Supplementary figures, reagents, and software packages:





Figure S1. Characterization of 4C4 positive and negative myeloid cells, supplemental to Figure 1. 7

8 (a) Representative images of OT and MB brain regions using the pan-myeloid reporter line 9 mpeg1.1:GFP-CAAX and the commonly used antibody 4C4 to label presumptive microglia at 28 10 days post fertilization (dpf). Scale: 100 μm. Optic tectum (OT), midbrain (MB), and hindbrain
 (HB). Insets of boxed areas show separate channels (top) and merged image (bottom) for each
 12 respective region. Scale: 10 μm. All images are representative of the n=3 replicates.

**(b)** Quantification of percent of *mpeg1.1:*GFP-CAAX<sup>+</sup> population also 4C4 positive. Dots 14 represent individual fish, data are mean  $\pm$  SD. One way ANOVA; \*\* p = 0.0047.

(c-d) Characterization of 4C4-negative myeloid cells. Representative images using the pan-myeloid reporter line Tg(*mpeg1.1:GFP-CAAX*) crossed to vascular reporter line Tq(*flk1:mCherry*), stained with the 4C4 antibody at 30 dpf. i) Insets highlighting mpeg1.1: GFP-CAAX<sup>+</sup> 4C4 negative border associated macrophages (BAMs) at the brain surface, ii) insets of representative mpeg1.1: GFP-CAAX+ 4C4-negative ramified parenchymal cell contacting a blood vessel; note, these cells lacked elongated perivascular macrophage morphology seen in mammalian PVMs. iii) Insets highlighting a mpeq1.1:GFP-CAAX<sup>+</sup> 4C4-negative, ramified, parenchymal, non-vessel contacting cell. Scale: 10 µm. All images are representative of the n=3 replicates. (e) Distribution of the 4C4-negative mpeg1.1:GFP-CAAX<sup>+</sup> cell population across the three morphologically and regionally defined subsets shown in C-D. Dots represent individual fish (n=4), data are mean ± SEM. Scale: 100 µm. Optic tectum (OT), midbrain (MB), and hindbrain (HB). 







Figure S2. Gating hierarchy and quality control of single cell sequencing data,
 supplemental to Figure 2.

- **(a)** Gating strategy to isolate cd45 positive cells for single-cell RNA sequencing data in Figure 2 66 a-g. The violet gate represents the total cd45-DsRed<sup>+</sup> population that was sequenced.
- 67 (b) Unsupervised clustering of juvenile *cd45* positive cells, replicated from Fig. 2b for reference.
- 68 (c) Select enriched genes in *cd45+mpeg1.1*<sup>-</sup> immune cell subsets calculated with the MAST DE

algorithm in Seurat. Size represents percent of cells expressing each gene while color
 represents normalized and scaled gene expression compared to all clusters; decreased
 expression: blue, expression unchanged: white, increased expression: red.

- 72 (d-e) Feature plot showing distribution of mitochondrial RNA content and genes recovered 73 (nCount) per cell.
- 74 (f) Comparison of independent biological replicates of *mpeg1.1*<sup>+</sup> cells pooled in Fig. 2c.
- 75 (g) UMAP plot highlighting levels of mpeg1.1 expression the  $mpeg1.1^+$  population from 2c.
- (h) UMAP plots showing the effects of changing the clustering resolution on unsupervised
   clustering in Seurat with the FindClusters function. Clustering resolutions 0.1, 0.3, and 0.5 are
   shown.
- (i). Volcano plot of differentially expressed genes between clusters JM1 (cluster 1) and JM0 (cluster 0) using a clustering resolution of 0.3. The MAST DE algorithm in Seurat was used to calculate log fold changes. Thresholds represented by dotted lines were set to adjusted p value  $<10^{-10}$ , log(2) fold change > 0.2.



## Figure S3: Feature plots of macrophage, microglia, and mammalian BAMs, supplementalto Figure 2.

(a) Feature plots from juvenile single-cell RNA dataset from Fig. 2C, showing canonical
 macrophage and microglia markers (top), and several proposed mammalian border associated
 macrophage (BAM) signature genes<sup>39,56</sup> with known homologs in fish (labels indicate
 mammalian/zebrafish homologs, bottom).







#### Figure S5. Neuronal engulfment in the OT and HB at 28 dpf, supplemental to Figure 4.

(a-b) Representative image and quantification of *NBT*:DsRed neuronal bodies within ramified or

amoeboid microglia (Sphericity > .6) from OT and HB brain regions. Two separate t-tests, \*\*p < 0.01, n=3 microglia per fish; n=3 represented as mean values. Scale bar: 10 µm. All images are representative of the n=3 replicates.



Figure S6. Human fetal microglia (Kracht et al., 2020) comparison to juvenile zebrafish microglia and pseudotime analysis, supplemental to Figure 4.

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(a) Pseudotime analysis with Monocle 3<sup>48</sup> on both juvenile and adult *mpeg1.1* positive cell
 populations. Left UMAP plot is colored by cluster, right colored by estimated pseudotime using
 dividing cells as a starting point.

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(b) Heatmap comparing juvenile zebrafish microglial clusters to a single-cell human fetal microglia dataset (Kracht et al 2020, table S3)<sup>49</sup>. Cells are colored by signature enrichment (estimated increase in average eigengene expression calculated with the AddModuleScore function in Seurat) in the listed zebrafish cluster compared to all other zebrafish clusters. (Twosided Wilcoxon Rank-Sum Test, \*p<10<sup>-50</sup>, \*\*p<10<sup>-100</sup>, \*\*\*p<10<sup>-200</sup>).

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#### List of key reagents and software packages

Antibodies     Aves Labs     Cat#GFP-1020, RRID:AB10000240       Rabit anti-DsRed 1:1000     Takara Bio     Cat#G32496;RRID:AB- 10013483       Mouse anti-synaptic vesicle glycoprotein 2A (SV2) 1:500     DHSB     RRID: AB_2315387       Matter anti-BrdU 1:500     Abcam     Cat#B32496;RRID:AB_305426       Mouse anti-AC4 1:200     Gift from Hitchcock Lab     Cat#30202321;RRID:AB_10013       Anti-Digoxigenin-AP, Fab     Fragments 1:500     Abcam       Artexa Fluor 488 goat anti- chicken 1:500     Thermo Fisher     Cat#4.       Alexa Fluor 555 goat anti- mouse     Thermo Fisher     Cat#A.       Alexa Fluor 555 goat anti- rabbit 1:500     Thermo Fisher     Cat# A.21422, RRID:AB_2536804       Alexa Fluor 647 goat anti- mouse     Thermo Fisher     Cat# A.21248; RRID:AB_2535816       Critical Commercial Assays     Thermo Fisher     Cat# A.21248; RRID:AB_2535816       Critical Commercial Assays     IoX Genomics     Library and gel bead kit-V3, 120267:Chip B kit:1000009       Sligma-Aldrich     Z# F4648     Z# F4648       TRNaphtol AS-MX Tablets     PrekinElmer     C# F4648       ProSense 680 Fluorescent Imagining Agent     Gene Expression Omnibus     GSE164771       Bulk RNA-sequencing of microglia from Uvenile and aduts     Gene Expression Omni	REAGENT or Software package	SOURCE	IDENTIFIER
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Alexa Fluor 647 goat anti-mouse 1:500     Thermo Fisher     Cat# A-21235; RRID:AB_2535804       Alexa Fluor 647 goat anti- Rat 1:500     Thermo Fisher     Cat# A-21248; RRID:AB_2535816       Critical Commercial Assays     Image: Cat# A-21248; RRID:AB_2535816       Critical Commercial Assays     Image: Cat# A-21248; RRID:AB_2535816       Critical Commercial Assays     Image: Cat# A-21248; RRID:AB_2535816       Chromium single cell gene expression platform, version 3     Sigma-Aldrich       SIGMAFAST Fast Red TR/Naphthol AS-MX Tablets     Sigma-Aldrich       ProSense 680 Fluorescent Imagining Agent     PerkinElmer       Deposited Data     Gene Expression Omnibus       Single cell RNA-Sequencing of microglia from juvenile and adults     Gene Expression Omnibus       Bulk RNA-sequencing of microglia from OT, MB, HB brain regions     Gene Expression Omnibus       Experimental Models: Organisms/Strains     57       Zebrafish: Tg(mpeg1.1:EGFP)     57       Zebrafish: Tg(cd45:DsRed)     33       Oligonucleotides     F: TAATACGACTCACTATAGGGG GACAGGAGAAACTCACAGG;R:	rabbit 1:500		21429:RRID:AB 2535850
1:500     RRID:AB_2535804       Alexa Fluor 647 goat anti- Rat 1:500     Thermo Fisher     Cat# A-21248; RRID:AB_2535816       Critical Commercial Assays     Image: Commercial Assays     Image: Commercial Assays       Chromium single cell gene expression platform, version 3     10x Genomics     Library and gel bead kit-V3, 120267:Chip B kit:1000009       SIGMAFAST Fast Red TR/Naphthol AS-MX Tablets     Sigma-Aldrich     C# F4648       ProSense 680 Fluorescent Imagining Agent     PerkinElmer     C#NEV10003       Deposited Data     Gene Expression Omnibus     GSE164771       Bulk RNA-sequencing of microglia from OT, MB, HB brain regions     Gene Expression Omnibus     GSE164772       Experimental Models: Organisms/Strains     57     ZFIN: ZDB-ALT-0000     Zebrafish: Tg(cd45:DsRed)       Oligonucleotides     In house     F: TAATACGACTCACTATAGGGG     F: TAATACGACTCACTATAGGGG	Alexa Fluor 647 goat anti-mouse	Thermo Fisher	Cat# A-21235:
Alexa Fluor 647 goat anti- Rat 1:500     Thermo Fisher     Cat# A-21248; RRID:AB_2535816       Critical Commercial Assays     Image: Commercial Assays     Image: Commercial Assays       Chromium single cell gene expression platform, version 3     10x Genomics     Library and gel bead kit-V3, 120267:Chip B kit:1000009       SIGMAFAST Fast Red TR/Naphthol AS-MX Tablets     Sigma-Aldrich     C# F4648       ProSense 680 Fluorescent Imagining Agent     PerkinElmer     C#NEV10003       Deposited Data     Gene Expression Omnibus     GSE164771       Single cell RNA-Sequencing of microglia from juvenile and adults     Gene Expression Omnibus     GSE164772       Bulk RNA-sequencing of microglia from OT, MB, HB brain regions     Gene Expression Omnibus     GSE164772       Experimental Models: Organisms/Strains     57     ZFIN: ZDB-ALT-0000       Zebrafish: Tg(mpeg1.1:EGFP)     57     ZFIN: ZDB-ALT-0000       Zebrafish: Tg(mpeg1.1:EGFP)     57     ZFIN: ZDB-ALT-0000       Ca74a ISH primers,     In house     F: TAATACGACTCACTATAGGGG GACAGGAGAAACTCAAGGCR: DACAGGACAACTCAAGGCR:	1:500		RRID:AB 2535804
Rat 1:500     RRID:AB_2535816       Critical Commercial Assays     Library and gel bead kit-V3, 120267:Chip B kit:1000009       SIGMAFAST Fast Red     Sigma-Aldrich     C# F4648       TR/Naphthol AS-MX Tablets     PerkinElmer     C#NEV10003       ProSense 680 Fluorescent     PerkinElmer     C#NEV10003       Deposited Data     Gene Expression Omnibus     GSE164771       Single cell RNA-Sequencing of microglia from juvenile and adults     Gene Expression Omnibus     GSE164772       Bulk RNA-sequencing of microglia from OT, MB, HB brain regions     Gene Expression Omnibus     GSE164772       Experimental Models:     Organisms/Strains     Organisms/Strains     Organisms/Strains       Oligonucleotides     In house     F:     TAATACGACTCACTATAGGGG       Advarda ISH primers,     In house     F:     TAATACGACTCACTATAGGGG	Alexa Fluor 647 goat anti-	Thermo Fisher	Cat# A-21248:
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Prosense 680 Fluorescent Imagining Agent     PerkinElmer     C#NEV10003       Deposited Data	TR/Naphthol AS-IVIX Tablets	De alvia Elas en	
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microglia from OT, MB, HB brain     regions       Experimental Models:	Bulk RNA-sequencing of	Gene Expression Omnibus	<u>GSE164772</u>
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Organisms/Strains     Zebrafish: Tg(mpeg1.1:EGFP)     57     ZFIN: ZDB-ALT-0000       Zebrafish: Tg(cd45:DsRed)     33     33     33       Oligonucleotides     In house     F: TAATACGACTCACTATAGGGG GACAGGAGAAACTCAAGG;R:	Experimental Models:		
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Zebrafish: Tg(cd45:DsRed)     33       Oligonucleotides        cd74a ISH primers,     In house       F:     TAATACGACTCACTATAGGGG       GACAGGAGAAACTCAAGG;R:     AATAACCOCTAAAGCOC	Zebrafish: Tg(mpeg1.1:EGFP)	5/ 22	ZFIN: ZDB-ALT-0000
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			GACAGGAGAAACTCAAGG:R:
			AATTAACCCTCACTAAAGGGC

		CATCCCAAACAACATGC
<i>ctsba</i> ISH primers,	In house	F: TAATACGACTCACTATAGGGA TGCAAGAGAGCAGTGG; R: AATTAACCCTCACTAAAGGGA AGTCCAATGAGCAGGTC
Seurat (Version 3.1.4)	58	https://satijalab.org/seurat
STAR (Version 2.5.4b)	59	https://github.com/alexdobin/ST AR/releases
DESeq2 package (Version 4)	60	hhtp://www.bioconductor.org/pa ckages/release/bioc/html/DESe2 .html
Harmony (Version 1.0)		
ImageJ	NIH	RRID: SCR_003070
Imaris	Oxford Instruments	RRID: SCR_007370
Prism	GraphPad	RRID: SCR_002798

#### 204

#### 205 Supplemental dataset excel files:

## 206207 Data S1: Pan hematopoietic single cell clusters in 28 dpf zebrafish brain

208 Legend: Differentially expressed genes per cluster for all juvenile (28 dpf) zebrafish CD45+ cells, as shown in Fig. 2b, S2b. First tab includes all clusters; further tabs are subsets of the first 209 210 tab for each individual cluster. Column 1 ("gene") = gene name; column 2 ("p val") = unadjusted p-value calculated with the MAST test in Seurat. Column 3 ("avg\_logFC) = average natural log 211 212 fold change for that gene between the labelled cluster (column 7, "cluster") and all other cells. 213 Column 4 ("pct.1") = fraction of cells in the labelled cluster expressing that gene. Column 5 214 ("pct.2") = fraction of cells outside of the labelled cluster expressing that gene. Column 6 215 ("p val adi") = Bonferroni adjusted p-value per gene. Column 7 ("cluster") = cluster shown in 216 UMAP plots referenced above. Filtered to show p val adj < 0.001, avg logFC>0.2. 217

#### 218 Data S2: Myeloid single cell clusters in 28 dpf zebrafish brain

219 Legend: Differentially expressed genes per cluster for all juvenile (28 dpf) myeloid 220 (mpeg1.1+) cells, as shown in Fig. 2C. First tab includes all clusters; further tabs are subsets 221 of the first tab for each individual cluster. Column 1 ("gene") = gene name; column 2 ("p\_val") = 222 unadjusted p-value calculated with the MAST test in Seurat. Column 3 ("avg logFC) = average 223 natural log fold change for that gene between the labelled cluster (column 7, "cluster") and all 224 other cells. Column 4 ("pct.1 [in cluster]") = fraction of cells in the labelled cluster expressing 225 that gene. Column 5 ("pct.2 [out of cluster]") = fraction of cells outside of the labelled cluster 226 expressing that gene. Column 6 ("p\_val\_adj") = Bonferroni adjusted p-value per gene. Column 7 227 ("cluster") = cluster shown in UMAP plots referenced above. Filtered to show  $p_val_adj < 0.001$ , 228 avg\_logFC>0.2.

## 229230 Data S3: Adult and juvenile single cell clusters

230

## Legend: Differentially expressed genes per cluster for all juvenile (28 dpf) and adult (12

233 months) myeloid (*mpeg1.1*+) cells, as shown in Fig. 2e. First tab includes all clusters; further

234 tabs are subsets of the first tab for each individual cluster. Column 1 ("gene") = gene name; 235 column 2 ("p\_val") = unadjusted p-value calculated with the MAST test in Seurat. Column 3 ("avg\_logFC) = average natural log fold change for that gene between the labelled cluster 236 237 (column 7, "cluster") and all other cells. Column 4 ("pct.1 [in cluster]") = fraction of cells in the labelled cluster expressing that gene. Column 5 ("pct.2 [out of cluster]") = fraction of cells 238 239 outside of the labelled cluster expressing that gene. Column 6 ("p val adj") = Bonferroni 240 adjusted p-value per gene. Column 7 ("cluster") = cluster shown in UMAP plots referenced 241 above. Filtered to show p\_val\_adj < 0.001, avg\_logFC>0.2.

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### Data S4: Region specific myeloid cell profiling in 28 dpf zebrafish brain

245 246 Legend: Differentially expressed genes calculated from bulk sequencing data comparing three 247 brain regions (tab 1: hindbrain (HB, lfc >0) vs midbrain (MB, lfc <0); tab 2: optic tectum (OT, lfc 248 >0) vs hindbrain (HB, lfc <0); tab 3: optic tectum (OT, lfc >0) vs midbrain (MB, lfc < 0). Statistics 249 were conducted using the DESeq2 package in R, and results were filtered to p\_adj < 0.05. 250 Column 1 ("Ensembl\_ID"): gene-specific Ensembl ID; Column 2 ("Gene\_ID"): gene name (if 251 available); Column 3 ("baseMean"): mean normalized gene expression over both samples, 252 corrected for size factors; Column 4 ("log2FoldChange"): Log base 2 fold change gene 253 expression in the first listed brain region compared to the second listed brain region. Positive 254 values indicate enrichment in the first listed brain region; Column 5 ("IfcSE"): log fold change 255 standard error estimate; Column 6 ("stat"): Wald test statistic value for the gene; Column 7 256 ("pvalue"); p-value associated with the test statistic; Column 8 ("padj"): Benjamini-Hochberg 257 (FDR < 0.05) corrected p-value.

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# Data S5: Differentially expressed genes between HB-enriched cluster JM4 and OT enriched cluster JM1.

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262 Legend: Differentially expressed genes between clusters JM4 and JM1, as shown in Fig. 4d. Column 1 ("gene") = gene name; column 2 ("p\_val") = unadjusted p-value calculated with 263 264 the MAST test in Seurat. Column 3 ("avg\_logFC) = average natural log fold change for that 265 gene between cluster JM4 and JM1. Positive values represent increases in cluster JM4 with 266 respect to JM1, while negative values represent genes increased in cluster JM1 with respect to 267 JM4. Column 4 ("pct.JM4") = fraction of cells in cluster JM4 expressing that gene. Column 5 268 ("pct.JM1") = fraction of cells in cluster JM1 expressing that gene. Column 6 ("p val adj") = 269 Bonferroni adjusted p-value per gene. Column 7 ("cluster") = cluster shown in UMAP plots 270 referenced above. Filtered to show genes with p\_val\_adj < 0.001, avg\_logFC>0.2.

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