio-protocol.

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The development of microglia

Microglia are the brain’s immune cells, and they play important roles in health and neurodegenerative disease. Kracht et al. performed single-cell analysis of human microglial gene expression and chromatin accessibility and compared the results with those of other studies of human and mice microglial development. By using in situ validation, these data identify fetal microglial subsets that appear to be distinct from adult human microglia, suggesting functional differences between the developing and mature brain.

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