Supplemental Information

Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures

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Supplemental Figures

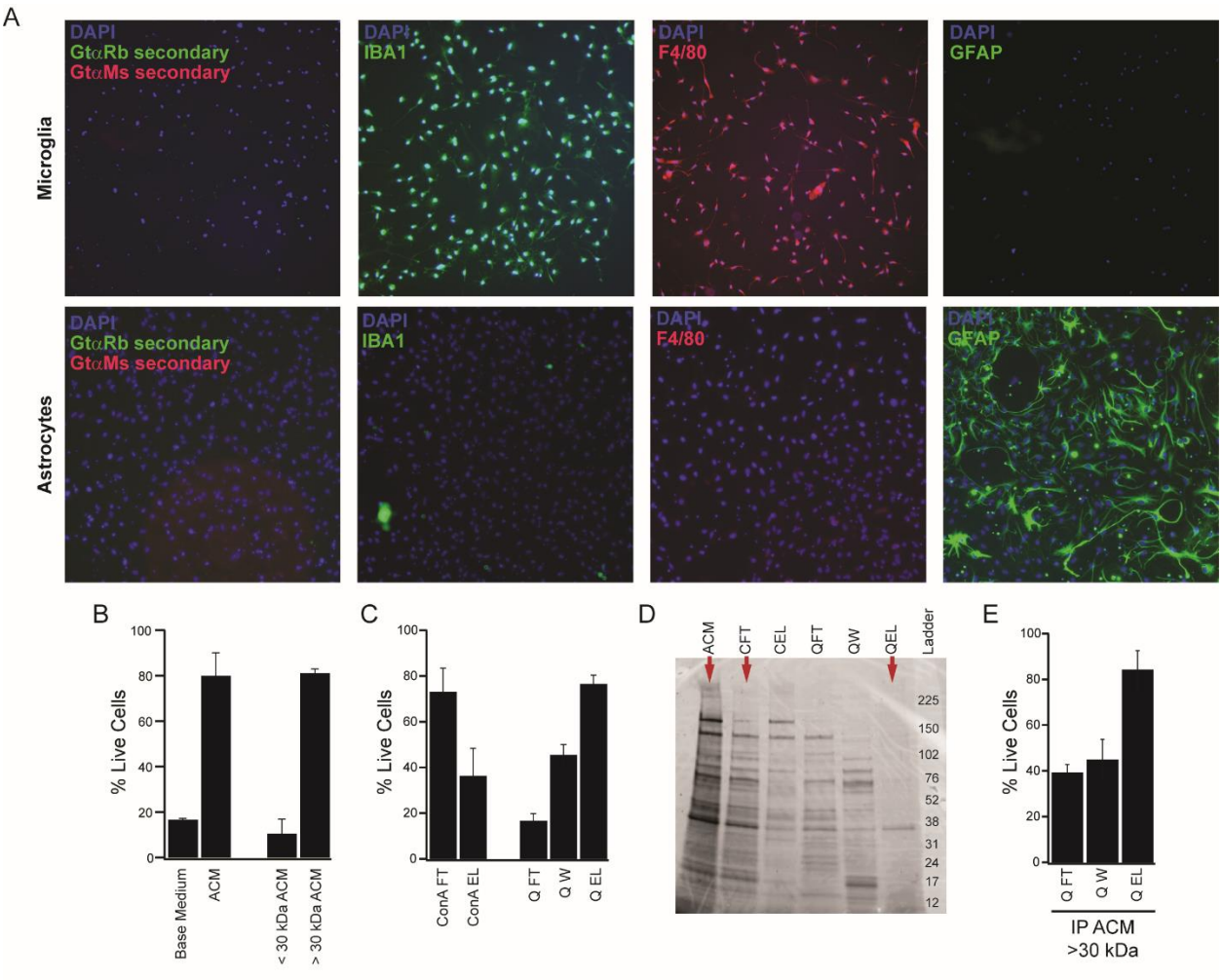

Figure S1. Identification of astrocyte-secreted microglial survival cues. *Related to Figure 1*

(A) Micrographs illustrating the purity of rat microglia (top) or astrocytes (bottom) (P8, 5 div), with macrophage-specific antibodies to Iba1 (middle) and F4/80 (right) and an astrocyte-specific antibody (GFAP) indicating high purity of microglia and astrocyte cultures. (B) Survival rates measured for P7-8 microglia after 5 days in culture showing that ACM survival activity is recovered in the retentate (> 30 kDa ACM) but not the flowthrough (< 30 kDa ACM) of a 30 kDa molecular weight cutoff centrifugal filter. (C) Survival activity of ACM fractionated over concanavalin A (conA) resin or Q-anion exchange resin. (D) SYPRO-orange stain of an SDS-PAGE gel illustrating the relative enrichment of a ~36 kDa band over several ACM fractionation steps. Fractions retaining peak survival activity are highlighted with red arrows. (E) The survival activity of ACM derived from immunopanned (IP) astrocytes and concentrated over a 30 kDa molecular weight cutoff filter behaves similarly to ACM derived from MD astrocytes.

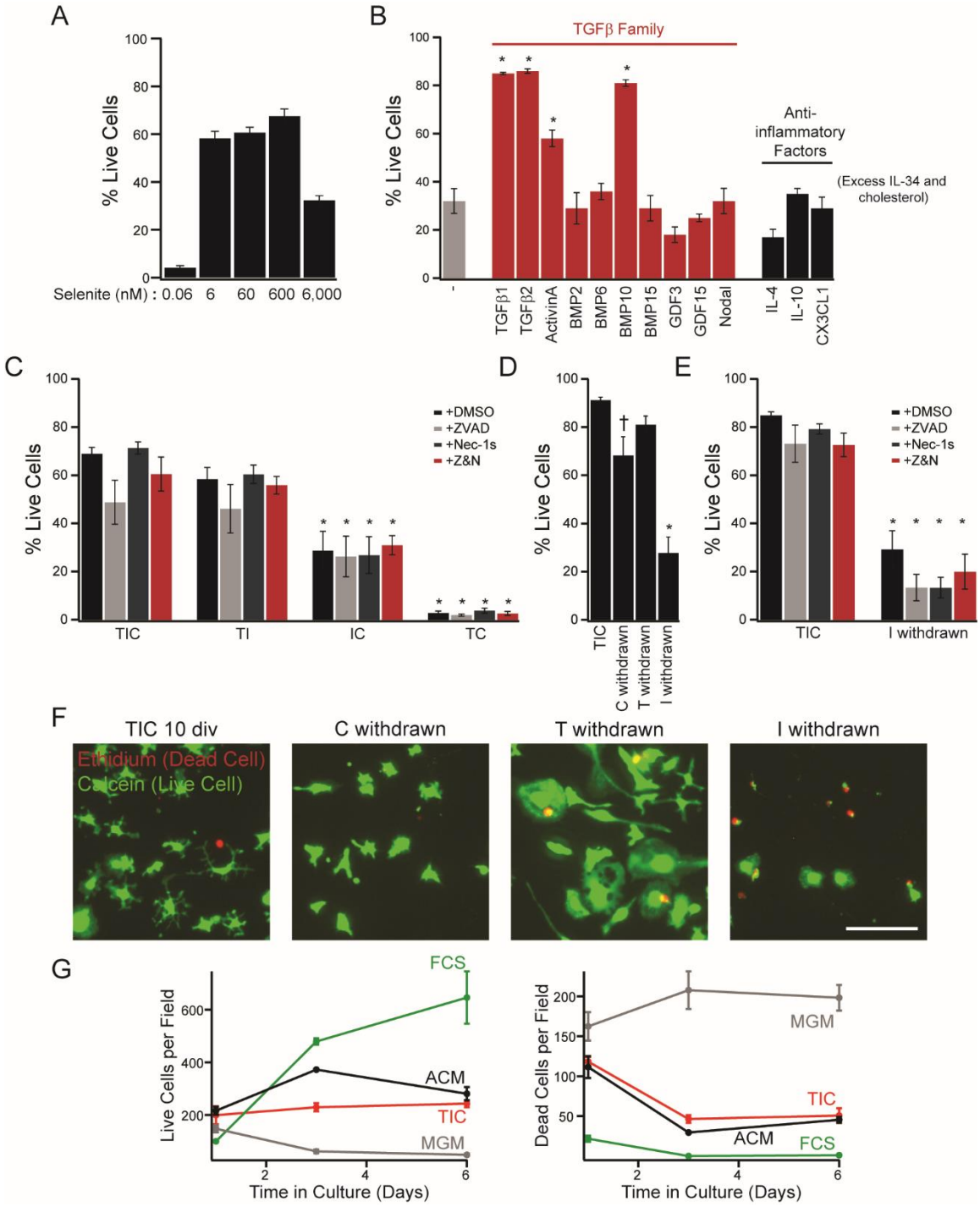
A
% Live Cells
Selenite (nM) : 0.06 6 60 600 6,000
B
TGFβ Family
% Live Cells
Anti-inflammatory Factors
(Excess IL-34 and cholesterol)
TGFβ1
TGFβ2
ActivinA
BMP2
BMP6
BMP10
BMP15
GDF3
GDF15
Nodal
IL-4
IL-10
CX3CL1
C
% Live Cells
+DMSO
+ZVAD
+Nec-1s
+Z&N
TIC
TI
IC
TC
D
% Live Cells
TIC
C withdrawn
T withdrawn
I withdrawn
E
% Live Cells
+DMSO
+ZVAD
+Nec-1s
+Z&N
TIC
I withdrawn
F
TIC 10 div
C withdrawn
T withdrawn
I withdrawn
Ethidium (Dead Cell)
Calcein (Live Cell)
G
Live Cells per Field
FCS
ACM
TIC
MGM
Time in Culture (Days)
Dead Cells per Field
MGM
TIC
ACM
FCS
Time in Culture (Days)

Figure S2. Additional features of defined-medium survival activity. *Related to Figure 2*

(A) Sodium selenite supplementation is necessary in defined-medium microglial cultures (P7, 5 div in TIC) but is toxic at high levels. (B) Survival-promoting impact of various TGF-β family members or anti-inflammatory factors in microglia (P7, 5div) cultures supplemented with IL-34 (100 ng/mL) and cholesterol (1.5 μg/mL). Factors were applied at the following concentrations: 10 ng/mL for TGF-β1 and TGF-β2; 100 ng/mL for BMPs, GDFs, Nodal, and IL-4; 250 ng/mL for Activin A; 1 μg/mL for IL-10 and CX3CL1. (C) Survival of microglia (P8-P13, 3-6 div) cultured in serum-free TIC medium or TIC lacking each individual survival factor (TI, IC, or TC). Cells were supplemented with the broad-spectrum caspase inhibitor, z-VAD-fmk (ZVAD, 10 μM, light gray), the RIPK1 inhibitor, necrostatin-1s (Nec-1s, 10 μM, dark gray), both drugs (Z&N, red), or vehicle (DMSO, black). (D-F) Microglia (P12-P14) were cultured in serum-free TIC medium for 7 days, then washed and supplied with TIC medium lacking individual TIC components for 3 additional days. (D) Survival after withdrawal of each factor. (E) Survival after IL-34 is withdrawn is not reversed by ZVAD or Nec-1s. (F) Micrographs of calcein-filled microglia illustrating that highly ramified microglial morphology requires the continued presence of all three TIC factors. (G) The total number of live cells (top) and dead cells (bottom) counted in the experiment shown in Fig. 2E, highlighting increased proliferation and clearance of dead cells in serum-containing cultures. * $P < 0.001$, † $P < 0.05$ one-way ANOVA across all groups with Dunnett's comparison to the leftmost condition on each graph.

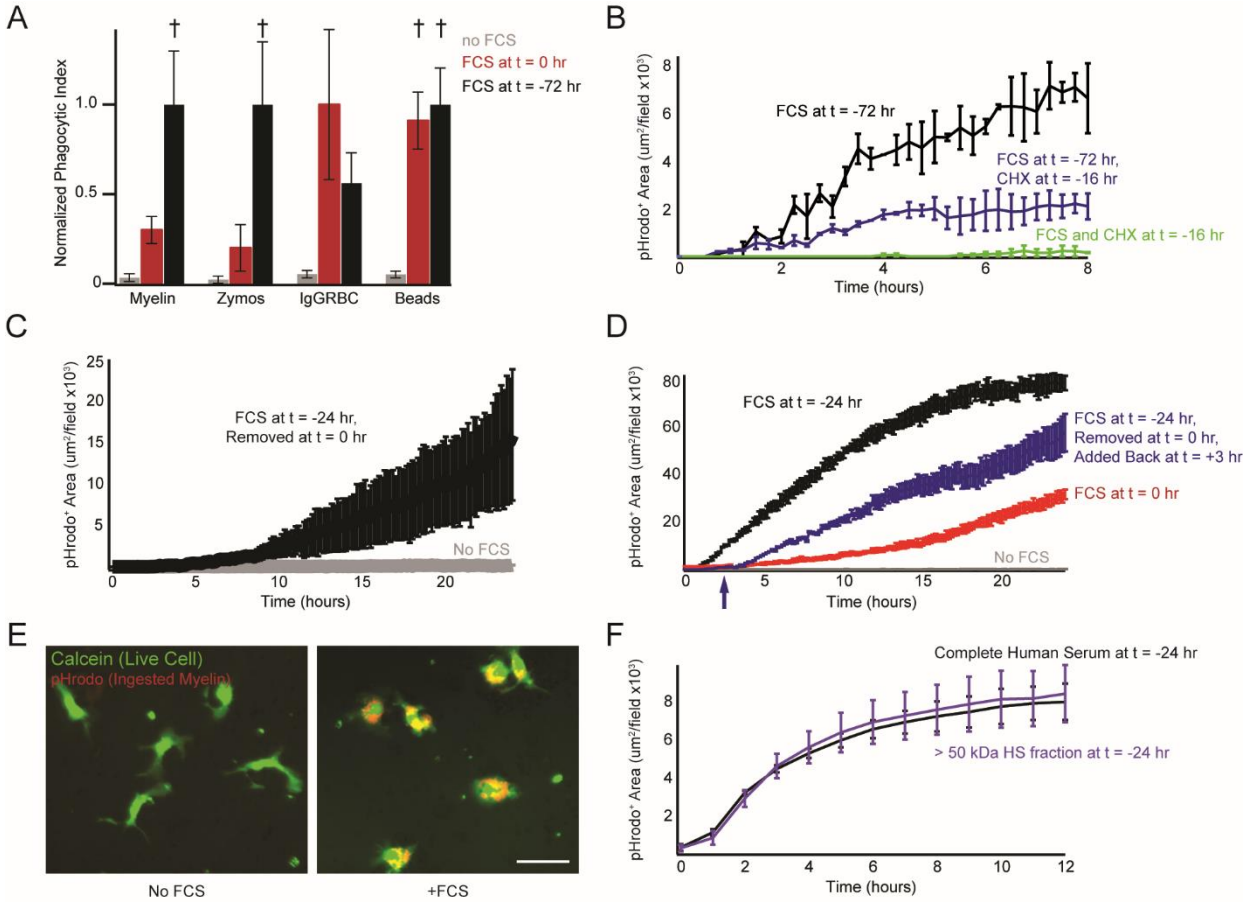

Figure S3. Serum unlocks latent phagocytic potential in microglia cultured in TIC. *Related to Figure 4*

(A) Microglial phagocytosis of multiple types of prey particles in the absence or presence of serum (FCS, 10%): myelin debris, zymosan (Zymos), IgG-opsonized erythrocytes (IgGRBC), or amine-coated polystyrene beads. Cells were initially cultured in the absence of serum, and serum was added either 3 days prior to adding prey (t = -72 hrs, black), only at the initiation of the phagocytosis assay (t = 0 hr, red), or not at all (gray). P15 rat microglia were fed at 6 div and images were collected after 20 hr of phagocytosis. (B) Microglia treated with cycloheximide (CHX, 40 μM) retain some phagocytic activity even 16 hours after CHX treatment, but only if the cells had been previously transformed by serum (blue). Cells exposed to CHX at the same time as serum exposure (green) and cells exposed to serum without CHX treatment (black) are shown for reference. (C) Comparison of myelin phagocytosis by serum-free microglia (gray) and microglia that were exposed to serum for 24 hours and then washed three times to remove serum just before the start of the assay (black). Serum-exposed cells maintain altered phagocytic capacity even after serum removal. (D) Reduced phagocytic activity in serum-exposed cells after removal of serum is immediately reversed by replacing the serum (blue), shown alongside the traces from Figure 5C for reference. (E) Micrographs of microglia (P21, 3 div) that were exposed to pHrodo-labeled myelin immediately after isolation. No pHrodo signal is observed in serum-free cultures (left) and robust myelin uptake is evident in serum-containing wells (right). (F) The phagocytosis-inducing factor from human serum (HS) is large or is associated with large particles. When applied to microglia (P10, 12 div) 24 hours before addition of myelin, human serum that was diluted 10-fold and re-concentrated over a 50 kDa MWCO centrifugal filter unit two consecutive times still induced phagocytosis to the same degree as complete human serum. * $P < 0.001$, † $P < 0.05$ one-way ANOVA with Dunnett's comparison. Scale bar in (E) is 40 μm.

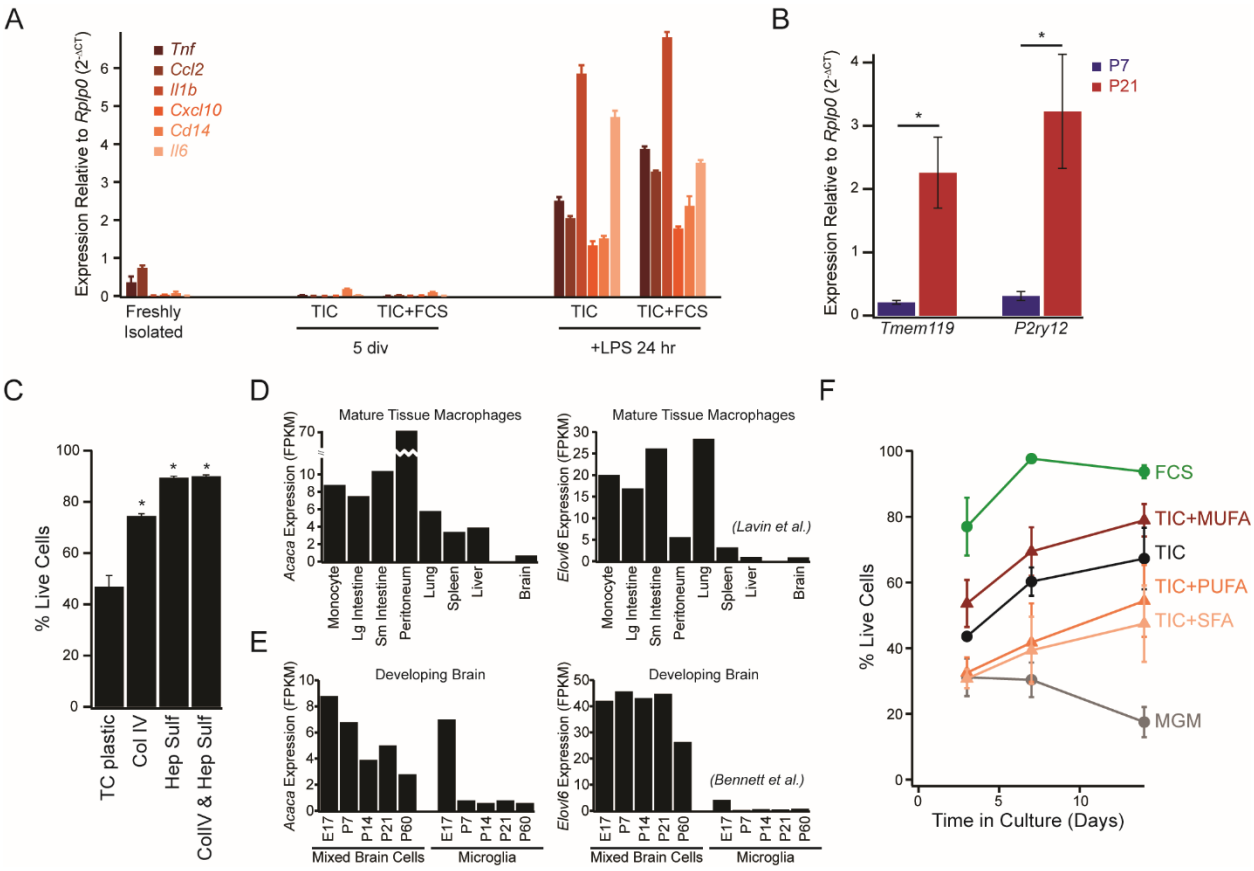

Figure S4. Mature microglia upregulate signature gene expression and require exogenous fatty acid supplementation. *Related to Figure 5*

(A) QPCR analysis of six canonical activation markers in freshly isolated or cultured microglia (P7, 5 div) with or without addition of LPS (1 ng/mL) during the final 24 hours. (B) QPCR analysis of two microglia signature genes, showing developmental upregulation between P7 and P21 by freshly isolated cells. (C) Survival of microglia (P21, 7 div) on uncoated tissue culture plastic (TC plastic) or TC plastic coated with collagen IV, heparan sulfate, or both collagen IV and heparan sulfate. Substrate coating facilitated survival of mature cells. (D) Expression of two key fatty acid biosynthesis genes *Acaca* and *Elovl6* across tissue macrophages isolated from the denoted organs according to published RNA-seq datasets (Lavin et al., 2015). Brain macrophages (mostly microglia) express low levels of both. (E) Expression of *Acaca* and *Elovl6* by mixed brain cell isolates or microglia harvested at different developmental time points according to published RNA-seq datasets (Bennett et al., 2016). Post-embryonically, microglia express low levels of both. (F) Time course of microglial survival in base medium (MGM, gray), TIC (black), TIC+10% FCS (green), or TIC with fatty acid supplementation (triangles, shades of orange). Fatty acids were added either as TIC + saturated fatty acids (SFA, 0.1 μg/mL stearic acid, 0.1 μg/mL palmitic acid, 0.001 μg/mL arachidic acid, SFA), TIC + monounsaturated fatty acids (MUFA, 0.1 μg/mL oleic acid, 0.001 μg/mL gondoic acid), or TIC + polyunsaturated fatty acids (PUFA, 0.01 μg/mL docosahexaenoic acid, 0.01 μg/mL arachidonic acid). Fatty acids were applied in proportion to their abundance in whole brain, and only the most abundant fatty acids were considered for addition. * $P < 0.001$, Student's *t*-test in (B) or one-way ANOVA with Dunnett's comparison to the TC plastic condition in (C).

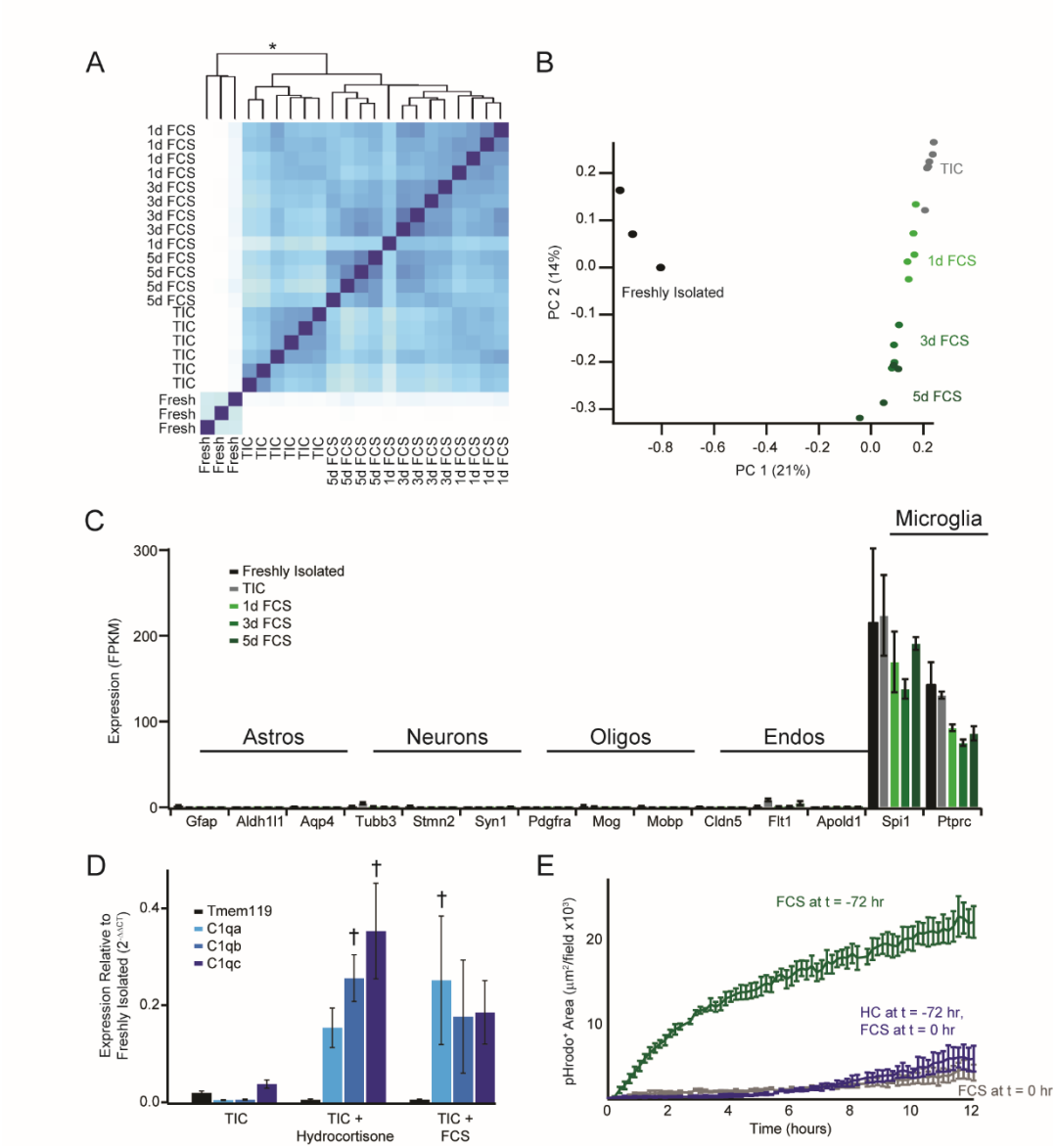

Figure S5. Analysis of microglial changes after serum exposure. *Related to Figure 5*

(A) Heatmap showing Euclidean distances between log-transformed RNA-seq replicates. Darker blue indicates stronger similarity. Freshly-isolated cells cluster apart from cultured cells. (B) Principal component analysis of RNA-seq replicates (top15% highest variance genes, normalized) showing clustering of cultured cells apart from freshly isolated cells (black) and gradual changes in culture with continued serum exposure (green). (C) Purity of sequenced microglia assed with cell-type specific marker expression. Both freshly isolated and cultured samples show minimal levels of transcripts highly-expressed by other neural cells (Astros- astrocytes, Oligos, oligodendrocytes, Endos- endothelial cells). (D) QPCR of Tmem119, C1qa, C1qb, and C1qc expression by cultured microglia (P17-21, 4-6 div in TIC) supplemented with 10% FCS or 1 μM hydrocortisone for 3 days. Either hydrocortisone or FCS stimulates C1q expression by cultured cells. (E) Hydrocortisone exposure does not promote phagocytosis of pHrodo-labeled myelin, despite inducing expression of complement components. Microglia (P21, 5div) were exposed to serum or hydrocortisone for 3 days before the assay. At the time of myelin addition, serum was supplemented to provide any additional serum-borne opsonins. Hydrocortisone (HC) treated cells (blue) behaved like untreated cells (gray), not serum-exposed cells (green). * AU $P < 0.01$ for primary branch confirmed by pvclust. † $P < 0.05$ one-way ANOVA with Dunnett's comparison to TIC alone condition.

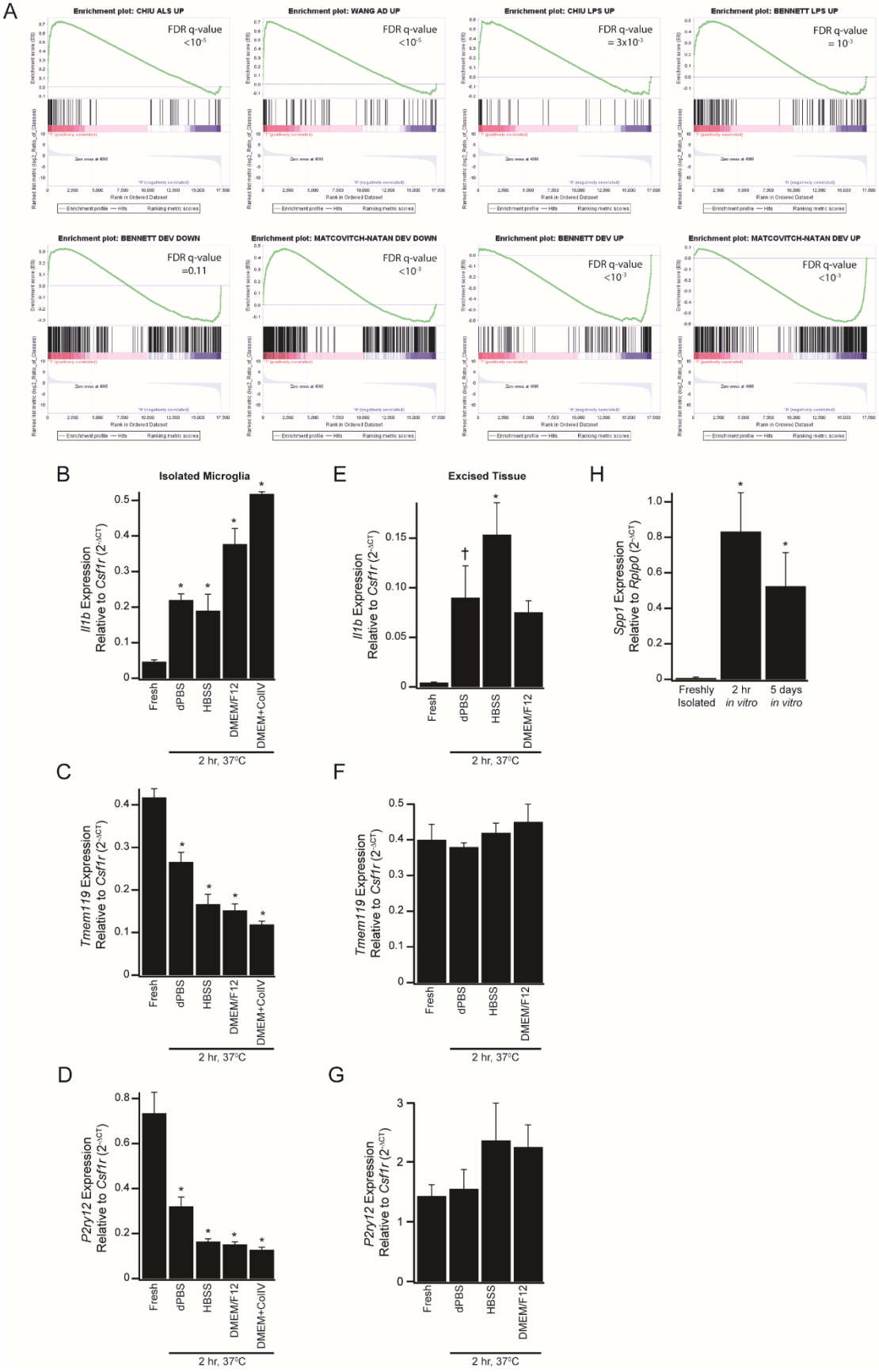
A
Enrichment plot: CHIU ALS UP
FDR q-value <10-5
Enrichment plot: WANG AD UP
FDR q-value <10-5
Enrichment plot: CHIU LPS UP
FDR q-value = 3x10-3
Enrichment plot: BENNETT LPS UP
FDR q-value = 10-3
Enrichment plot: BENNETT DEV DOWN
FDR q-value =0.11
Enrichment plot: MATCOVITCH-NATAN DEV DOWN
FDR q-value <10-3
Enrichment plot: BENNETT DEV UP
FDR q-value <10-3
Enrichment plot: MATCOVITCH-NATAN DEV UP
FDR q-value <10-3
B
Isolated Microglia
Il1b Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
DMEM+CollV
2 hr, 37°C
E
Excised Tissue
Il1b Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
2 hr, 37°C
H
Spp1 Expression Relative to Rplp0 (2-ΔCT)
Freshly Isolated
2 hr in vitro
5 days in vitro
C
Tmem119 Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
DMEM+CollV
2 hr, 37°C
F
Tmem119 Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
2 hr, 37°C
D
P2ry12 Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
DMEM+CollV
2 hr, 37°C
G
P2ry12 Expression Relative to Csf1r (2-ΔCT)
Fresh
dPBS
HBSS
DMEM/F12
2 hr, 37°C

Figure S6. Analysis of microglial gene expression changes in culture. *Related to Figure 6*

(A) GSEA results for enrichment of gene lists from previously-published microglial expression profiles (see supplemental methods) (Bennett et al., 2016; Chiu et al., 2013; Matcovitch-Natan et al., 2016; Wang et al., 2015) with log2-ratio ranked expression data comparing freshly isolated microglia and microglia cultured in serum-free TIC (P21, 8div). (B-D) QPCR analysis showing upregulation of *Il1b* and downregulation of *Tmem119* and *P2ry12* mRNA by microglia plated on uncoated or collagen IV coated (+ColIV) tissue culture plates for two hours at 37°C in different media (dPBS, HBSS, or DMEM/F12). (F-G) QPCR analysis showing upregulation of *Il1b* but no change in *Tmem119* or *P2ry12* mRNA in excised brain tissue held at 37°C for two hours in different media. (H) QPCR analysis showing rapid and sustained upregulation of *Spp1* by cultured microglia compared to freshly isolated cells. * $P < 0.01$, † $P < 0.05$ one-way ANOVA with Dunnett's comparison to freshly-isolated controls.

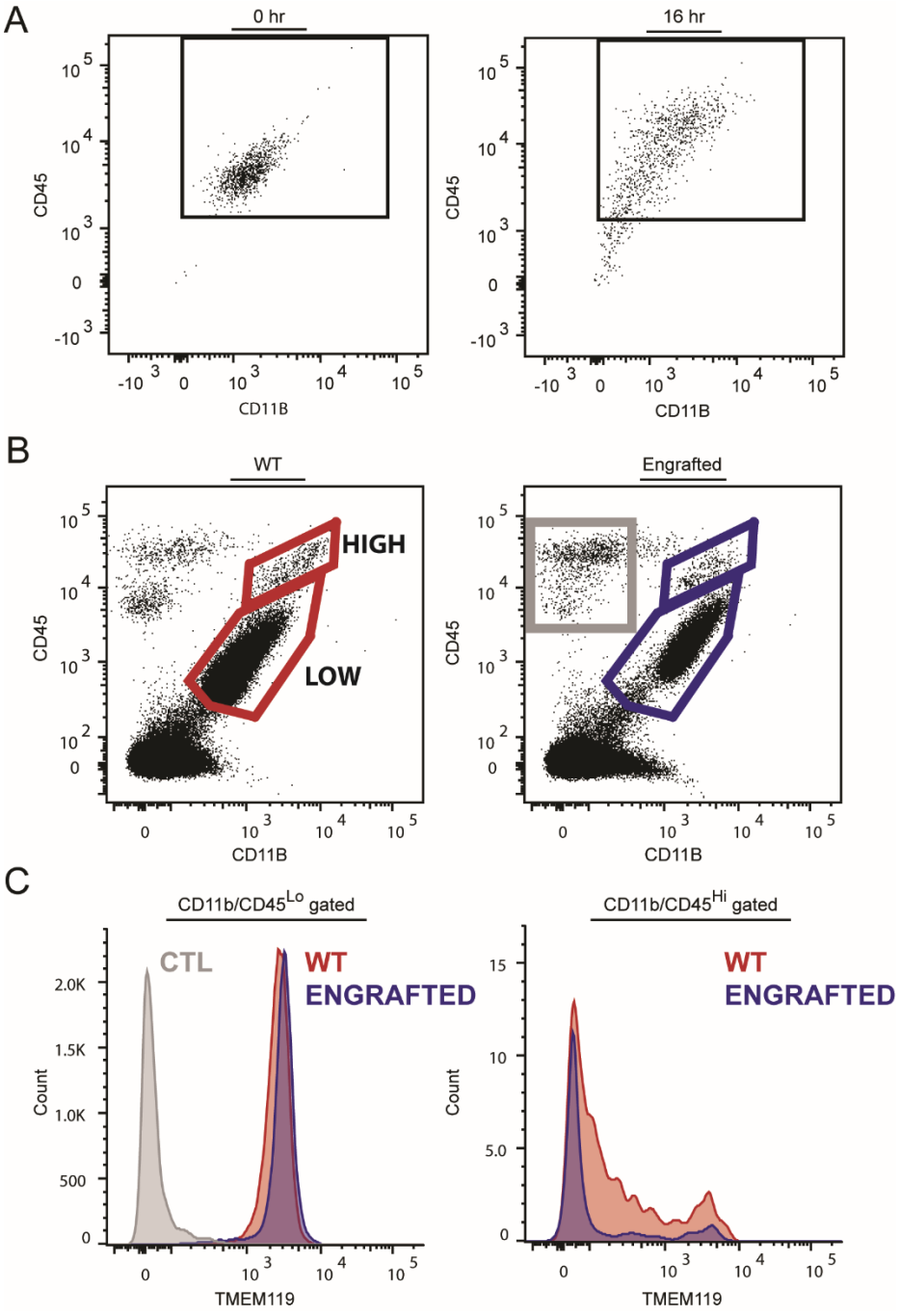

Figure S7. Gating strategy for analysis of engrafted cells. *Related to Figure 7*

(A) Gating strategy for CD11B^{+} purified cells assessed *in vitro* at 0 hr (left) or 16 hr (right). (B) Gating strategy for whole brain single cell suspensions from WT (left) and microglia-transplanted CSF1R$^{-/-}$ animals (right), showing CD11B^{+}/CD45 high and low gates. (C) Nearly all CD45^{Lo} cells show high Tmem119 immunoreactivity, while most CD45^{Hi} cells have reduced or absent Tmem119 signal, as compared to the CD11B^{-}/CD45 single-positive control population.

Protein (*Gene Name*)	MW	IP EL	IP FT	MD EL	MD FT
ApoE (*Apoe*)	36 kDa	84	34	88	16
ApoJ (*Clu*)	51 kDa	76	14	34	10
Biglycan (*Bgn*)	42 kDa	69	19	43	0
Neurocan (*Ncan*)	136 kDa	64	3	36	0
Phosphacan (*Ptprz1*)	255 kDa	48	6	33	0
APP (*App*)	87 kDa	28	7	53	3
Gal3-BP (*Lgals3bp*)	64 kDa	26	1	17	0
Fibromodulin (*Fmod*)	43 kDa	13	1	11	0
Syndecan-4 (*Sdc4*)	22 kDa	12	0	3	0
Brevican (*Bcan*)	96 kDa	10	0	23	0
Psap (*Psap*)	61 kDa	10	1	4	0
Glypican 1 (*Gpc1*)	62 kDa	9	5	19	1
AEBP1 (*Aebp1*)	128 kDa	8	0	7	0
Glypican 4 (*Gpc4*)	63 kDa	7	3	21	4
M-CSF (*Csf1*)	62 kDa	7	2	9	2

Table S1. Mass spectrometry detection of proteins in biochemically enriched ACM survival activity. *Related to Figure 1*

The relative abundance of each protein detected is shown as a composite score combining total ion current and exponentially modified protein abundance index (emPAI) scores. TIC and emPAI scores were normalized within each condition (ACM derived from immunopanned, IP, or McCarthy-DeVellis, MD, astrocyte cultures), setting the maximum value to 100 for each score; the average of the two normalized scores is reported in the table. The active eluate fraction (EL) and inactive flowthrough fraction (FT) of the final step hydrophobic interaction column were compared for the top 15 most abundant proteins detected in the immunopanned astrocyte ACM's EL (IP EL). Molecular weight (MW) of each protein is shown for reference.

Species	Gene	F Sequence	R Sequence	Size (bp)	Start (Base #)	NCBI Reference
rat	*Csf1r*	gcctacagcgttacaactgg	agagccgttcacaggtatcc	248	1121	NM_001029901.1
rat	*P2ry12*	accgatacctgaagaccacc	tgactatctcgtgccagacc	210	503	NM_022800.1
rat	*Sparc*	agaccttcgactcttcctgc	tgttgccctcatctctctcg	207	395	NM_012656.1
rat	*Spp1*	ctgtgtcctctgaagaaacgg	caaaacgtctgcttgtgtgc	215	285	NM_012881.2
rat	*Rplp0*	agtacctgctcagaacaccg	aacatgttcagcagtgtggc	170	439	NM_022402.2
rat	*Eef1a1*	gtcctgattgttgctgctgg	tctgactgtatggtggctcg	140	413	NM_175838.1
rat	*Tnf*	ccaccacgctcttctgtcta	gggcttgtcactcgagtttt	143	275	NM_012675.3
rat	*Il1b*	tggcagctacctatgtcttgc	gcagtgcagctgtctaatgg	153	276	NM_031512.2
rat	*Ccl2*	ctgtgctgaccccaataagg	cattcaaaggtgctgaagtcc	128	297	NM_031530.1
rat	*Tmem119*	tcctgattggttcagagttgg	aatccagaatggctgtgacc	157	1792	NM_001107155.1
rat	*Cxcl10*	tgtccctgtttctcctgacc	catagctgcctgagggaaga	150	485	NM_139089.1
rat	*Cd14*	tcccactctcagaatctaccg	ctgcggatctgagaagttgc	160	42	NM_021744.1
rat	*Il6*	tctctccgcaagagacttcc	cctccgacttgtgaagtggt	150	73	NM_012589.2

Table S2. QPCR primer sequences. *Related to STAR Methods*

Supplemental Movies and Dataset Legends

Movie S1. Cell motility of microglia cultured in TIC. *Related to Figure 2*
Phase images of P20 microglia at 7 div. Each frame is 30 min apart and movies are played at 3 frames per second.

Movie S2. Cell motility of microglia cultured in TIC and serum. *Related to Figure 2*
Phase images of P20 microglia at 7 div exposed to 10% FCS for 24 hr before imaging. Each frame is 30 min apart and movies are played at 3 frames per second.

Movie S3. Interaction of serum-free microglial cultures with pHrodo-labeled myelin debris. *Related to Figure 4*
Phase images of P15 microglia at 7 div interacting with pHrodo-labeled myelin debris (red). Each frame is 10 min apart and movies are played at 12 frames per second. Minimal pHrodo signal is observed.

Movie S4. Interaction of serum-exposed microglial cultures with pHrodo-labeled myelin debris. *Related to Figure 4*
P15 microglia at 7 div exposed to 10% FCS for 24 hr before addition of pHrodo-labeled myelin debris (red). Each frame is 10 min apart and movies are played at 12 frames per second. Robust pHrodo signal is observed even before the added myelin has settled to the bottom of the well.

Movie S5. Cell motility of optimized microglial cultures. *Related to Figure 5*
Phase images of P24 microglia at 14 div plated on collagen-coated Primaria tissue culture plates in TIC with monounsaturated fatty acid and heparan sulfate supplementation. Each frame is 3 min apart and movies are played at 6 frames per second. Timestamp in lower-right corner denotes hours:minutes.

Dataset S1. RNA-seq FPKM tables and gene lists used for GSEA and analysis. *Related to Figure 5*