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Supplemental Information

Neuroinflammation and EIF2 Signaling Persist despite Antiretroviral Treatment in an hiPSC Tri-culture Model of HIV Infection

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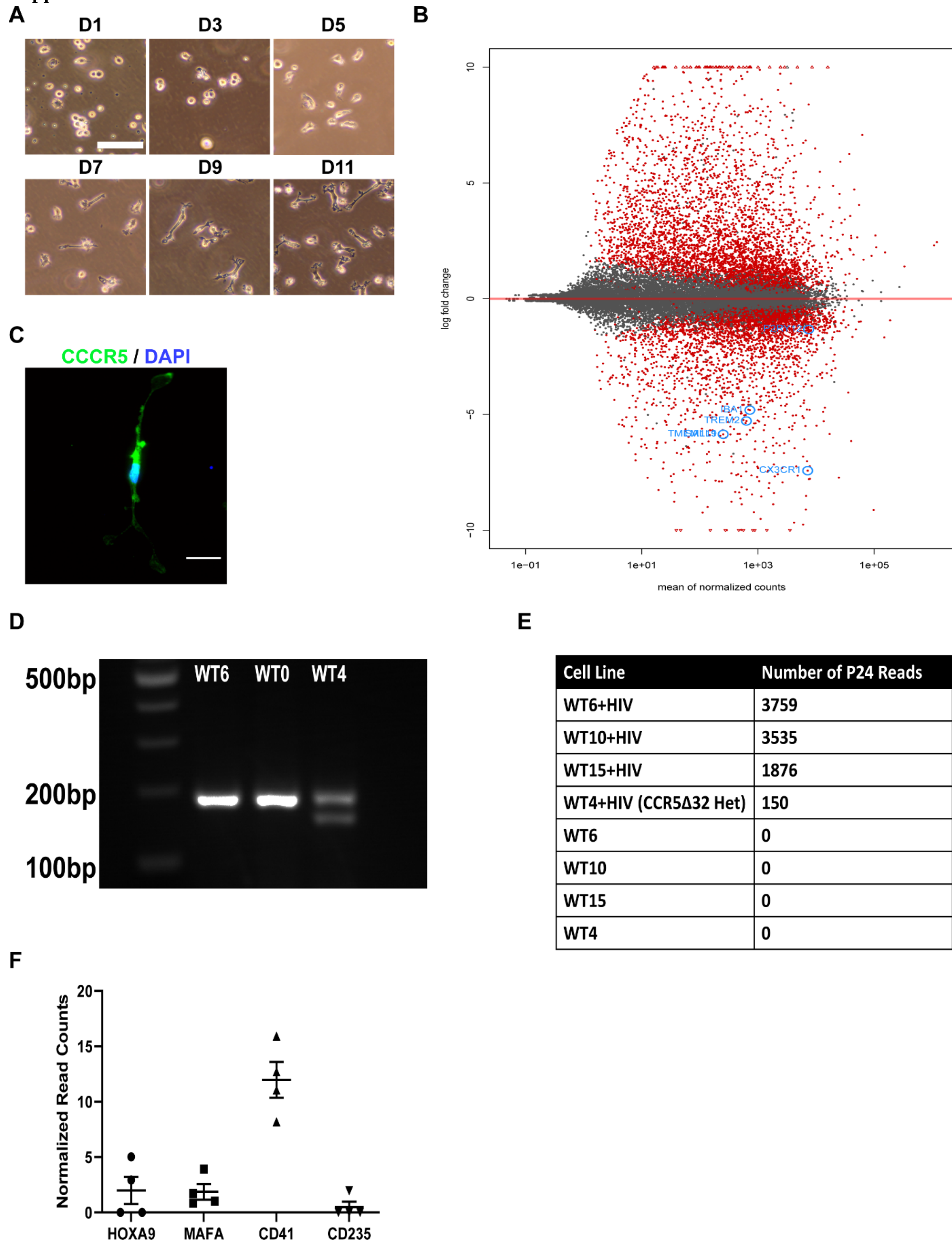
Supplemental Data



Cell Line	Number of P24 Reads
WT6+HIV	3759
WT10+HIV	3535
WT15+HIV	1876
WT4+HIV (CCR5Δ32 Het)	150
WT6	0
WT10	0
WT15	0
WT4	0

Figure S1: iMg and CCR5Δ32 mutation characterization. Related to Figures 1 and 2
(A) Brightfield images of iMg differentiation, depicting ramification by D11. Scale bar represents 50µm.

(B) MA plot of bulk RNAseq data comparing iMg (baseline) to MDMs. n=3 Benjamini-Hochberg FDR-0.05.
(C) Immunostaining showing CCR5 expression (green) on D11 iMg. Scale bar represents 25um.
(D) PCR of CCR5Δ32 shows WT4 is heterozygous for the mutation.
(E) Infected WT4 has dramatically less raw reads of P24 from Bulk RNAseq compared to infected WT6 and WT10 iMg.
(F) Normalized read counts showing lack of expression of myeloid progenitor markers in iMg bulk RNAseq. n=4 cell lines. Error bars represent SEM.

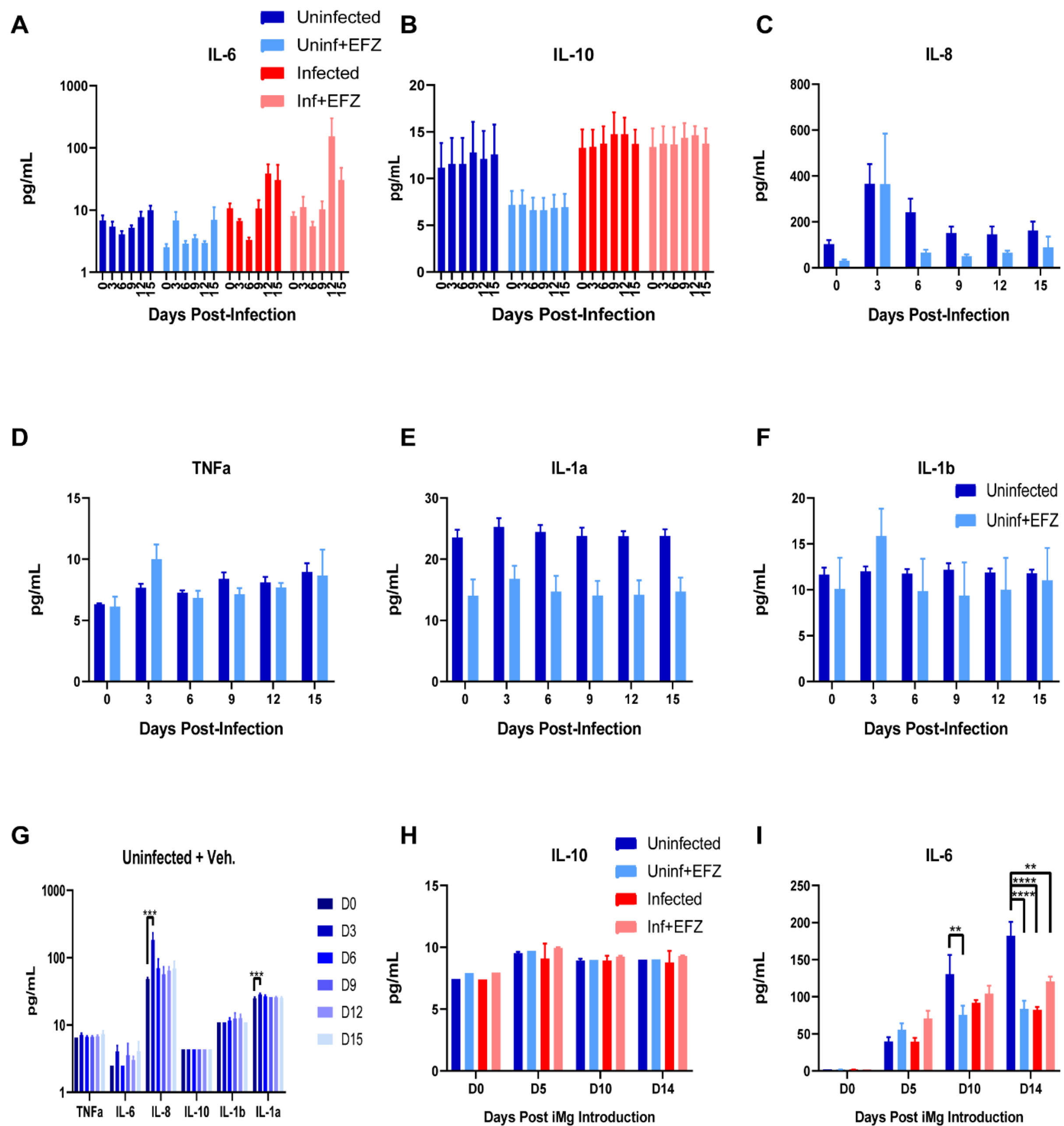


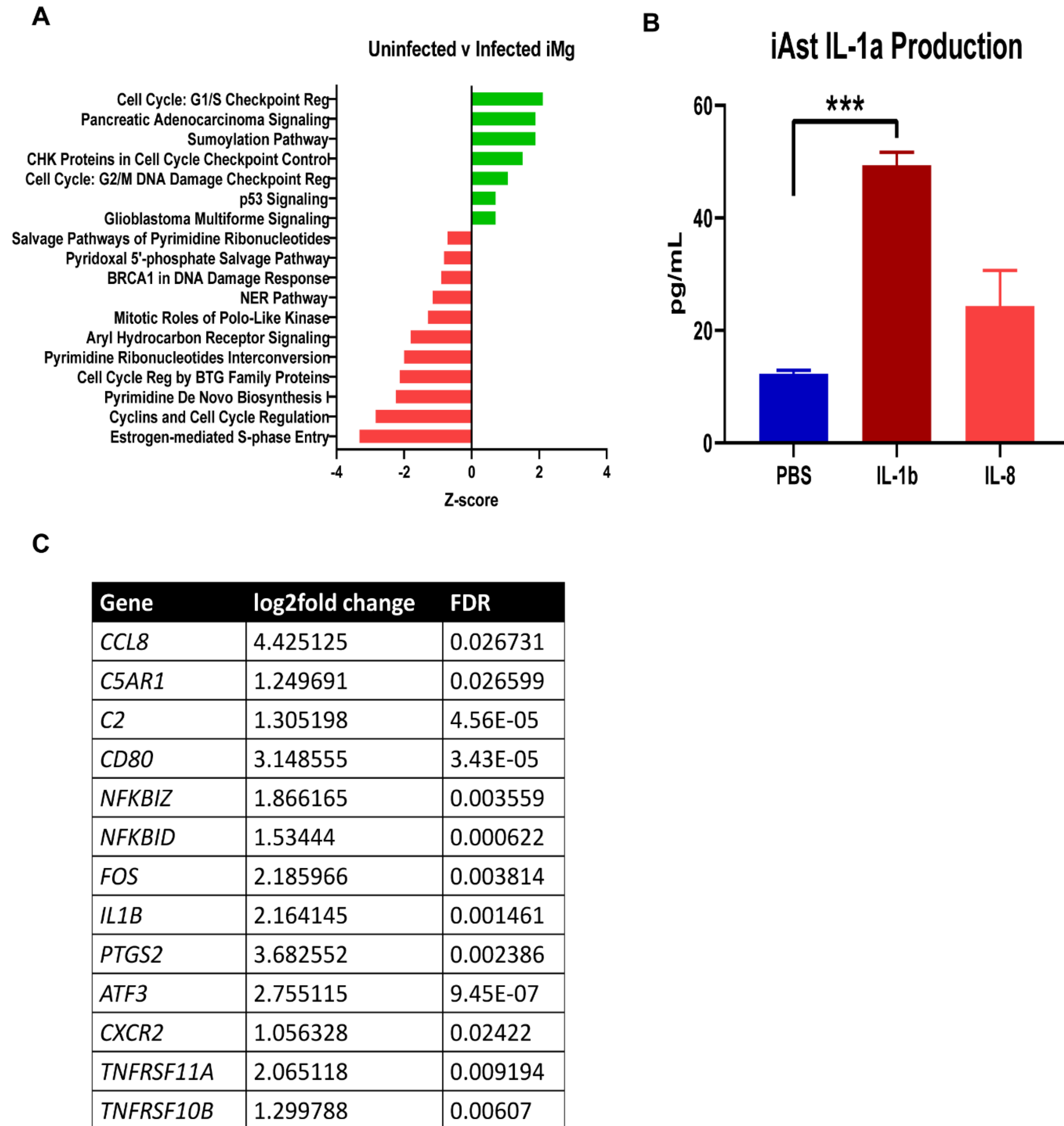
Figure S2: Additional cytokine expression during infection and with vehicle control. Related to Figures 2 and 7

(A&B) IL-6 (A) and IL-10 (B) production in mono-culture did not change for any of the four conditions. n=3 independent differentiations of WT6 (n=4 Uninf and Uninf+EFZ, n=3 Inf and Inf+EFZ), one-way ANOVA, Dunnett's post hoc analysis, error bars represent SEM.

(C-F) cytokine production in Uninf and Uninf+EFZ mono-cultures. IL-8 (C), TNFa (D), IL-1a (E), and IL-1b (F) production in mono-culture did not change for Uninf or Uninf+EFZ. n=4 independent differentiations of WT6, one-way ANOVA, Dunnett's post hoc analysis, error bars represent SEM.

(G) Uninf+ Veh. had minimal but significant increases in IL-8 and IL-1a production at D3, but no increase at D12 where infected cultures had significant increases. n=3 independent differentiations of WT6, one-way ANOVA, Dunnett's post hoc analysis, ***p<0.001, error bars represent SEM.

(H&I) No change in tri-culture IL-10 (H) for any of the four conditions, but IL-6 (I) production increased over time for Uninf, and. n=3 independent differentiations of WT6, two-way ANOVA, Tukey's post hoc analysis, **p<0.01; ****p<0.0001, error bars represent SEM.



Gene	log2fold change	FDR
CCL8	4.425125	0.026731
C5AR1	1.249691	0.026599
C2	1.305198	4.56E-05
CD80	3.148555	3.43E-05
NFKBIZ	1.866165	0.003559
NFKBID	1.53444	0.000622
FOS	2.185966	0.003814
IL1B	2.164145	0.001461
PTGS2	3.682552	0.002386
ATF3	2.755115	9.45E-07
CXCR2	1.056328	0.02422
TNFRSF11A	2.065118	0.009194
TNFRSF10B	1.299788	0.00607

Figure S3: Bulk RNAseq analysis of uninfected versus infected iMg in mono-culture and IL-1a production in iAst. Related to Figures 2 and 3

(A) Ingenuity Pathway Analysis of Uninfected v Infected iMg bulk RNAseq. n=3 cell lines, Fisher's exact <0.05. Benjamini-Hochberg FDR 0.05.

(B) iAst produced IL-1a in response to 8hr exposure to IL-1b (10ng/mL), but not IL-8 (10ng/mL). n=3 cell lines, one-way ANOVA, Dunnett's post hoc analysis, ****p<0.0001, error bars represent SEM.

(C) Select inflammation related genes upregulated in iMg+HIV bulk RNA-seq. Log2fold change at least 1 and FDR <0.05.

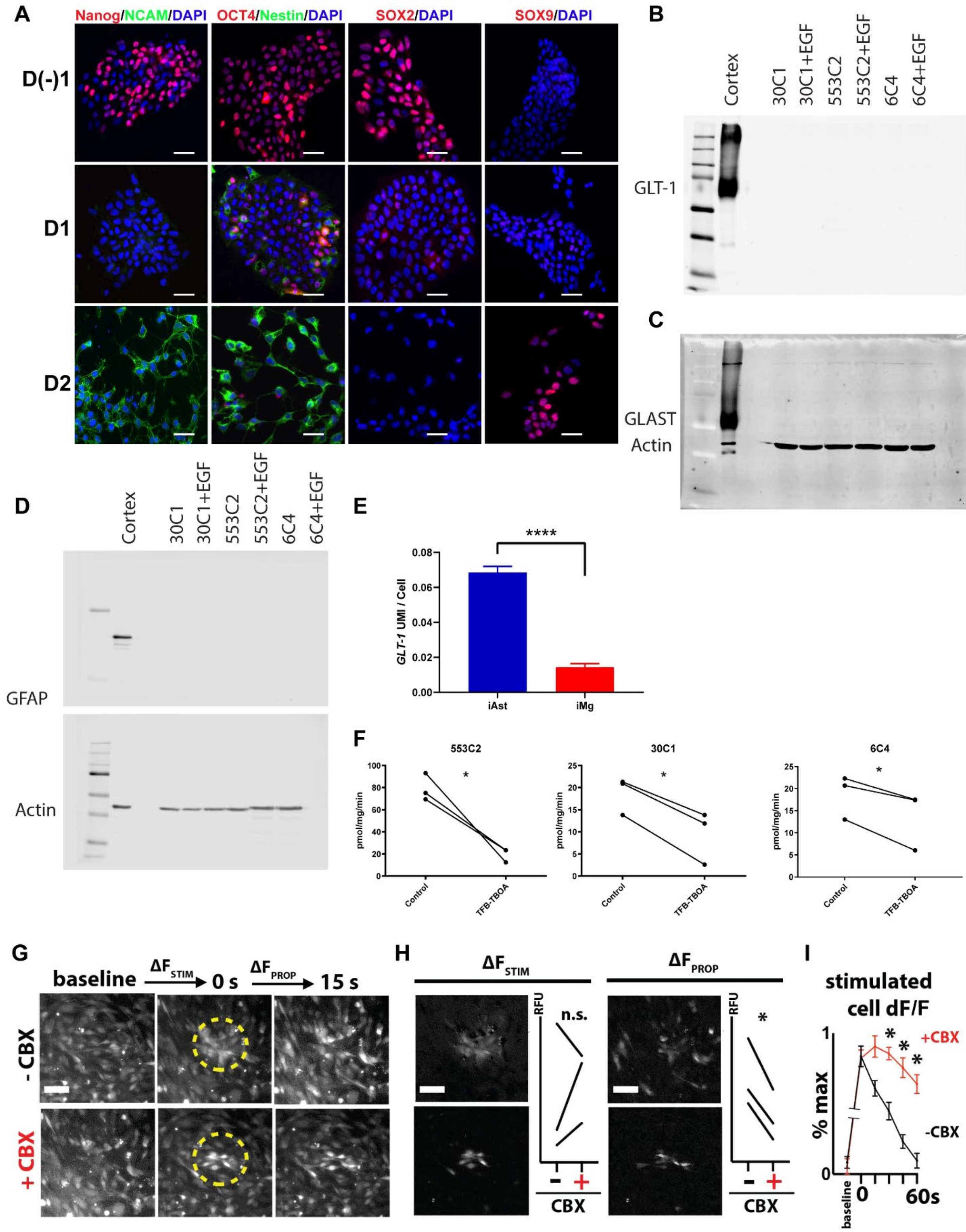
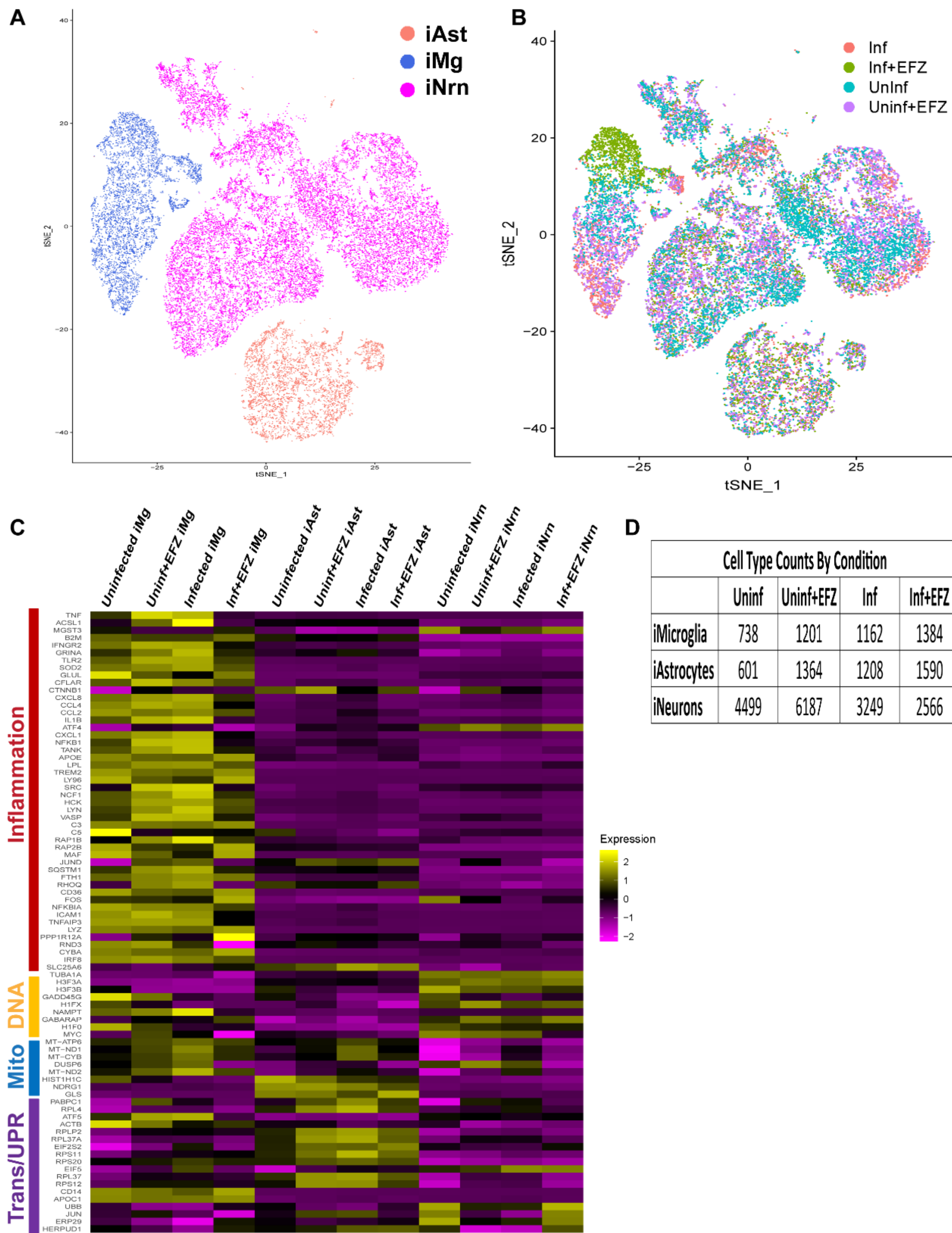


Figure S4: Characterization of iAsts. Related to Figure 3

(A) By day 2 of the NGN2 differentiation (post puromycin selection), cells express have lost pluripotency markers (Nanog, OCT4, and Sox2) and begin to express neural progenitor markers Nestin and NCAM, as well as, the astrocyte marker Sox9. Scale bar represents 25µm.
(B-D) Western blot showing iAst do not express glutamate transporter GLT-1 (B), GLAST (C), or GFAP (D) in mono-culture.
(E) scRNAseq analysis shows that, in tri-culture, iAst express *GLT-1*. iAst n=4,763; iMg n=4,485. two-tailed t test, ****p<0.0001, error bars represent SEM.
(F) Glutamate uptake assay showing uptake by three iAst lines and impaired uptake with exposure to glutamate transporter inhibitor TFB-TBOA. n=3 independent experiments for each cell line. Two-tailed paired t test. *p<0.05.
(G) Representative images of trans-cellular calcium propagation in iAst mono-culture ± 100µM carbenoxolone (CBX). ΔFstim is the change in fluorescence from baseline to stimulation within the central region. Dotted circle denotes initial stimulation area. ΔFprop is the change in the surrounding region's fluorescence between 0 and 15 seconds. Center region radius = 100µm; Surround region radius = 200 µm. Scale bar represents 50µm.
(H) Representative ΔFstim and ΔFprop images, along with summary graphs that include all three lines. CBX does not significantly influence connexin-independent ΔFstim, while CBX does significantly reduce fluorescence propagation (ΔFprop). RFU=relative fluorescent units. n=3 cell lines (3 technical replicates per line). Two-tailed paired t test. n.s.= not significant, *p<0.05.
(I) Change in dF/F of individual cells that were mechanically stimulated. CBX significantly decreases decay rate of dF/F. n=3 cell lines (3 technical replicates per line). Two-way ANOVA, Sidak's post hoc analysis, error bars represent SEM. *p<0.05.



Cell Type Counts By Condition				
	Uninf	Uninf+EFZ	Inf	Inf+EFZ
iMicroglia	738	1201	1162	1384
iAstrocytes	601	1364	1208	1590
iNeurons	4499	6187	3249	2566

Figure S5: iMg exhibit the largest inflammatory response by gene expression among the three cell types in tri-culture. Related to Figures 4 and 5

(A) t-SNE plot of all cells from all conditions excluding the undesignated cluster.

(B) t-SNE from (A) broken down by condition.

(C) Heatmap of inflammation, DNA accessibility, mitochondria, and translation/UPR related genes in iAst, iNrn, and iMg in all four conditions: Uninf, Uninf+EFZ, Inf, Inf+EFZ.

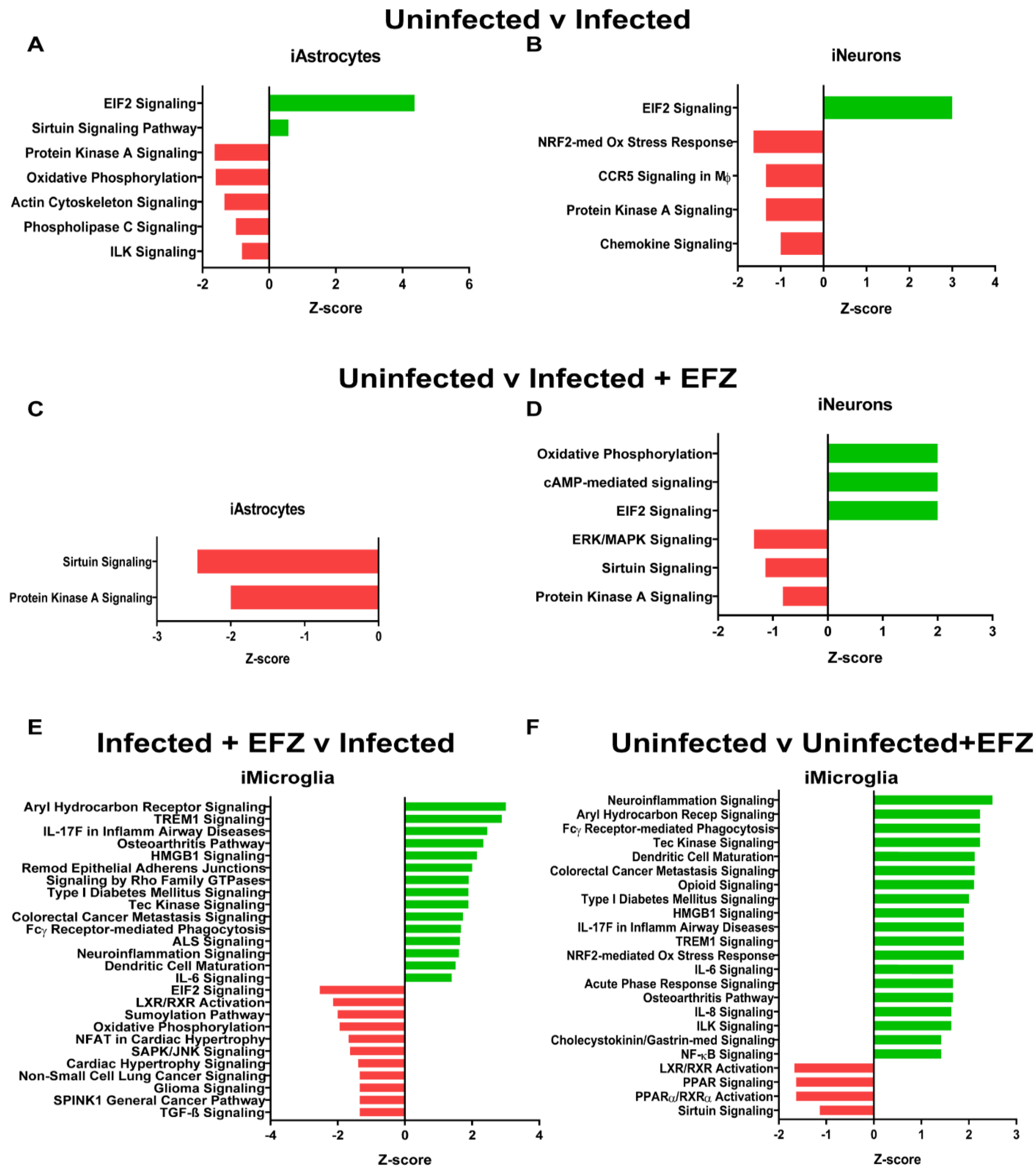


Figure S6: iAst and iNrn Ingenuity pathway analysis for Uninf v Inf, Uninf v Inf+EFZ, and Inf+EFZ v Inf. Related to Figures 5 and 6.

(A&B) Top affected pathways from Ingenuity pathway analysis of iAst (A) and iNrn (B) in the uninfected versus infected conditions. Uninfected is baseline. Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.
(C&D) Top affected pathways from Ingenuity pathway analysis of iAst (C) and iNrn (D) in the uninfected versus infected + EFZ conditions. Uninfected is baseline. Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.
(E&F) Top affected pathways from Ingenuity pathway analysis of Inf+EFZ v Inf iMg (E) and Uninf v Uninf+EFZ iMg (F) Inf+EFZ is baseline in (E) and Uninf is baseline in (F). Benjamini-Hochberg FDR 0.05, Fisher's exact <0.05, z-score cutoff ± 0.5.

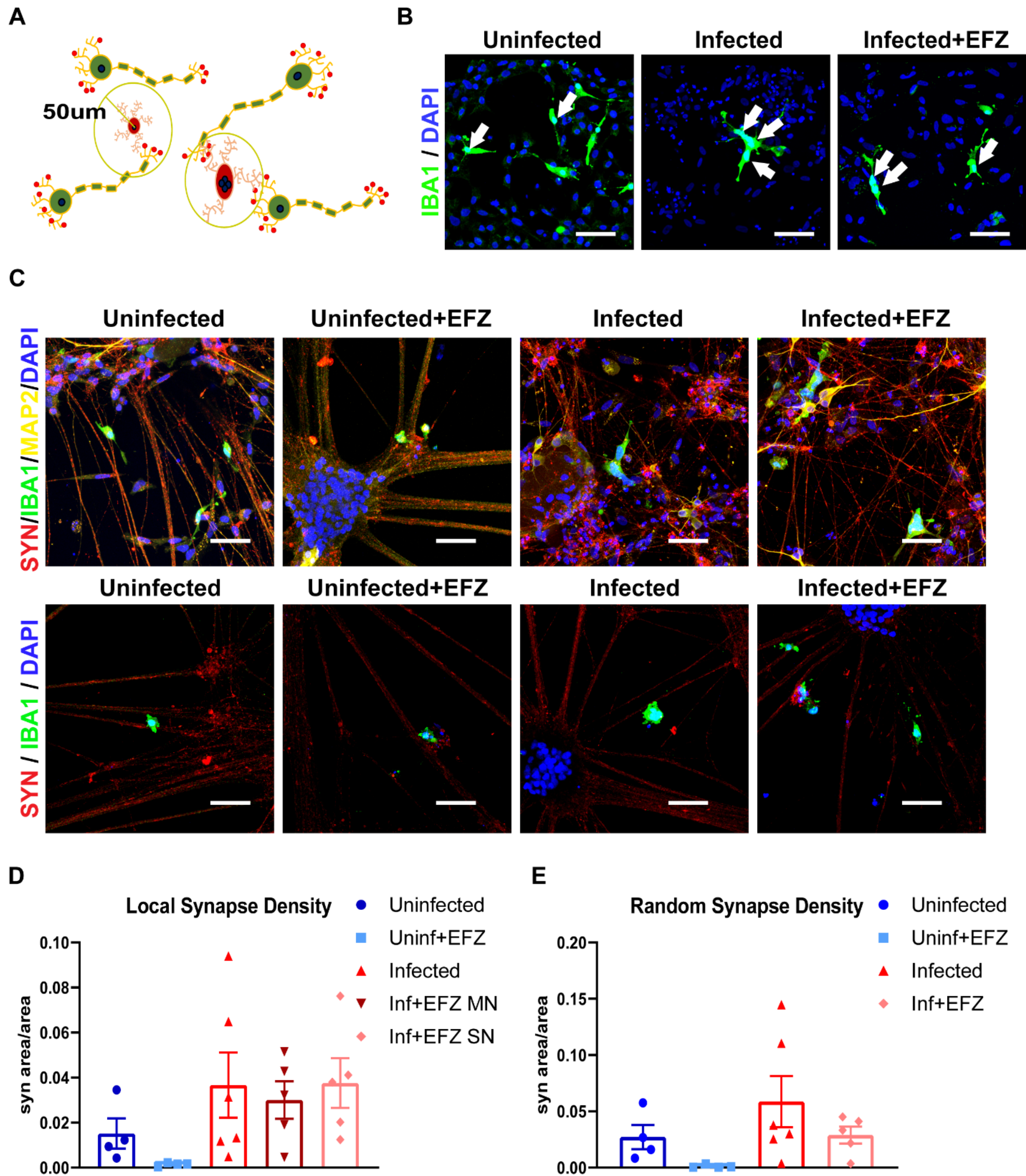


Figure S7: No change in synaptic density local to microglia or throughout the culture. Related to Figure 6.

(A) Cartoon representation of synaptic density analysis

(B) Immunostaining of IBA1+ (green) iMg in Uninf, Inf, and Inf+EFZ tri-cultures depicting multi-nucleated iMg in the infected condition and multi- and single-nucleated iMg in the Inf+EFZ condition. Scale bar represents 50 µm.

(C) Representative images for overall health and analysis of local synapse density measured by density of synaptophysin+ (red) staining within a 50um radius of IBA1+ (green) iMg. MAP2+ neurons (yellow) and DAPI (blue). Scale bar represents 50 µm.

(D) No significant difference in local synapse density across conditions compared to control. n= 4 independent differentiations of WT6 (Uninf=4, Uninf+EFZ=4, Inf=6, Inf+EFZ=5). One-way ANOVA, Dunnett's post hoc analysis, error bars represent SEM.

(E) No significant difference in random synapse density across conditions compared to control. One-way ANOVA, Dunnett's post hoc analysis, error bars represent SEM.

Supplemental Experimental Procedures

Human induced pluripotent stem cell lines
HiPSC lines for the iNrn (6c4) and iAst (6c4, 553c2, and 30c1) were generously received from Herbert M. Lachman, MD., Einstein University, Bronx, New York. All lines were reprogrammed with episomal vectors. All lines trained over to a feeder-free system with Stem MACS iPS-Brew XF media (Miltenyi Biotec 130-104-368). Lines were tested for mycoplasma using Lookout mycoplasma PCR detection kit (Sigma MP0035). iPSC lines for the iMg (CHOPWT4, CHOPWT6, CHOPWT10, CHOPWT15) were cultured by the Human Pluripotent Stem Cell Core (CHOP). All lines were reprogrammed with Sendai viral vectors (Cytotune 2.0, Invitrogen).

Detailed CMP differentiation protocol
As described in (Paluru et al., 2014), differentiation began once iPSCs reached ~70% confluency. All three base media were supplemented with 2 mM glutamine, 50µg/ml ascorbic acid (Sigma), 150µg/ml transferrin (Roche Diagnostics), and 400 µM monothioglycerol (MTG) (Sigma). The base media were RPMI (Invitrogen), StemPro-34 (SP-34) (Invitrogen), and serum free differentiation (SFD) media. The cultures were maintained at 37 °C in an environment of 5% CO2,5%O2, and 90%N2. Days 0–1 RPMI with 5 ng/ml BMP4 and 50 ng/ml VEGF, and 1µM CHiR (Tocris 4423); Day 2 RPMI with 5 ng/ml BMP4, 50 ng/ml VEGF and 20 ng/ml bFGF; Day 3 SP34 with 5 ng/ml BMP4,50 ng/ml VEGF and 20 ng/ml bFGF; Days 4–5SP34 with 15 ng/ml VEGF and 5 ng/ml bFGF; Day 6 SFD with 50 ng/ml VEGF, 100 ng/ml bFGF, 50 ng/ml SCF, and 25 ng/ml Flt3L;Days 7–9 SFD with 50 ng/ml VEGF, 100 ng/ml bFGF, 50 ng/ml SCF, 25 ng/ml Flt3L. Fresh media mixes (2 ml/well) were added each day. By day 6, media was increased to 4 ml/well. From days 7 to 9, single cells shed off the adherent layer into the medium and were collected. CMPs were frozen at 1-3 million cells per vial in 90%FBS and 10% DMSO.

iAstrocyte passaging
After puromycin selection on DIV1 of NGN2 differentiation, NPCs are replated on DIV2 at 100,000 cells/cm^2. Once cells reach 95% confluency, iAstrocytes were washed 2x with PBS and then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) for 5 min at 37ºC and then spun down at 1,000 rpm for 5 min and split 1:3. Cells were plated to plastic with no additional coating. This was consistent through the entire differentiation.

iAstrocyte cryopreservation
iAstrocytes were washed 2x with PBS and then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) for 5 min at 37ºC and then spun down at 1,000 rpm for 5 min. Supernatant was aspirated and cell were resuspended in 90% Sciencell Astrocyte Medium and 10% DMSO and frozen at 1 million cells per vial.

HIV infection
iMicroglia were differentiated to D11. On D11, they were exposed to 50ng/mL of JAGO strain HIV (UPenn Center of Aids Research; CFAR) for 24 hours. Media was fully exchange after 24 hours and collected. Full media exchanges occur every 3 days for 15 days. If antiretroviral treatment was used, it was started on day 6 of infection and added with each media exchange. Efavirenz (U.S. Pharmacopeia 1234103) was used at 20nM and Darunavir (Prezista TMC114) at 4.5µM.

RNA extraction
Cells were washed twice with RT PBS, then lifted with StemPro accutase (Thermo Fisher Scientific A11105-01) and spun down at 1,500 rpm for 5 min. Fresh cells were processed through Qiashredder (Qiagen 79654) and RNeasy mini kit (Qiagen 74104) and frozen at -80ºC.

Immunofluorescence
Cells were washed twice with PBS, then fixed in 4% PFA (VWR TCP0018) for 15 min at RT. Cells were washed 3x in PBS for 5 min at RT before being stored in PBS at 4°C. Cells are blocked in 5% BSA (Sigma A9418) and 0.1% Triton X-100 (sigma X100) for 1 hr at RT. Sections use 0.3% Triton-X 100 in blocking buffer. Primary and secondary antibodies were diluted in blocking buffer. Cells were incubated in primary antibodies overnight at 4°C. Cells were washed 3x 5min each at RT in PBS-T (0.1% Tween20) (Sigma P9416). Secondary was performed at RT in the dark for 1 hour. Cells were washed 3x 5min each at RT in PBS-T, then mounted with Prolong gold antifade (Life Technologies P36930).

TMEM119 and DA1E IgG control antibodies required antigen retrieval. Before blocking, cells were heated to 100° C for 4 minutes in 10mM sodium citrate/0.05% tween20, pH 6, then incubated for 15 minutes at RT. After 15 minute incubation, cells were blocked as normal.

Antibodies used for immunofluorescence: Chicken anti-MAP2 (Abcam ab5392, RRID:AB_2138153, 1:500); Mouse anti-PSD-95 clone K28/43 (NeuroMab 75-028, RRID:AB_2292909, 1:500); Mouse anti-Synaptophysin clone SY38 (Millipore MAB5258-20UG, RRID:AB_95185, 1:250); Mouse anti-Nestin clone 10C2 (Millipore MAB5326, RRID:AB_11211837, 1:200); Rabbit anti-Thrombospondin-1 (Abcam ab85762, RRID:AB_10674322, 1:250); Mouse anti-Glutamine Synthetase (Millipore MAB302, RRID:AB_2110656, 1:500); Rabbit anti-SOX9 (Abcam ab185230, RRID:AB_2715497, 1:250); Rabbit anti-CX3CR1 (Abcam ab8021, RRID:AB_306203, 1:500); Rabbit anti-TMEM119 (Abcam ab185333, RRID:AB_2687894, 1:200); Rabbit anti-IBA1 (Wako 019-19741, RRID:AB_839504, 1:500); Rabbit anti-P2RY12 (Alomone Labs APR-020, RRID:AB_11121048, 1:100); Rat anti-LAMP1 (Abcam ab25245, RRID:AB_449893, 1:500); Mouse anti- HIV1 p24 [39/5.4A] (Abcam ab9071, RRID:AB_306981, 1:500); Rabbit anti-Human Nanog (Cell Signaling Technology 3580, RRID:AB_2150399, 1:800); Mouse anti-NCAM Clone 2-2b (Millipore MAB5324, RRID:AB_95211, 1:250); Rabbit anti-OCT-4 (Cell Signaling Technology 2750, RRID:AB_823583, 1:200); Rabbit anti-SOX2 (Millipore AB5603, RRID:AB_2286686, 1:100); Rabbit anti-CCR5 (Thermo Fisher Scientific pa5-29011, RRID:AB_2546487, 1:500); Mouse anti-GLAST (Miltenyi Biotec 130-095-822, RRID:AB_10829302, 1:50); Mouse anti-GFAP (Sigma-Aldrich SAB1405864, RRID:AB_10739114, 1:10,000); Mouse anti-Beta-Actin (Cell Signaling Technology 3700, RRID:AB_2242334, 1:10,000); Rabbit anti-EAAC1 (Santa Cruz Biotechnology sc-25658, RRID:AB_2190727, 1:50); Rabbit GLT-1 (Jeff Rothstein Lab 1:5,000); Rabbit anti-DA1E IgG XP isotype control (Cell Signaling Technology 3900S, RRID: AB_1550038, 1:200); Goat anti-mouse IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific A-11029, RRID:AB_138404, 1:500); Goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific A-11034, RRID:AB_2576217, 1:500); Goat anti-mouse IgG (H+L) Alexa Fluor 568 (Thermo Fisher Scientific A-11004, RRID:AB_2534072, 1:500); Goat anti-rabbit IgG (H+L) Alexa Fluor 568 (Thermo Fisher Scientific A-11036, RRID:AB_10563566, 1:500); Goat anti-rat IgG (H+L) Alexa Fluor 568 (Molecular Probes A-11077, RRID:AB_141874, 1:500); Goat anti-rat IgG (H+L) Alexa Fluor 680 (Molecular Probes A-21096, RRID:AB_141554, 1:500); Goat anti-Chicken IgY (H+L) DyLight 680 (Thermo Fisher Scientific SA5-10074, RRID:AB_2556654, 1:500)

iAstrocyte/ iMicroglia immunofluorescence counting

Images were obtained with a Nikon eclipse N1 scope equipped with LED-based epifluorescence. The optical fractionator workflow mode of Stereo Investigator 64 bit was then used to generate random areas of the wells to image. 3-5 images were obtained per well. Images were then transferred to image J where the channels were manually merged. After merging images, the "cell count" plugin of imageJ was used to manually quantify the total number of DAPI (+) cells, and the number of co-labeled DAPI+ astrocyte marker(+) or microglia marker (+) cells. Cells were manually counted positive by higher intensity fluorescence over negative secondary control. In the P24 staining experiments, multinucleated cells were counted by the number of DAPI+ nuclei per cell mass, as these were initially multiple cells that became infected and went through syncytia.

Local Synapse Density

Images were analyzed on ImageJ. The iMicroglia were outlined and had a 50µm radius circle drawn from the center of the cell with ROI manager. The total synapse density was measured using the analyze particles plugin. The plugin was used for the ROI of the cell and the surrounding circle. The area of the cell and the area of the particles were subtracted from the total area of the circle and the total particles. The remaining total area of the particles was divided by the remaining total area of the circle to calculate local synapse density.

iAstrocyte Glutamate Uptake

iAst plated in 12-well dishes were placed in a 37°C waterbath and rinsed twice with 1ml of either sodium or choline containing buffer (5mM Tris Base, 10mM HEPES, 2.5mM KCl, 1.2mM CaCl2 2H2O, 1. mM MgCl 6H2O, 1.2mM K2HPO4, 10mM Dextrose and 140mM NaCl or 140 mM choine chloride). The cells were then incubated with 0.5µM [3H]-Glutamate for 5 min in the absence or presence of 3mM TFB-TBOA (Tocris; cat #2532) for 5 minutes. Assays were ceased with the addition of 1 mL 4°C choline-containing buffer then solubilized in 1 mL of 0.1 N sodium hydroxide. An aliquot (500 µl) was combined with 5 ml of EcoLite (MP Biomedicals; cat #SKU 0188247501) and radioactivity was measured using a Beckman scintillation counter. Na+-dependent transport was calculated as the

difference in radioactivity in the presence or absence of sodium and normalized to the amount of protein per well (Lowry protein assay).

Calcium wave propagation assay

iAstrocytes were tested for gap junction-dependent calcium wave propagation similarly to (Fujii, Maekawa, & Morita, 2017). iAstrocytes were grown on Matrigel (Corning 354230) (1:25 DMEM) coated glass coverslips at 90,000cells/cm^2 and grown for one week in Astrocyte media (Sciencell 1801). Cells were washed 2x with RT HBSS (Corning MT21023CV) and then incubated in HBSS with 4µM Fluo-4 AM (Thermo-Fisher Scientific F14201) for 30min at 37° C. Cells were then washed 3x with HBSS and then left in in HBSS for imaging. Cells treated with carbenoxolone, a gap junction blocker, were incubated in HBSS with 100µM carbenoxolone for 10 minutes before imaging. All epifluorescence images were acquired with an exposure of 500ms under 10x magnification using a Nikon eclipse Ti-U microscope. The first baseline image was acquired prior to mechanical stimulation of cells with a 3-5MOhm borosilicate pipette pulled with a Sutter P-97 Micropipette Puller. Pipettes were slowly advanced toward individual cells using a micromanipulator. t=0 images were captured immediately following visual confirmation of cell stimulation. Images were acquired every subsequent 15 seconds for 60 seconds.

Calcium wave analysis

The time-series of six images were combined into a virtual stack of spatially registered images using the ImageJ plugin Turboreg (rigid body translation). Both ΔFsti and ΔFprop were calculated using regional changes in fluorescence. ΔFstim was defined as change in total fluorescence intensity from baseline to t=0sec within the Center/Stimulation Region (within 100um of stimulation point). ΔFprop defined as change in total fluorescence intensity from t=0sec to t=15sec within the Surround Region (the adjacent outer-ring region extending 100um from the Center Region). Because mechanical stimulation of the cells with the micromanipulator increased risk of shifting the imaging plane, ΔFprop for the surrounding region was defined as F(t=15) – F(t=0).

To determine the rate of efflux from the initially stimulated cells, an average dF/F trace was calculated for each replicate trial. The technical replicates were then averaged to produce one dF/F trace per cell line. For each replicate experiment, individual dF/F cell traces (dF/F= (F-F0)/F0 = (F-F(t=0)) / F(t=0)) were calculated and averaged to determine each replicate's average dF/F trace. ROIs for individual cells were determined using ImageJ's magic wand package for semi-automated segmentation of maximum Z-projections for each stack.

Bulk RNA-seq

RNA was extracted using previously described with RNeasy mini kit (Qiagen 74104) and frozen at -80°C. Samples were sent to the Center for Applied Genomics (CAG) for sequencing. In short, the Illumina TruSeq Stranded Total RNA library kit (Illumina RS-122-2201) for RNA-seq was utilized for preparation of the libraries for sequencing. Libraries were produced using liquid handler automation with the PerkinElmer Sciclone instrument. This procedure started with a ribosomal RNA (rRNA) depletion step. The depletion step uses target-specific oligos with specialized rRNA removal beads to remove both cytoplasmic and mitochondrial rRNA from the total RNA. Following this purification, the RNA was fragmented using a brief, high-temperature incubation. The fragmented RNA was then reverse transcribed into first-strand cDNA using reverse transcriptase and random primers. Second strand cDNA was generated using DNA Polymerase which was then used in a standard TruSeq Illumina-adapter based library preparation. Library preparation consisted of four main steps: unique adapter-indexes were ligated to the RNA fragments, AmpureXP bead purification occurred to remove small library fragments, the libraries were enriched and amplified using PCR, and the libraries underwent a final purification using AmpureXP beads. Upon completion, library quality was assessed using an automated electrophoresis instrument, the PerkinElmer Labchip GX Touch, and qPCR using the Kapa Library Quantification Kit and Viia7 real-time PCR instrument. Libraries were diluted to the appropriate sequencer loading concentration and pooled accordingly to allow for the desired sequencing depth. RNA libraries were sequenced in one lane of the Illumina HiSeq2500 sequencer using the High Output v4 chemistry and paired-end sequencing (2x100bp).

Single Cell RNA-seq cell preparation

Cells were incubated in 0.25% Trypsin+EDTA at 37°C for 8 minutes, put through a cell strainer, and spun down in ice-cold PBS at 4°C for 5 min at 1500 RPM. Cells were resuspended in ice-cold DPBS without Mg^{2+} and Ca^{2+}+0.04% BSA. Up to 20,000 cells were sent for sequencing per sample. Samples were sent to CAG for sequencing.

Bulk RNA-seq analysis

RNA-seq reads were demultiplexed using the DRAGEN genome pipeline (Goyal et al., 2017). FASTQ files were aligned to hg19 reference using the STAR (v.2.6.1) (Dobin et al., 2013) aligner with default settings. Generated BAM files were read into the R statistical computing environment. Gene counts were obtained using the GenomicAlignments package. Differential expression analysis was performed using the R/Bioconductor package DESeq2 which uses a negative binomial model (Love, Huber, & Anders, 2014). Analysis was performed using standard parameters with the independent filtering function enabled to filter genes with low mean normalized counts. The Benjamini-Hochberg adjustment was used to estimate the false discovery rate (Padj) and correct for multiple testing. Genes were then analyzed using the Ingenuity IPA software (QIAGEN Inc.). Additional published RNA-seq data was utilized for comparative analysis from project accession SRP092075 (Abud et al., 2017). Datasets were obtained and converted to fastq format using the Sequence Read Archive (SRA) tool provided by NCBI. Fastq-formatted data was analyzed similarly to the bulk RNA-seq samples using the DRAGEN pipeline and integrated into the experimental R data object.

Single Cell RNA-seq analysis

Next-generation sequencing libraries were prepared using the 10x Genomics Chromium Single Cell 3' Reagent kit v2 per manufacturer's instructions. Libraries were uniquely indexed using the Chromium i7 Sample Index Kit, pooled, and sequenced on an Illumina Hiseq sequencer in a paired-end, single indexing run. Sequencing for each library targeted 20,000 mean reads per cell. We had a mean of 39,227 reads per cell post-normalization with 2,165 median genes per cell. Data was then processed using the Cellranger pipeline (10x genomics, v.3.0.2) for demultiplexing and alignment of sequencing reads to the GRCh38 transcriptome and creation of feature-barcode matrices. Individual single cell RNAseq libraries were aggregated using the cellranger aggr pipeline. Libraries were normalized for sequencing depth across all libraries during aggregation. Secondary analysis on the aggregated feature barcode matrix was performed using the Seurat package (v.3.0) within the R computing environment. Briefly, cells expressing less than 200 or more than 5000 genes were excluded from further analysis. Additionally, cells expressing >20% mitochondrial genes were excluded from the dataset. Log normalization and scaling of features in the dataset was performed prior to principal component dimensionality reduction, clustering, and visualization using tSNE. Cell types were identified using expression of canonical cell markers in microglia (AIF1, SPI1, CD4), neurons (MAP2, SYN1), and astrocytes (THBS1, SOX9). Differentially expressed genes and identification of cluster or cell type specific markers were identified using a wilcoxon rank sum test between two groups. P-value adjustment was performed using bonferroni correction based on total number of genes in the aggregated dataset. Genes were then analyzed using the Ingenuity IPA software (QIAGEN Inc.).

qRT-PCR

RNA was extracted by RNeasy mini kit (Qiagen 74104). cDNA was generated using SuperScript VILO Master mix (Thermo Fisher Scientific 11755050). RNA expression was measured using Taqman probes (Thermo Fisher Scientific) for *CX3CR1* (Hs01922583_s1), *P2RY12* (Hs01881698_s1), *TMEM119* (Hs01938722_u1), *THBS1* (Hs00962908_m1), and *GAPDH* (Hs02786624_g1). 30ng of cDNA was used per well, with three technical replicates per probe per sample. qRT-PCR is run on an Applied Biosystems 7900HT Fast Real-Time PCR System. All expression levels were normalized to *GAPDH* expression.

Reverse transcriptase activity

10 µl per well of supernatant was placed into a 96-well microtiter plate to be analyzed for RT activity. 50 µl of RT cocktail was added per well into the 96-well microtiter plate. RT cocktail consists of: 50mM Tris (Amresco J837) pH 7.8, 75mM KCl (Ambion 9610), 2mM Dithiothreitol (DTT) (Sigma D0632), 5mM Magnesium Chloride (Ambion 9530G), 5ug/mL Polyadenylic acid (GE Healthcare 27-4110-01), 2.5ug/mL pd (t)12-18 (Oligo dT) (USB Corporation #19817), 0.05% NP-40 (Calbiochem 492016), 10 µCi/ml Thymidine 5'-triphosphate, ALPHA-[32P] / [32P] TTP (Perkin Elmer BLU005A250MC). Samples were incubated at 37° C overnight. 30 µl of RT reaction mixture was placed onto pre-marked DE81 paper (Whatman 3658915) and air dried for 15 min at RT. Paper was washed 4x, 5 min each with 2x SSC (Roche-Apply science 11 666 681 001) by submerging in a tray on a rotating platform. Paper was then washed 1x, 1 min in 100% ethanol and air dried in an oven at 80-100° C (25-30 min). Each paper sample was placed into a scintillation vial. 5 ml Betaflour (National Diagnostics LS-151) was added to each scintillation vial. 32P was counted on a scintillation counter, yielding CPM.

PCR for CCR5Δ32 mutation

DNA was isolated with DNeasy blood and tissue kit (Qiagen 69504). DNA Oligos were generated by IDT. Forward primer: 5' – CAAAAAGAAGGTCTTCATTACACC – 3'. Reverse primer: 5' –

CCTGTGCCTCTTCTTCTCATTTCG – 3'. Primers were reconstituted to 100uM. Mastermix consists of 10uL KAPA PCR buffer, 7uL H20, 1uL 10uM forward primer, 1uL 10uM reverse primer. 1uL DNA added separately. 1.5% agarose gel was run for 1hour at 120v. The gel was imaged on a Biorad Universal Hood II Gel Doc System.

Western Blot

iAst plated in 12-well dishes were rinsed twice with PBS containing 0.1 mM Ca^{2+} and 1.0 mM Mg^{2+} (PBS Ca^{2+}/Mg^{2+}) then lysed in 200ul of radioimmunoprecipitation (RIPA) buffer for 1 hour while rotating on a shaker at 4ºC. Cortical and hippocampal tissue was harvested from adult C57BL/6 mice and solubilized in 5 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% SDS, containing protease and phosphatase inhibitors, including 1 mg/ml leupeptin, 250 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mM iodoacetamide, 10mM NaF, 30mM Na pyrophosphate, 1mM Na^3VO^4). All lysates were centrifuged at 17,000g for 20 min to remove cellular debris and nuclei. The supernatants were analyzed for total protein using the Pierce protein assay kit according to the manufacturer's instructions. Lysates are diluted 1:1 with 2X Laemmli buffer and either boiled at 95ºC for 5 min (GFAP, Actin) or kept at 25ºC for 45 minutes (GLAST, GLT-1, EAAC1). iAst lysates (20ug) or cortical/hippocampal lysates (5ug) were then resolved by SDS PAGE using 10% BioRad minigels and transferred to Immobilon-FL membranes (Millipore Cat# IPFL00010). Membranes were incubated with blocking buffer (1% non-fat dry milk in TBS-T) for 1 hour at 25ºC prior to probing with primary antibodies diluted in blocking buffer overnight at 4ºC: rabbit anti-GLT-1 (C-terminal directed 1:5,000; Rothstein et al 1994), mouse anti-GLAST (Miltenyi Biotec Cat# 130-095-822, 1:50), rabbit anti-EAAC1 (Santa Cruz Cat# SC-25658, 1:50), mouse anti-GFAP (Cell Signaling Cat#3670S, 1:1,000), and mouse anti-Actin (1:10,000 dilution, Cell Signaling, Cat# 3700S). Membranes were washed with blocking buffer 3 times for 10 min at 25ºC. After the washes, membranes were probed with anti-mouse or anti-rabbit fluorescently conjugated secondary antibodies (LI-COR Biosystems, 1:10,000) for 45 minutes at 25ºC. The membranes were washed 3 times for 10 minutes each then visualized using a LI-COR Odyssey.

Synaptophagocytosis analysis

Confocal images of microglia were analyzed in IMARIS software. IBA1 labeling for the microglia was surfaced, as well as synaptophysin staining. Total volume was taken for the microglia and the synaptophysin staining inside the cells. Total synaptophysin volume per cell was then divided by the cell volume defined by IBA1 labeling. At least 5 cells per condition per biological replicate were counted across at least 4 biological replicates. Each biological replicate is an average of the individual cells counted in that replicate. The average per condition is the average of the biological replicates.

Cytokine analysis

Supernatants were tested for 6 analytes (TNFa, IL-6, IL-8, IL-10, IL-1b, IL-1a) on a custom Human magnetic Luminex plate (R&D systems LXSAHM; run by Penn Mental Health AIDS Research Center (PMHARC)). The plate was run by the Penn Mental Health Aids Research Center on a MAGPIX powered by Luminex XMAP technology.

iAstrocyte cytokine exposure

iAstrocytes in mono-culture were exposed to IL-1b (R&D Systems 201-LB-005) or IL-8 (R&D Systems 208-IL-010) at 10ng/mL or PBS vehicle control for 8 hours. Supernatants were collected and sent for cytokine analysis.

Human astrocytes

Three separate donors for human astrocytes were obtained from Sciencell. Cells were plated on PLL-coated plates (Sigma Aldrich P6282) and grown for two weeks in Astrocyte media (Sciencell 1801) and then RNA was extracted.

Monocyte-derived macrophage differentiation

We receive donated buffy coat from New York Blood Center from three donors (D471, D446, D470). Buffy coat was diluted 1:1 with PBS (without Ca^{2+} and Mg^{2+}) (Invitrogen 14190144). 15 mLs of Ficoll (Sigma Aldrich 26878-85-8) was added to 50mL conical tubes. 25mLs of Buffy Coat/PBS was slowly layered onto the Ficoll. Samples were spun at 1200 rpm for 45 minutes with no brake. The peripheral blood mononuclear cell (PBMC) layer was removed and placed into a new 50mL conical tube, and the volume was brought up to 50mL with PBS. The PBMCs are spun at 450Xg for 10 minutes. The supernatant was discarded, and the pellet resuspended in 10mLs of Red Blood Cell Lysis buffer (Sigma Aldrich 11814389001). Cells were shaking at RT for 10 minutes. Volume was brought up to 50mLs in

PBS and spun at 450Xg for 10minutes. The supernatant was discarded, and the pellet was resuspended in DMEM with 10% FBS + Gentamicin (Thermo Fisher Scientific 15750060). The PBMCs were plated on 6-well tissue culture plate for 5 days. On day 5, a full media exchange was performed and added 10ng/mL Human GM-CSF (Gold Biotechnology 1120-03-20). A half media exchange was performed on day 7. At day 10, RNA was collected from macrophages.

RNA seq analysis Statistics

Benjamini-Hochberg procedure was performed on all bulkRNAseq and sing cell RNAseq data, with an FDR cutoff of 0.01 for bulk RNAseq and 0.05 for scRNAseq. Pathway analysis was performed with Ingenuity Pathway Analysis. All pathways had a Fisher's exact below 0.05.

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