Supplemental Information

Single-cell atlas reveals meningeal leukocyte heterogeneity in the developing mouse brain

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Materials and Methods

HI brain injury model

The day of birth was considered PND0, and mice underwent HI on PND4 to model brain injury in preterm infants. Briefly, the pups were anesthetized with isoflurane (5% for induction and
3% for maintenance) and underwent unilateral left carotid ligation followed by xylocaine administration as a topical anesthetic. After recovery in the dam’s cage for 1 hour, the pups were transferred to a humidified hypoxic chamber for 70 minutes (10% O₂ in N₂, 36.0 ± 0.5°C) (Rice et al. 1981; Albertsson et al. 2014; Nazmi et al. 2018). The pups were then returned to the dam’s cage until being sacrificed. Naïve littermates were used as controls.

**Meningeal dissection**

Six hours after HI, transcardiac saline perfusion was performed after intraperitoneal injection of thiopental (Pentocur, Thiopental, 50 mg/ml; Abcur AB, Helsingborg, Sweden). The meninges (arachnoid and pia mater) were peeled off the brain under a surgical microscope. Neuronal nuclear protein (NeuN) is a neuronal nuclear antigen that is commonly used as a biomarker for neurons. The effectiveness of the meningeal removal was confirmed by comparing NeuN-stained neonatal mouse brain before (Fig. S1a, d) and after (Fig. S1b, e) the removal of the meninges, which shows no parenchymal damage from the meningeal peeling off. We were able to remove the meninges in their entirety, without parenchymal contamination, as confirmed by the lack of NeuN⁺ cells compared to the cortical layers of the brain (Fig. S1c). The removed meninges were then placed in Hank’s Balanced Salt Solution (no Ca²⁺, MgCl₂; Gibco, Life Technologies) on ice.

**Fluorescence-activated cell sorting**

Fluorescence-activated cell sorting (FACS) was used to sort leukocytes that were positive for cluster of differentiation 45 (CD45) from the control and HI mouse meninges. As a transmembrane protein, CD45 is expressed in all cells of the hematopoietic lineage, except erythrocytes and plasma cells (Hathcock et al. 1992; Altin and Sloan 1997). To ensure consistency, and to make sure that the experiments were repeatable, we performed the experiments in two separate runs with one naïve and one HI sample per run. Each sample was pooled from 12 pups, with
equal numbers of females and males in each sample. The samples were processed to single cell suspensions as previously described (Derecki and Kipnis 2014). Afterwards, the cells were incubated with Fc block (clone 2.4G2; cat. 553142; BD Pharmigen) followed by staining with primary antibody against CD45 (1:50, FITC; clone 30-F11; cat. 553080; BD Pharmigen) and 7-AAD as the cell viability marker. The cells were then sorted on a BD Aria Fusion sorter to obtain a live CD45\textsuperscript{int+high} cell population (Fig. S1F). Immediately after sorting, the samples (approximately 10,000 cells/sample) were prepared for single-cell gel beads-in-emulsion.

**Single-cell RNA-sequencing sample preparation and analysis**

The sample cDNA was aligned to the mouse reference genome (mm10), and CellRanger 2.2.0 (10x Genomics) identified about 4,000–6,000 cells/sample with a post-normalization of 37,818 mean reads/cell and a median unique molecular identifier of 10,000 counts/cell (Table S1). Q30 was \(>90\%\) in all samples, and the sequencing saturation was 60\% overall. Seurat 3.027 was used to further analyze the cell clusters. Cells with between 200 and 6000 expressed genes and with mitochondrial counts below 7\% were kept for downstream analysis. The data were normalized with a scale factor of 10,000 and were log transformed before identifying the 2,000 most highly variable genes. All genes were scaled using the default settings in Seurat (including cell cycle bias) before performing the principal component analysis. Cells were then clustered using a graph-based clustering method (standard Seurat v3.0 and 4.0 workflow) using the dimensions of 18 principal components and a resolution of 0.2. The clusters were visualized in a Uniform Manifold Approximation and Projection (UMAP) plot. Differentially expressed gene analysis was performed in Seurat using the Wilcoxon Rank Sum test, and UMAP was used to reduce the dimensions of the Seurat results. Doublets were identified using DoubletFinder (McGinnis et al. 2019), which were found mainly in the microglia-like cluster and were removed before further analysis.
**Cluster annotation**

Gene lists from CellMarker (Zhang et al. 2019) were used to manually annotate the different cell populations, including cells from the immune system and CNS as well as progenitor cells. In addition, a second method using a newly generated automated annotation with CHETAH (de Kanter et al. 2019) was performed. Using a scRNA-seq data set as a reference, CHETAH assigned labels to cells using a classification tree based on the reference cell subtypes. Here, the scRNA-seq compendium of the Tabula Muris Consortium was used (Tabula Muris Consortium 2018). The immune and brain cells of all 20 organs and tissues were selected and grouped into 20 main cell types. When the confidence score for a specific cell annotation was higher than 0.1, the cell was assigned to a specific node until the final subtype was identified. If the confidence score was less than 0.1, the cell was labeled as unassigned or intermediate depending on whether the closest node of the tree was the top one (unassigned cell) or one of the branches (intermediate cell, labeled as the closest node). The results generated by the two methods were compared by importing a simplified CHETAH classification per cell into the Seurat UMAP mapping cells using cell barcodes.

**Data aggregation with an existing database for BAMs and microglia-like cells**

To compare the BAMs in neonatal meninges with the adult mouse meningeal BAMs, we aggregated our BAM cluster with data representing the SD-BAMS and D-BAMs, respectively, from adult mice\(^3\). After the data aggregation, cells in the BAM cluster were reclastered using the Seurat v3.0 graph-based clustering method and were visualized in UMAP. We also compared our meningeal microglia-like cell cluster with parenchymal microglia from age-matching P4/5 mice (Hammond et al. 2019). This aggregation was performed as for the BAMs using Seurat v4.0.

**SCORPIUS trajectory analyses**
To investigate the relationships between the different subclusters of BAMs, microglia-like cells, and monocytes, we performed SCORPIUS (Cannoodt et al. 2016) analyses on the clusters using the suggested workflows and Pearson’s correlation.

**GO enrichment analysis**

GO biological processes were investigated using gProfiler (Reimand et al. 2016) based on the differentially expressed genes obtained from each pair of comparisons. The GOPlot (Walter et al. 2015) R package was used to visualize the most relevant GO term results, and adjusted p-values and z-scores (calculated in GOPlot) were reported. For all searches, only significantly regulated genes (adjusted p-value < 0.05) were used, and the order of the queries was based on gene fold change.

**Ingenuity Pathways Analysis**

IPA (Qiagen) was used to predict pathway regulation, upstream analysis, and regulators. Only genes with p < 0.05 and a q-cutoff of 0.01 were used for the analysis. For analyzing BAMs and comparing with BAMs in adult mice, a cut-off of an absolute fold change > 1.5 was used based on the IPA’s suggested number of genes to input. For canonical IPA pathways, an absolute z-score ≥2 indicated the predicted activation of the pathway. For absolute z-score < 2, the pathways were considered to be mildly activated. P-values were exported from IPA based on Fischer’s exact test (right-tailed for upstream analysis). All prediction graphs were exported from IPA, except for the canonical pathways analyses that were generated in GraphPad Prism 8 (La Jolla, California, USA) based on the data exported from the IPA.

**Immunofluorescent and immunohistochemical staining**

After fixation, the meninges were blocked with 5% donkey serum and the tissues were stained with rabbit anti-mouse CD206 (1:200, cat. ab64693, Abcam), rat anti-mouse Lyve1 (1:250, clone ALY7, cat. 14-0443-82, Invitrogen), and goat anti-mouse Iba-1 (1:250, cat. ab5076,
Abcam) or rat anti-mouse L6g (1:1000, BD Pharmigen, clone 1A8, cat. 551459) and rabbit anti-mouse MPO (1:150, Abcam cat. ab95359) primary antibodies overnight at +4°C. Alexa Fluor donkey anti-goat 488®, donkey anti-rat 594®, and donkey anti-rabbit 647® (all 1:1500, Invitrogen) secondary antibodies were incubated for 1 h at room temperature. After staining the DNA with DAPI, the slides were mounted with ProLong Gold anti-fade reagent (P36930, Invitrogen).

For immunohistochemical staining of Ly6g and NeuN, the meninges were fixed on the slides, while the brain sections underwent antigen retrieval. The samples were blocked with 5% goat or horse serum after blocking endogenous peroxidase with hydrogen peroxide. The sections were then incubated either with rat anti-mouse Ly6g (1:1000, BD Pharmigen, clone 1A8, cat. 551459) or mouse anti-mouse NeuN (1:250, Millipore MAB377), followed by incubations with goat anti-rat or horse anti-mouse secondary antibodies (both 1:250, Vector, cat. cat. BA-9400 and BA-2000-1.5 respectively). After ABC Elite incubation, the sections were incubated with 0.5 mg/ml 3,3'-diaminobenzidine in NiSO₄, β-D-glucose, NH₄Cl, and β-D-glucose oxidase (all from SigmaAldrich, Sweden). For the quantification of meningeal neutrophils after HI, we used a 20× objective lens on a modified Leica microscope (Leica DM6000 B, Germany) with the newCAST software (Visiopharm, Denmark), equipped with a motorized stage (Ludl MAC 5000, United States) and a digital camera (Leica DFC 295, Germany). Briefly, Ly6g⁺ cells in either meninges ipsilateral to the injury side after HI or naïve meninges were counted in regions of interest covering 20% of the total meningeal area and randomly selected through the newCAST software. The density (cells/mm²) was then calculated.
Supplementary Figures

**Figure S1. Meningeal dissection and sorting methods.** A-E) NeuN immunohistochemical staining of PND4/5 mouse brain before (A, D; meningeal layer indicated by red arrows) and after meningeal removal (B, E); and the negligible few NeuN-positive cells on a meningeal sheet separated from the parenchyma (C). D and E are higher magnification pictures of A and B, respectively. Scale bars: A, B: 400 µm; D, E: 100 µm. F) FACS sorting strategy of CD45+ meningeal leukocytes from naïve and HI animals.
Figure S2. Signature gene expression for BAMs and microglia-like cells. A–D) We used signature gene markers for different BAM subtypes from adult meningeal BAMs. UMAP plot representing the expression of common BAM marker genes (Apoe, Ms4a7, Ms4a6c, Lyz2, Clec4a1, Ifitm2, Tgfb1, Cybb, Mndal, Ifi27l2a, Ifitm3, Cd36, Aldh2, Pla2g7, Pf4, Msr1, Aoah, Dab2; A), SD-BAMs (Ccr1, Lyve1, Ednrb, Colec12, Prps2, Ptgs2, P2rx7, Egfl7; B), MHCII
low BAM signature genes (*Nrp1*, *Blvrb*, *Smagp*, *Vcam1*, *Mrc1*, *Dse*, *Cd163*, *Cd38*, *Gpx3*, *Cp*, *Stard8*, *Maf*, *Stab1*, *Cbr2*, *Igf1*, *Igfbp4*, *Snx2*, *F13a1*, *Gas6*, *Ninj1*; C) and D-BAMs (*Cfp*, *Pla2g2d*, *Ccl8*, *Irf7*, *Crip1*, *Ccl9*, *Clec4b1*, *Ccr2*, *Vim*, *Lsp1*, *Lgals3*; D). E–G) *Mrc1* (E) and *Lyve1* (F) gene expression in the microglial-like subclusters. MHCII low BAM signature genes expressed in the major microglial-like cluster (subcluster 8; G). H–K) The expression of the *Sall1* (H), *Aif-1* (Iba-1; I), *Itgam* (J), and *Itgb2* (K) genes in the meningeal microglia-like population. L–Q) The expression of signature genes for parenchymal microglial-like subpopulation present at PND4/5, as previously described by Hammond et al. (Hammond et al. 2019), and the resulting annotation of the same subpopulation in the aggregation analysis of parenchymal microglia and meningeal microglia-like cells (Q). R, S) The expression of the signature genes typical of parenchyma microglia subcluster 6, mainly present at E14.5 as identified by Hammond et al. (Hammond et al. 2019), in the aggregation data (*Ms4a7*, *Ccr1*, *Ms4a6c*; R) and in the neonatal dataset expressed as violin plots of the individual genes (S).
Figure S3. Neonatal meningeal monocytes show changes of cytoskeleton and immune-related processes when compared to adult meningeal monocytes. A) UMAP plot representation of neonatal, adult SD- and D-monocytes, with splitting view in the right panel. B) Volcano plot representing the differentially expressed genes between neonatal monocytes and adult combined SD- and D-monocytes (grey area represents FC > 1.5, p < 0.05). C) GOPlot representation of relevant GO terms from the gProfiler analysis, with the table abbreviations below. The
outer circle represents how many genes are up or downregulated in the GO term. The inner circle has a double function – the height of the bar indicates the negative log10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. D) IPA up and downregulated pathways in neonatal monocytes compared to SD and D-monocytes.
Figure S4. Gene marker analysis for all subclusters generated from the aggregated data analysis using neonatal meningeal microglia-like cells and previously reported P4/5 parenchymal microglia by Hammond et al. (Hammond et al. 2019). A) Heatmaps showing the signature gene expression for each subcluster identified in the microglial aggregation analysis between parenchymal microglia and meningeal microglia-like cells. B) UMAPs showing the expression of some signature genes present in the heatmaps.
Figure S5. GOPlot results for microglial-like cell subclusters 0, 1, 2 and 4 (A-D). The top 10 GO terms are shown. The outer circle represents how many genes are up or downregulated in the GO term. The inner circle has a double function – the height of the bar indicates the
negative log10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. The differentially expressed genes (DEGs) were considered as genes with p-value < 0.05, adjusted p-value < 0.01, and absolute fold change equal or greater than 1.5.
Figure S6. GOPlot results for microglial-like cell subclusters 5, 6, 7, and 9 (A-D). The top 10 GO terms are shown. The outer circle represents how many genes are up- or downregulated in the GO term. The inner circle has a double function – the height of the bar indicates the negative log10 adjusted p-value (the taller, the more significant), and the color of the bar
represents the z-score. The differentially expressed genes (DEGs) were considered as genes with p-value < 0.05, adjusted p-value < 0.01, and absolute fold change equal or greater than 1.5.
**Figure S7.** GOPlot results for microglial-like cell subclusters 10–13 (A-D). The top 10 GO terms are shown. The outer circle represents how many genes are up- or downregulated in the GO term. The inner circle has a double function – the height of the bar indicates the negative
log10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. The differentially expressed genes (DEGs) were considered as genes with p-value < 0.05, adjusted p-value < 0.01, and absolute fold change equal or greater than 1.5.
Figure S8. Characterization of the smaller leukocyte populations in the neonatal meninges. A-D) Neutrophil marker gene expression of S100a8 (A), S100a9 (B), Lrg1 (C), and Plscr1 (D). E) Cd3e gene expression in the T/ILC cluster. F, G) Re-clustering of ILCs (F) and typical signature gene expression of ILCs (G).
Figure S9. **T cell characterization and immunoprofiling analysis.** A, B) T cell subclusters in UMAP plot (A) and dot plot showing the gene expression levels for typical markers of T cell subtypes (B). C) Cell cycle gene expression levels in T cell subclusters. D-F) T cell receptor A (TRA) and B (TRB) expression in all clusters (D), and separately for TRA (E) and TRB (F). **Abbreviations:** Th: T helper; Treg: T regulatory; Tfh: T follicular helper.
Figure S10. B cells characterization and immunoprofiling analysis. A, B) B cell subclusters in UMAP plots (A) and dot plot showing the gene expression levels for typical markers of B cell subtypes (B). C) Cell cycle gene expression levels in B cell subclusters. D-G) UMAP representing the expression of Ig-H, Ig-K, and Ig-L in all clusters (D) and separately for Ig-H (E), Ig-K (F), and Ig-L (G).
Figure S11. Changes in total BAMs and monocytes after HI. A, G) BAMs (A) and monocytes (G) show subtle changes after HI compared to naïve animals. B, H) Volcano plots showing the differentially expressed genes with p < 0.05 and fold change > 1.5 (grey quadrant) for BAMs (B) and monocytes (H). C, I) Gene ontology analysis for BAMs (C) and monocytes (I) with gProfiler: the inner circle has a double function – the height of the bar indicates the negative log10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. D-F, J, K) Canonical pathway analysis (z-score >1.5) (D, J) and regulators (E, F, K) for BAMs (D-F) and monocytes (J, K).
Figure S12. GOPlot results for BAM subclusters 0-3 (A-D). The top 10 GO terms are shown.

The outer circle represents how many genes are up- or downregulated in the GO term. The inner circle shows the number of genes involved in the GO term.
circle has a double function – the height of the bar indicates the negative log 10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. The differentially expressed genes (DEGs) were considered as genes with p-value < 0.05, adjusted p-value < 0.01, and absolute fold change equal or greater than 1.5.
Figure S13. GOPlot results for BAM subclusters 4-7 (A-D). The top 10 GO terms are shown.

The outer circle represents how many genes are up- or downregulated in the GO term. The inner
circle has a double function – the height of the bar indicates the negative log10 adjusted p-value (the taller, the more significant), and the color of the bar represents the z-score. The differentially expressed genes (DEGs) were considered as genes with p-value < 0.05, adjusted p-value < 0.01, and absolute fold change equal or greater than 1.5.
Figure S14. Changes in microglia-like cells after HI. A-F) Developmental white matter-associated genes (A-C) and disease-associated genes (D-F) are differentially expressed in the microglia-like after HI. The Wilcoxon Rank Sum test was used to test if the expression differed significantly between the naive and HI conditions.
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