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Supplemental information

Single-cell RNA-seq analysis reveals compartment-specific heterogeneity and plasticity of microglia

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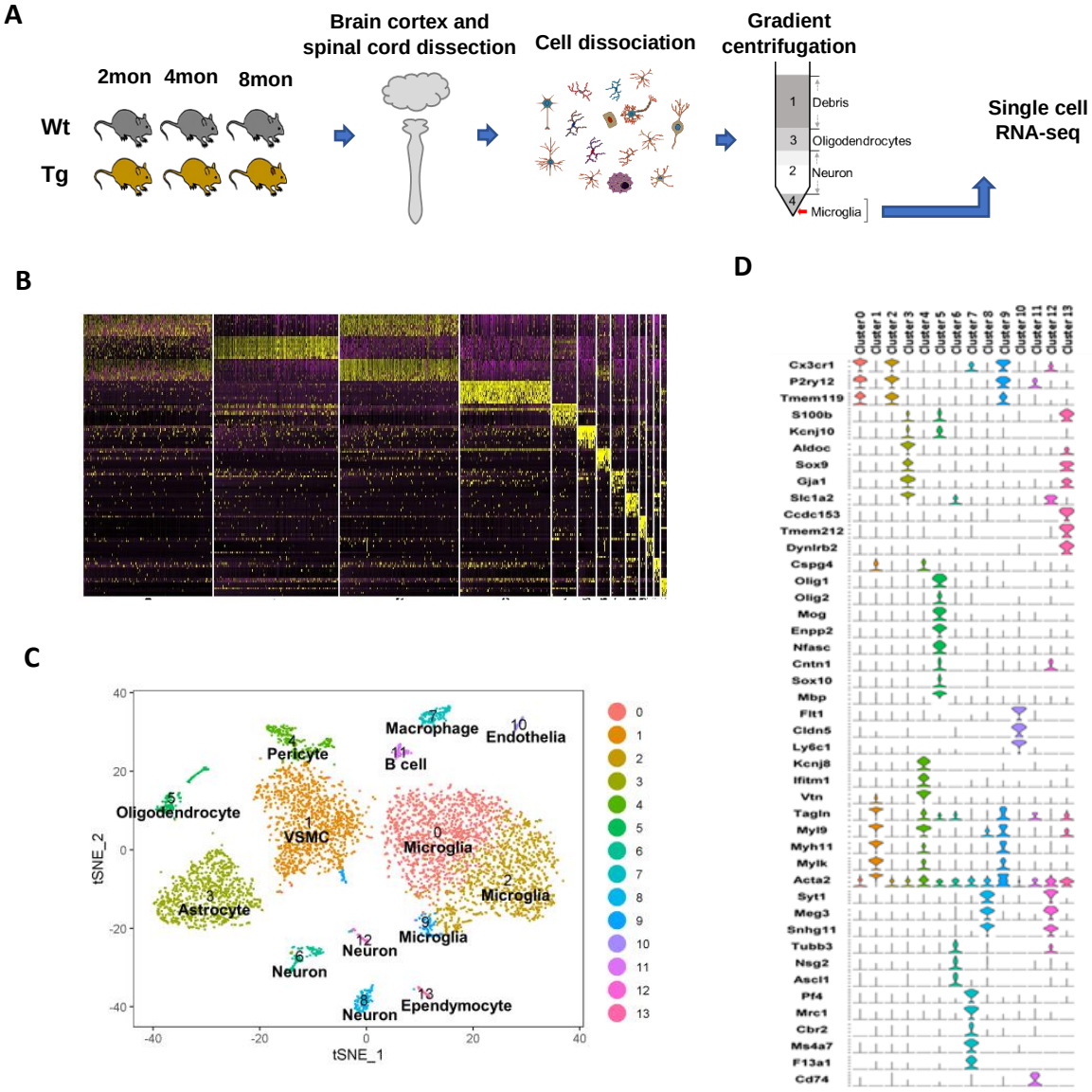

Figure S1. Workflow of the single cell RNA-seq (scRNA-seq) experiment and data analysis of cortical and spinal microglia of Wt and gp120 transgenic (Tg) mice (Related to Figure 1). A: A diagram showing the workflow of cell dissociation and scRNA-seq**.** Microglia-enriched fraction 4 was processed for the scRNA-seq. **B**. Expression heatmaps of the top 10 variable genes in each cluster demonstrated a well separated cluster. **C**. t-SNE plots displaying different cell types identified. VSMC: vascular smooth muscle cell. **D**: Combined violin plots of cell type-specific gene markers for individual clusters.

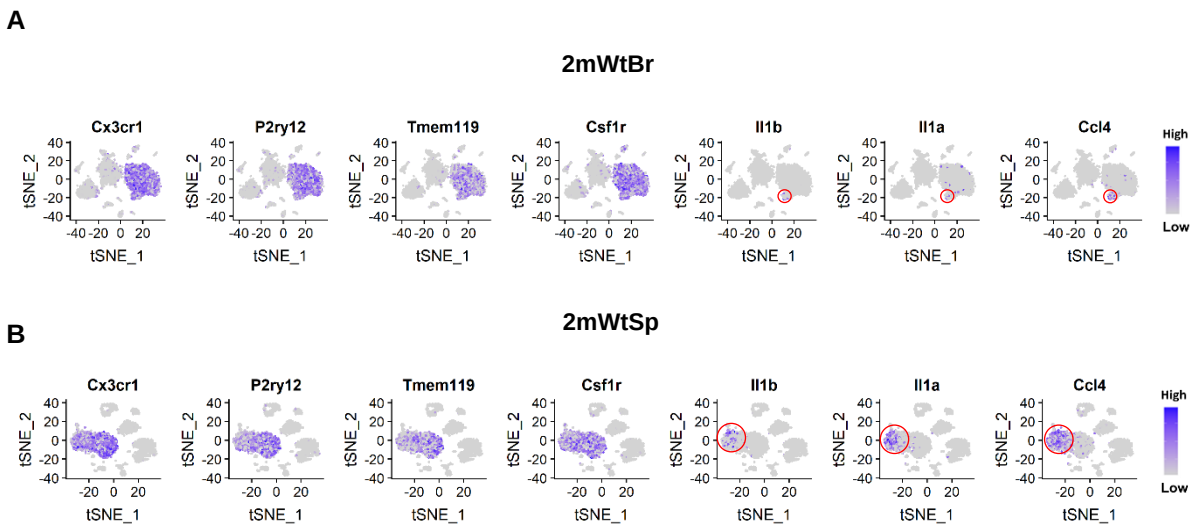

Figure S2. Feature plots of microglial marker genes and inflammatory genes showing marked differences of IFLAM-M population sizes in the cortex (2mWtBr) and the spinal cord (2mWtSp) of Wt mice at two-month-old (Related to Figure 1). Feature plots of pan-microglial marker genes Cx3cr1, P2ry12, Tmem119 and Csf1r identifying the microglial populations in the t-SNE plots of cortical (A) and spinal (B) cells. Feature plots of IFLAM-M signature genes (Il1a, Il1b, Ccl4) showing the population of IFLAM-M (red circled) in the cortex (A) and spinal cord (B). Blue: high expression. Gray: low expression.

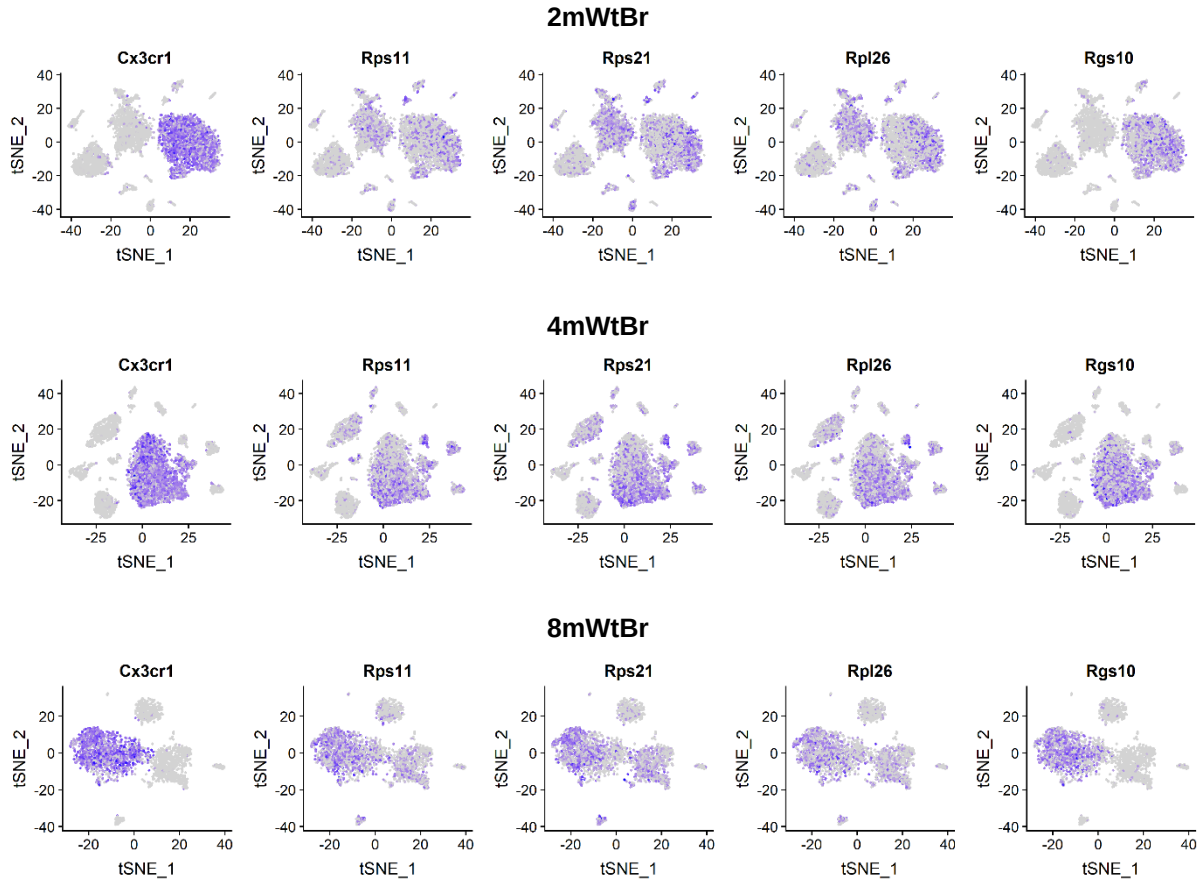

Figure S3. Feature plots showing the slightly lower expression of genes coding ribosomal proteins (e.g. Rps11, Rps21, Rpl26) and Rgs10, the gene coding a member of regulator of G protein signalling family17 in HOM-M2 microglia from the two-, four-, and eight-month-old Wt brain cortex (Related to Figure 2). The plot of Cx3cr1 shows the whole microglial clusters in each dataset.

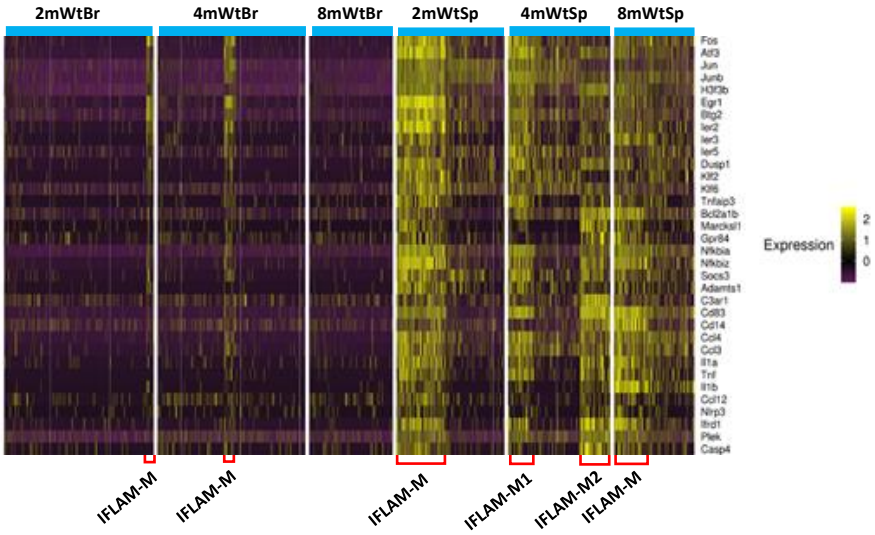

Figure S4. Gene expression heatmaps showing the IFLAM-M populations (red brackets) in the cortices and spinal cords of Wt mice at two-, four- and eight-month-old (Related to Figure 2).

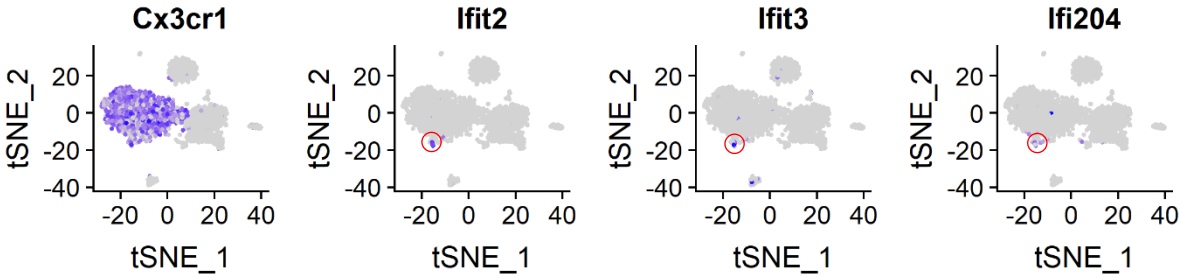

Figure S5. Feature plots show a small cortical microglial cluster with the signature of interferon-response genes (e.g. Ifit2, Ifit3, and Ifi204) in the eight-month-old Wt brain cortex (**Related to Figure 2).** The plot of Cx3cr1 demonstrates the whole microglial clusters.

Transparent Methods

Methods

Animals

Gp120 Tg mice (from Dr. Marcus Kaul, Sanford-Burnham Medical Research Institute) express HIV-1 LAV gp120 under the control of the glial fibrillary acidic protein (GFAP) promoter(Toggas et al., 1994). All animal procedures were performed according to protocol 0904031B approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch, and all methods were performed in accordance with the relevant guidelines and regulations.

Cell dissociation from the brain cortices and spinal cords

We used the same tissue dissociation procedures to prepare brain and spinal cell dissociation in parallel. To isolate cells for scRNA-seq, Wt and gp120 Tg mice were euthanized with 14% urethane followed by decapitation. The brain cortices and whole spinal cords from the Wt and Tg mice of two, four, and eight months (n=2, mixed gender) were rapidly dissected out respectively, the brain meanings were removed thoroughly and temporarily stored in 6ml cold Hibernate A/B27 (HABG) medium. HABG was prepared by adding 30 ml of Hibernate A (BrainBits, cat. no. HA), 600µl of B27 (ThermoFisher, cat. no.17504044), 88 µl of 0.5mM Glutamax (Invitrogen, cat. no. 35050-061), penicillin-streptomycin (ThermoFisher, cat. no.15070063) to final concentration of 2%, and DNase I (ThermoFisher, cat. no. AM2224) to final concentration of 80 U/ml. After completion of dissection, the cortical or spinal tissues were separately placed in 100 mm petri dishes on ice, cut into 0.5 mm pieces with a scalpel, and transferred to a 6 well culture plate containing 6ml papain digestive medium for each sample. Digestive medium was freshly prepared by dissolving 12 mg papain powder (Worthington, cat. no. LS003119) in 6 ml Hibernate A without calcium (BrainBits, cat. no. HACA) with 15 µl 0.5 mM Glutamax, and DNase I at a final concentration of 80 U/ml. The digestive medium was activated at 37 °C for 20 to 30 min. Tissues were digested in a 37°C incubator for 1 hr (agitating every 20 min), and then transferred with the digestive medium into a 15 ml Corning tube on ice, followed by addition of 6 ml cold HABG medium. The tissues were subsequently slowly triturated on ice with a 2 ml Pasteur Pipet for 10 to 15 times. Then triturated the cells 10 to 15 times using a fire-polished narrow-bored glass pipette into single cells. The digestion was ended by adding 5 ml mixed digestion ending solution to the tissue. The ending solution was prepared by mixing 9 ml hibernate medium without calcium with 1 ml reconstituted albumin-ovomucoid inhibitor solution (Worthington, cat. No. LK003182). The dissociated cell suspension was passed through a 70 µm strainer (MACS, cat. no. 130-098-462), and centrifuged at 400×g for 5 min at 4°C. After discarding the supernatant, the cell pellets were gently resuspended in 6 ml cold HABG.

Separation of cells by density gradient centrifugation

Microglia in the cell suspension were enriched according to a published procedure(Brewer and Torricelli, 2007). Briefly, we diluted and prepared 4 ml OptiPrep (Sigma, cat. no. D1556) density gradient in a 15 ml Corning tube following the published protocol(Brewer and Torricelli, 2007). A total of 6 ml cell suspension was carefully pipetted onto the top of the density gradient and centrifuged at 800×g (Beckman, Cs-16R Centrifuge) for 30 min at 22 °C (with acceleration and deceleration rates set at 0). The top layer containing cell debris was carefully aspirated. Fraction 1 was enriched with debris, and fractions 2 and 3 were enriched with oligodendrocytes and neurons respectively (Fig. S1a)(Brewer and Torricelli, 2007). The bottom fraction (~500 µl) enriched with microglia(Brewer and Torricelli, 2007) was collected, mixed with 5 ml cold HABG medium and centrifuged for 10 min at 400×g (Beckman, Cs-16R Centrifuge) at 4°C. The cell pellet was gently resuspended in 3.1 ml cold D-PBS and was processed for additional removal of myelin and cell debris with Debris Removal Solution (Miltenyi Biotec, cat. no. 130-109-398), following the manufacturer's instructions. Cells were

suspended in HABG medium. The viability was checked using trypan blue staining. Cell preps with viability >95% were shipped (overnight on ice) to Cincinnati Children's Hospital Medical Center (to Dr. Potter) for droplet-based scRNA-seq.

Droplet-based single-cell RNA-seq

The droplet-based scRNA-seq was based on a protocol from Macosko *et. al* (http://mccarrolllab.org/wp-content/uploads/2015/05/Online-Dropseq-Protocol-v.-3.1-Dec-2015.pdf). Hydrophobic-treated microfluidic devices were ordered from Nanoshift (Richmond, CA). A total of 1.5 mL of cells (120k cells/ml) were flowed through the device along with 1.5 mL barcoded beads (Chemgenes, 175k beads/ml) and droplet generation mineral oil (QX200, Bio-Rad Laboratories). Resulting emulsion was collected in a 50 ml tube at room temperature; subsequently, the emulsion was allowed to hybridize on ice for 45 min prior to droplet breakage. The excess oil was removed, and the emulsion was transferred to a 50 mL glass conical. 40 mL of cold 6X SSC was added to the emulsion, along with 1 ml perfluorooctanol with vigorous shaking to break droplets. Following droplet breakage, beads were collected from the supernatant into two 50 ml conical tubes. The tubes were spun at 1200 g for 5 min, pelleting the beads. The supernatant was subsequently removed, and the beads were transferred to a 1.5 ml low-adhesion tube. Following this, the beads were rinsed once with 6x SSC, once with 5X RT buffer, and once with 1X RT buffer. Captured mRNAs hybridized to the beads were then reverse transcribed for 30 min at room temp. followed by 1.5 hr. at 42 °C with rotation in RT mix with 2000 U Maxima H Minus Reverse Transcriptase (ThermoFisher, MA) in 200 µl total volume. Reverse transcription was followed by an exonuclease treatment for 45 min to remove unextended bead primers. The nucleic acid on the beads were then PCR amplified (four cycles at 65 °C and 12 cycles at 67 °C annealing temperature with 3000 beads per 50 µl reaction). The cDNA from the PCR reaction was purified using 0.7x volume of SPRIselect beads (Beckman Coulter, cat. no. B23318). The quantity and quality of cDNA was measured using an Agilent Bioanalyzer hsDNA chip. To generate a library, cDNA was fragmented and amplified (12 cycles) using the Nextera XT DNA Sample prep kit with three separate reactions of 600, 1200 and 1800 pg input cDNA. The libraries were pooled and purified twice using 0.7X volume of SPRIselect beads. The purified libraries were quantified using an hsDNA chip and were sequenced on the Illumina HiSeq 2500 using the sequencing parameters described in the DropSeq protocol. The libraries were run on a single lane targeting 150 million reads per library.

Computational methods

Drop-seq FASTQ files were processed using the standard pipeline (Drop-seq tools version 1.12 from McCarroll lab, http://mccarrolllab.org/wp-content/uploads/2016/03/Drop-seqAlignmentCookbookv1.2Jan2016.pdf). Generated read counts were analysed in R version 3.4.4 (R Core Team 2018) via Seurat 2.2.1 (https://CRAN.R-project.org/package=Seurat)(Butler et al., 2018) following its standard pre-processing and clustering workflow (https://satijalab.org/seurat/v3.1/pbmc3k_tutorial.html). By default, Seurat implements a global-scaling normalization method “LogNormalize” that normalizes the gene expression measurements for each cell by the total expression, multiplies this by a scale factor (10,000 by default), and log-transforms the result. We set the filter criteria of min.genes = 300, and mitochondrial gene proportion ≤ 10% as a QC control for scRNA-seq data to remove the dead and the low-quality cells. From the distribution of reads, the data was filtered to a minimum of 300 cells per gene and 30 genes per cell. This was followed by UMI and mitochondrial filtering and normalization prior to selecting all highly variable genes falling within a selected cut-off window for PCA clustering. Statistically significant PCs (p-value < 0.001) were used in cluster determination to produce heatmaps and t-SNE plots at a resolution of 0.6. Clusters were annotated based on the expression level of canonical marker genes and gene expression visualized using feature maps. Differentially expressed genes across these clusters were generated by the FindAllMarkers function (using the MAST test) of Seurat, returning only genes with adjusted p-values < 0.05. These genes were then used to search

for enriched pathways via IPA (Ingenuity Pathways Analysis, QIAGEN Inc.). Seurat violin plots that were consolidated using graphical packages in R (Bodenhofer et al., 2011; Claus and Wilke, 2019). A number of helper functions were also employed ranging from the data input, manipulation and display (Bates and Maechler, 2014; Wickham et al., 2017; Xie, 2012) to color schemes(Neuwirth, 2014).

RNAscope *in situ* hybridization (ISH) and immunofluorescent (IF) staining

Mice were anesthetized with euthanized with 14% urethane and transcardially perfused with 30 ml cold 1xPBS. The cortex and the spinal cord were collected, and fixed in freshly prepared 4% PFA for 24 h at 4°C. The tissues were then immersed in 30% sucrose in 1xPBS at 4°C until they sank to the bottom, embedded in OCT mounting medium, and stored in an air-tight container at –80°C prior to sectioning. The mounted tissue blocks were equilibrated to –20°C in a cryostat for ~1 hour prior to sectioning. 10–20 µm cryo-sections were cut, mounted onto SuperFrost® Plus slides, and stored in airtight slide boxes in Ziplock bags at –80°C until use. Probes for the gene *Cst7* (498711-C2) and *Irf7* (cat. no. 534541-C3) were purchased from Advanced Cell Diagnostics (Newark, CA). ISH procedures, including tissue pre-treatment and probe hybridization, were performed according to the protocol of RNAscope® Multiplex Fluorescent V2 Assay provided by the manufacturer (Advanced Cell Diagnostics). Immediately after the last wash of ISH, the sections were rinsed with 1xPBS (3x5 mins), followed by immunostaining of Iba1 and nuclear staining by DAPI as described (Dual ISH-IHC, Advanced Cell Diagnostics). The stained sections on glass slides were mounted with ProLong Gold Antifade Mountant (Invitrogen, cat. no. P36930) and stored overnight at 4°C before imaging (Zeiss LSM 880 confocal microscope system). Anti-Iba1 antibody (abcam, cat. ab178847) was used at 1:200 dilution. Images were viewed using Zeiss LSM 880 with Airyscan (Carl Zeiss Microscopy GmbH) and Nikon A1R MP (Nikon Instruments Inc.). The 3-D images were processed using Imaris 9.6 software (Oxford Instruments).

Canonical pathway analysis

To evaluate the effects of HIV-1 gp120 on microglial function, upregulated genes with an adjusted p-value of < 0.05 (Benjamini-Hochberg corrected) from each cluster were imported into Ingenuity Pathway Analysis (IPA) (QIAGEN Inc.). IPA's Canonical Pathways Analysis was performed to visualize the activity of individual pathways. The significance (p-value) of the association between the dataset and the specific canonical pathway was computed using the Fisher's exact test (https://chhe.research.ncsu.edu/wordpress/wp-content/uploads/2015/10/IPA-Data-Analysis-training-slides-2016_04.pdf) with Benjamin-Hochberg multiple testing correction at $p < 0.05$ as the threshold of significance. The activation z-score was used to identify the biological pathway that were activated or inactivated. An absolute z-score of ≥ 2 was considered significant, with z-score ≥ 2 for activated pathways and a z-score≤ -2 for inactivated pathways.