Appendix

Single-cell transcriptomics reveals distinct inflammation-induced microglia signatures

Sousa et al.

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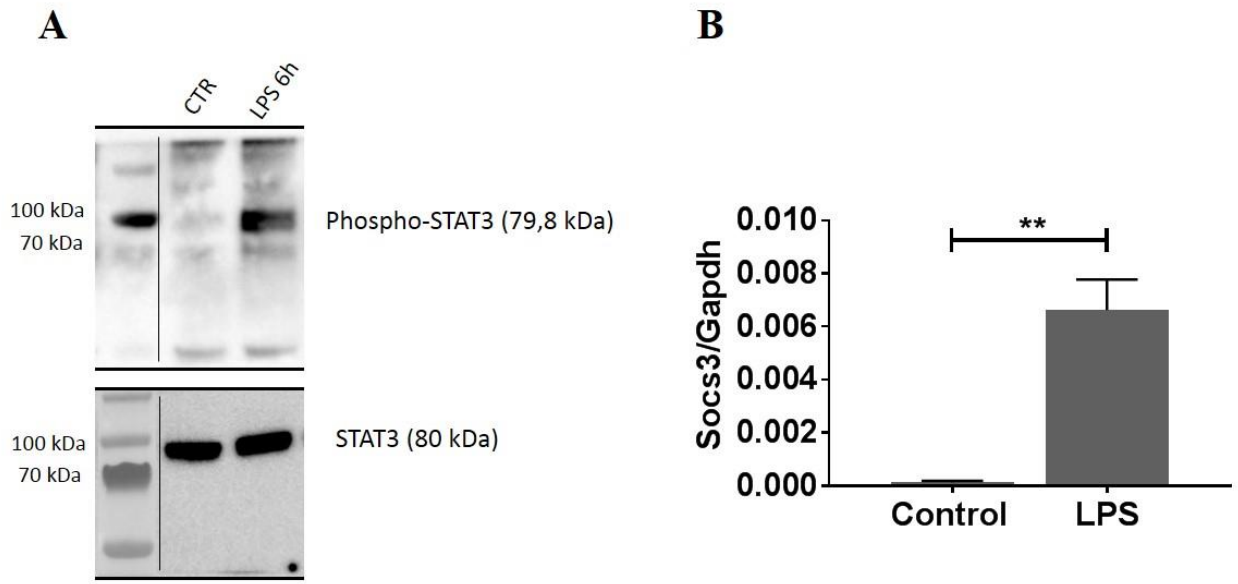
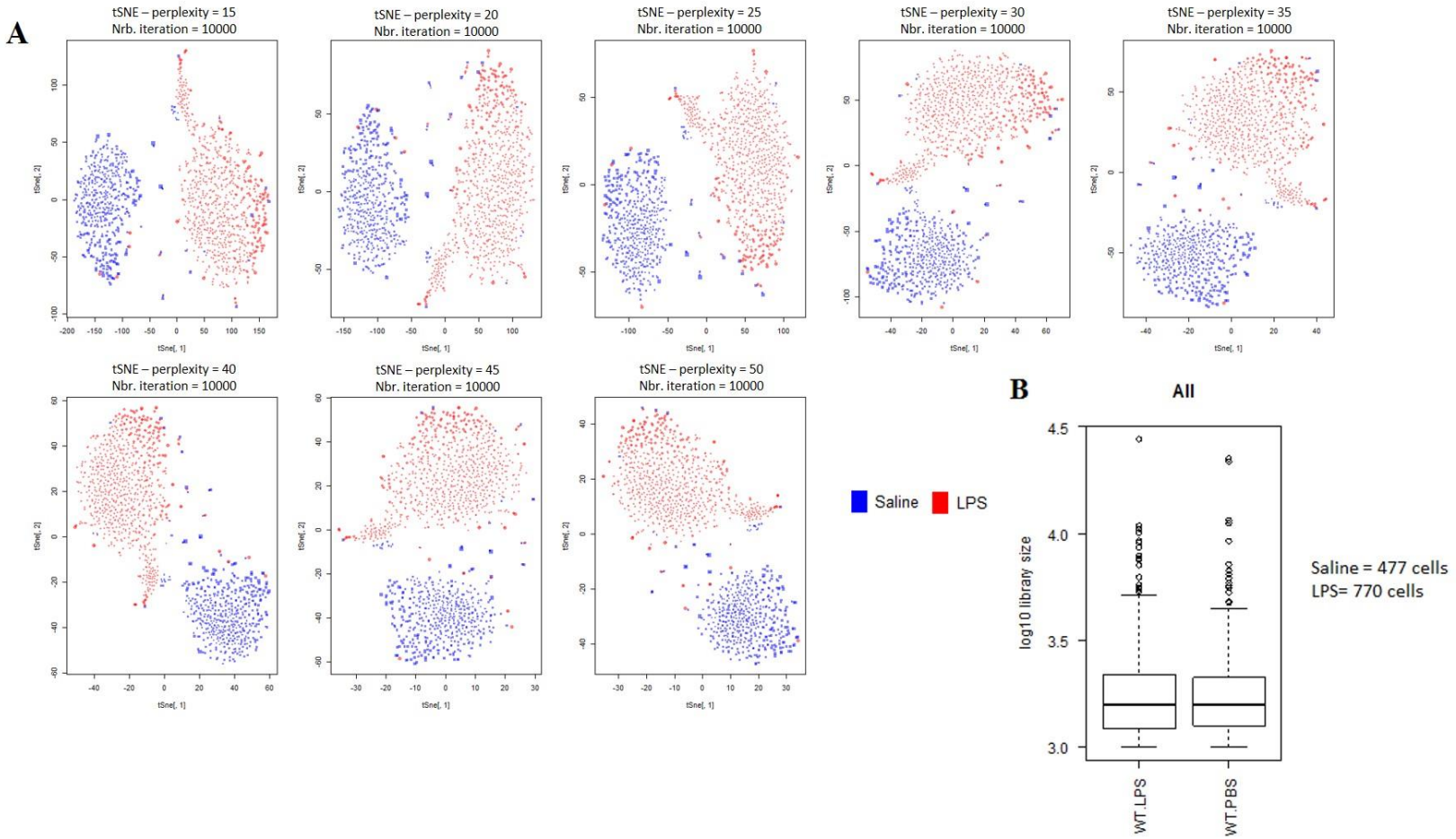
Appendix Fig. S1

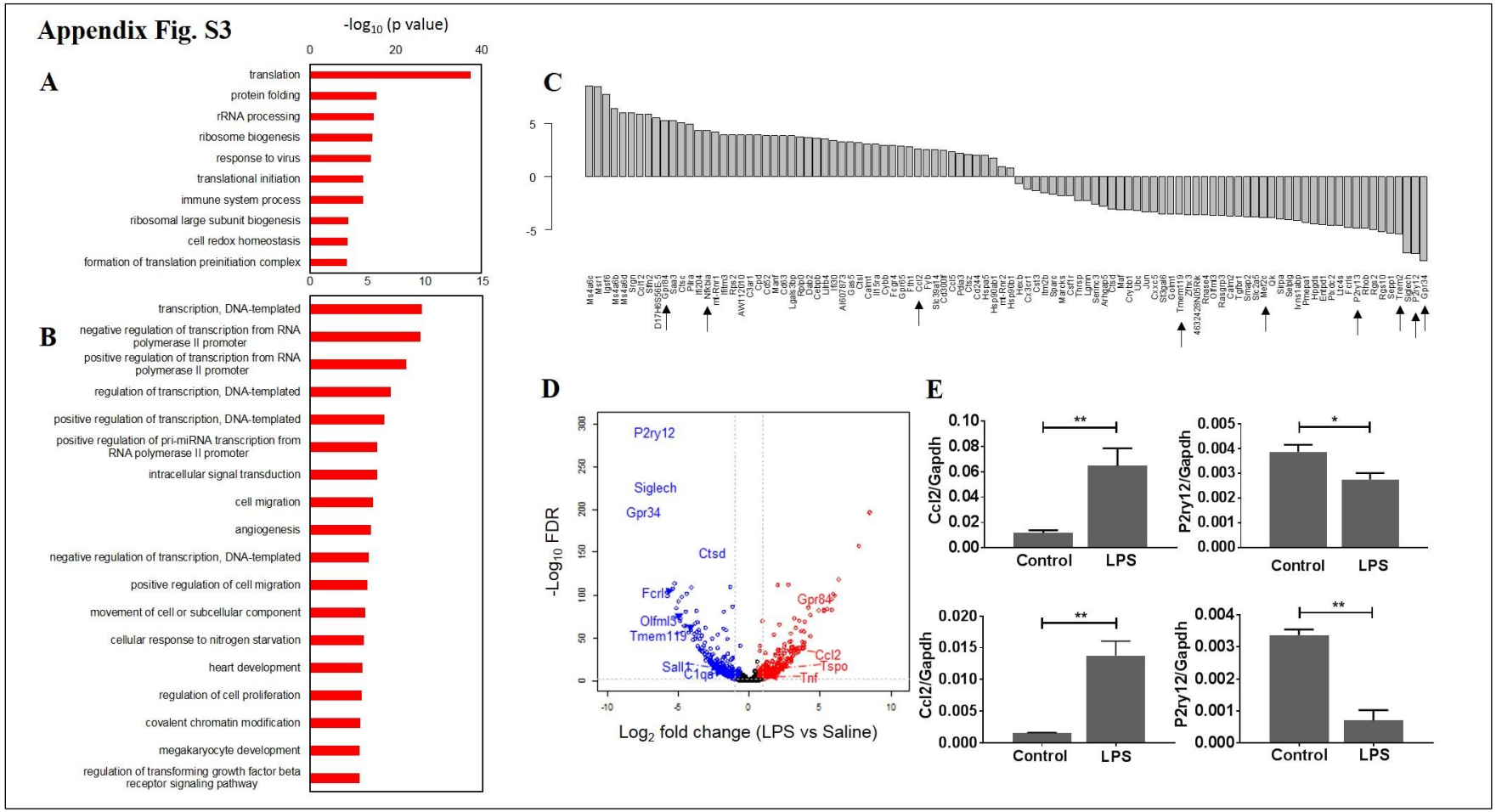


Appendix Figure S1. STAT3 phosphorylation and *Socs3* expression levels are upregulated following LPS exposure. (A) Western blot analysis representative of 2 independent experiments showing the phosphorylated form of STAT3 (upper panel) and total STAT3 levels (bottom panel). Total proteins were isolated from neonatal cultivated microglia treated for 6 hours with LPS (1 ng/ml) or left untreated. **(B)** Neonatal cells were stimulated for 6 hours with LPS (1 ng/ml) or left untreated. Expression levels of *Socs3* were analysed by qPCR. Bars represent mean of relative expression (*Gapdh* as housekeeping gene) ± SEM (**$p < 0.01$ by two-tailed Student's test; n=3).

Appendix Fig. S2

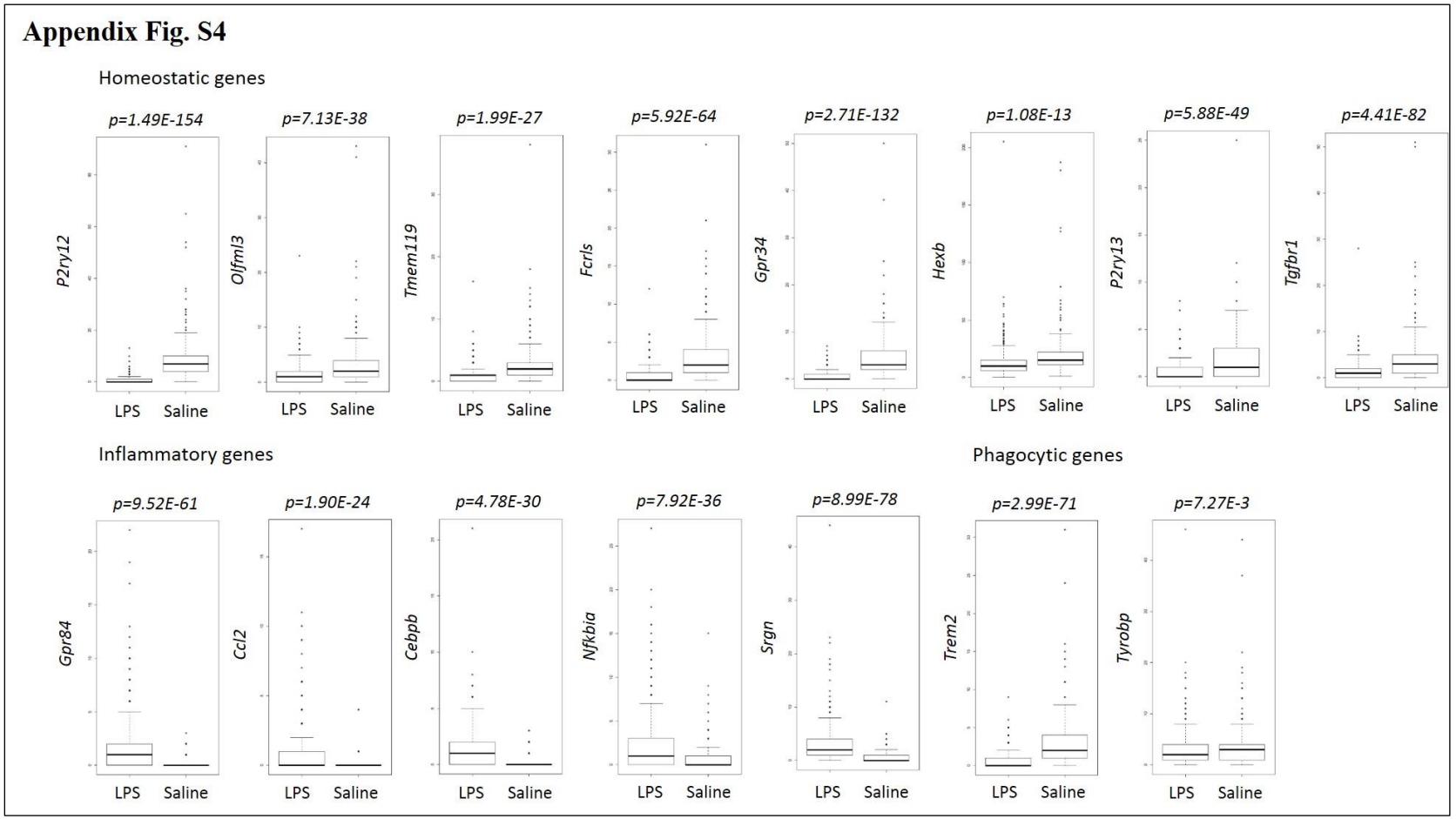


Appendix Figure S2. Evaluation of the perplexity parameter and library size. (A) The perplexity parameter, an estimate of the number of effective neighbours, does not affect the 2D-tSNE clustering representation. For subsequent 2d-tSNE analysis, a perplexity of 30 was selected. **(B)** Library size for 477 cells in the saline and 770 cells in the LPS samples.



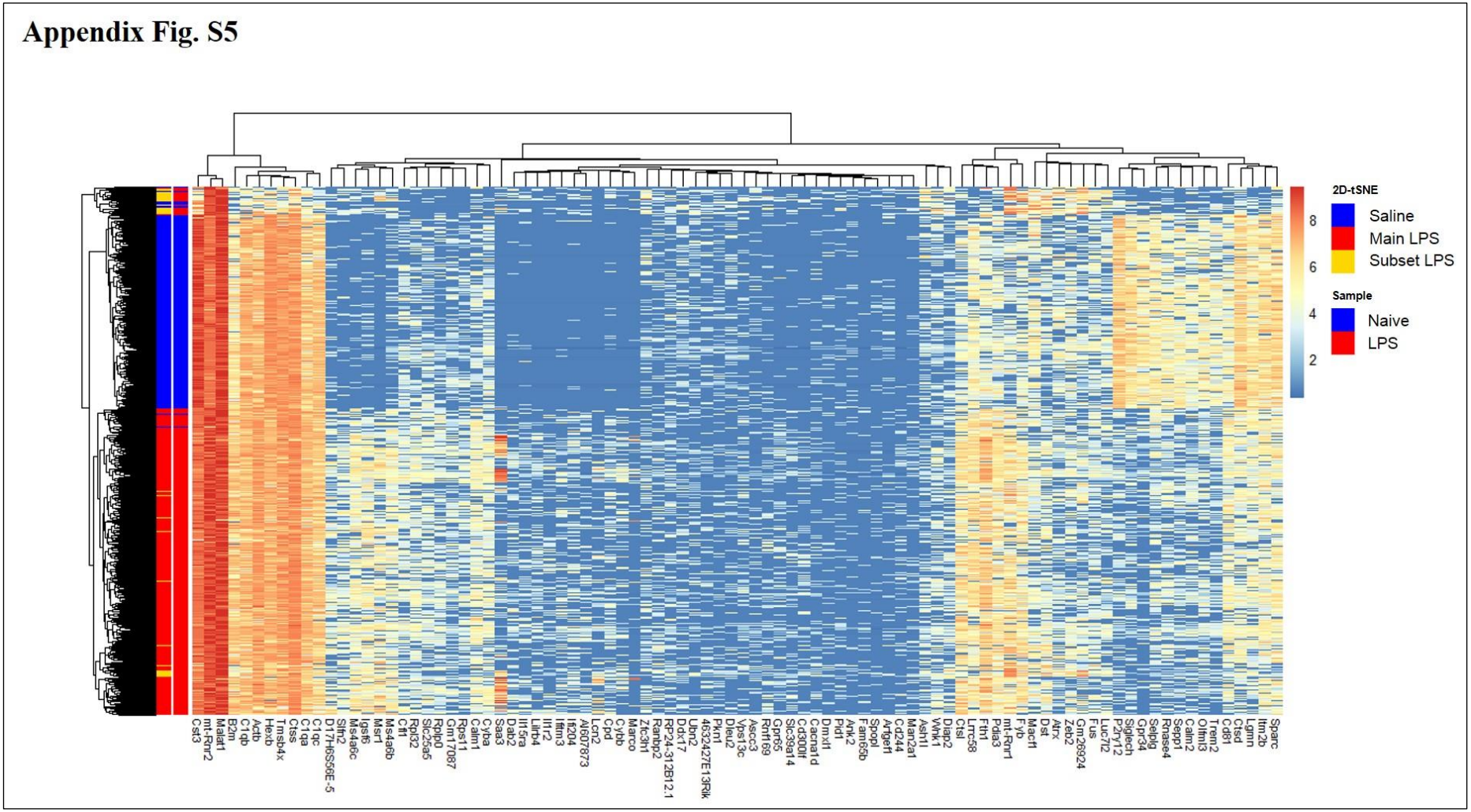
Appendix Figure S3. Microglia isolated from LPS-injected mice are activated. (A-B) Top biological processes identified by Database for Annotation, Visualization and Integrated Discovery (DAVID) resulting from **(A)** upregulated and **(B)** downregulated genes under LPS treatment versus control conditions. **(C)** Bars plot of log2 fold changes between 770 microglial cells isolated from LPS-injected mice and 477 microglia in steady state (top 50 up-regulated and top 50 down-regulated genes; FDR<0.05). **(D)** Volcano plot showing the fold change of genes (log2 scale) between microglia isolated from LPS-injected mice to steady state microglia (x axis) and their significance (y axis, -log10 FDR). A selection of highly significant upregulated (*Gpr84*, *Ccl2*, *Tspo*, *Tnf*) or downregulated (*P2ry12*, *Siglech*, *Gpr34*, *Ctsd*, *Fcrls*, *Olfml3*, *Tmem119*, *Sall1*, *C1qa*) genes is included. P values were determined by Mann-Whitney U test with FDR correction. **(E)** Primary adult microglia (upper panels) were

cultivated in the presence of TGF-β (50 µg/ml) and M-CSF (10 ng/ml), while neonatal cells (bottom panels) were stimulated for 24 hours with TGF-β (50 µg/ml) followed by 6 hours stimulation with LPS (1 ng/ml) or left untreated. Expression levels of *Ccl2* and *P2ry12* were analysed by qPCR. Bars represent mean of relative expression (*Gapdh* as housekeeping gene) ± SEM (*p < 0.05; **p < 0.01 by two-tailed Student's test; n=3).



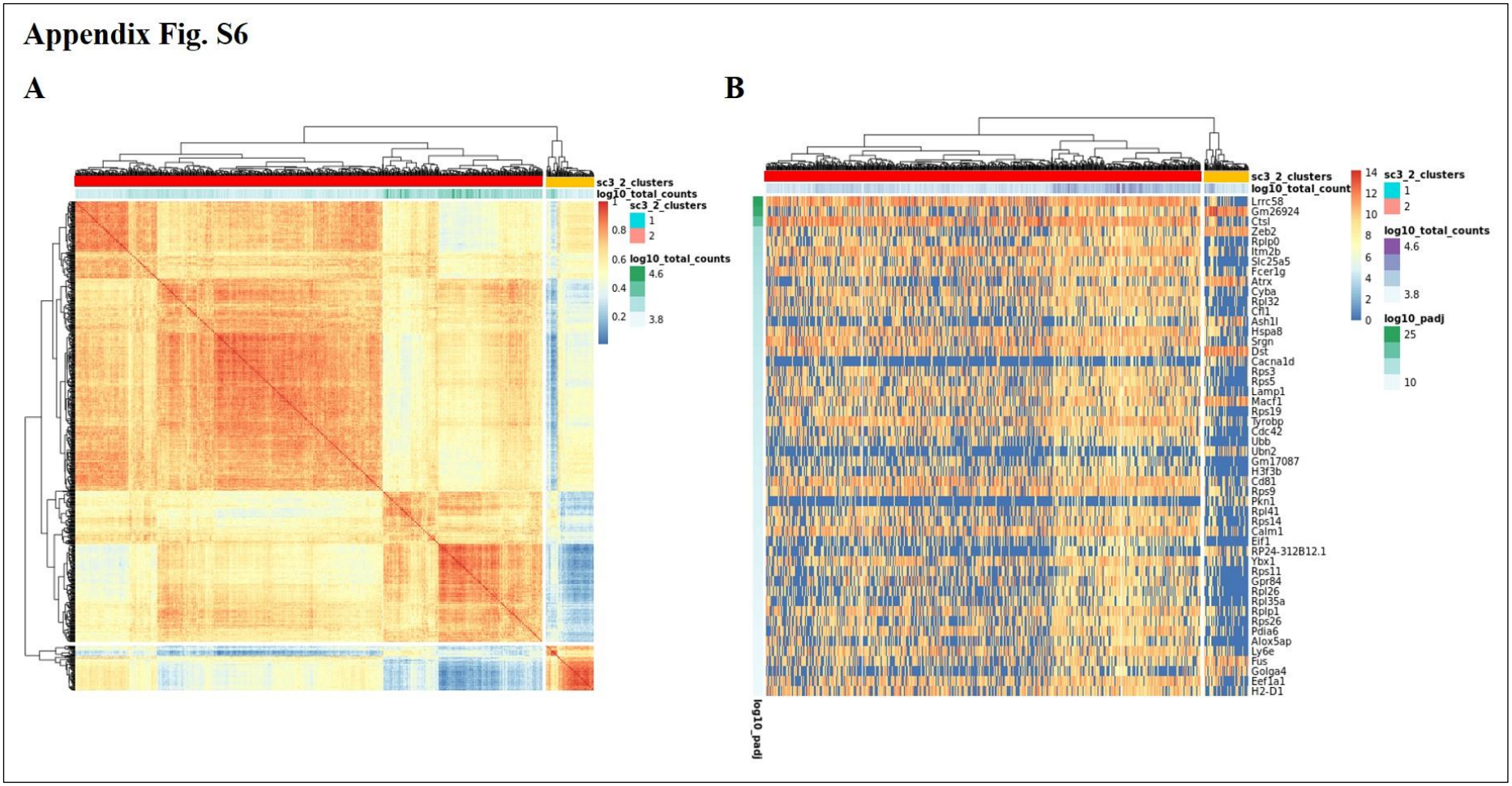
Appendix Figure S4. Comparison between microglia isolated from LPS-injected and control mice at single-cell level. Bar plots showing mean expression (UMI count) of selected genes across LPS and control clusters.

Appendix Fig. S5



Appendix Figure S5. Microglia heterogeneity at the single-cell level. Heatmap showing clustering analysis of 1,247 single cells, featuring top 100 differentially expressed genes (FDR<0.05) between all the clusters identified by 2D-tSNE (naïve, blue; main LPS, red; subset LPS, yellow).

Appendix Fig. S6



Appendix Figure S6. Validation of the existence of the identified inflammatory subpopulations by the "SC3" method. (A) Clustering analysis performed using the "SC3" package to identify clusters of cells showing similarity in their transcriptomic profiles (hierarchical clustering, log2 outlier score, log10 total counts and gene expression levels). K-means clustering distinguished 2 clusters under inflammatory conditions with an average silhouette width of 0.49. **(B)** Heatmap showing top 50 differentially expressed genes driving the segregation of cells into the two identified clusters by "SC3" (adjusted p value<0.05).

Appendix Table S1. List of top differentially expressed genes unique to "main LPS", "subset LPS" or DAM versus naive (FDR<0.05; upregulated genes, Log2FC≥3; downregulated genes, Log2FC≤-3).

Up DAM	Down DAM	Up Main LPS	Down Main LPS	Up Subset LPS	Down Subset LPS
Itgax	Zw10	Rps2	Rasgrp3	Gm26924	Lamp1
Mamdc2	Cebpd	Manf		Golga4	Gm17087
Fam20c	Trnt1	Pdia4		Zfc3h1	Cd68
Ccl4	D14Abb1e	Calm1		RP24-312B12.1	Rps14
Lmbrd2	Stambpl1	Pdia6		Stab1	Itm2c
Egr2	Gpam	C5ar1		Cacna1d	Eif1
Csf1	E2f3	Gnl3		Ash1l	H3f3b
Clec7a	Fyco1			Ascc3	Cd81
Baiap2l2	Mtx3			Atrx	Ubb
Ank	Gbp7			Ttc14	Lrrc58
Zfp692	Rbm7			Chd7	
Axl	Dcun1d2			Myo9a	
Igf1	C1rl				
Gpnmb	Ftsj2				
Ildr2	Sdccag3				
Fxyd6	Rassf1				
Psat1	Ovca2				
St14	E230001N04Rik				
Birc5	Gnb4				
Cdca8	Mrpl37				
H2-Q7	Ppfibp2				
Etl4	BC005561				
Ifit2	Hist1h2be				
Slc1a2	Ggta1				
Pycrl	Dbp				
Lgi2	Pdlim5				
Ero1lb	SCARNA16				
Nceh1	Diexf				
Ch25h	Zfp141				
Got1	Klf2				
	Filip1l				
	Clec4a2				
	Mex3b				
	Zfp398				
	Ggt5				
	Ccl24				
	Rmnd5b				
	Tgm2				
	Nsun6				

	3110062M04Rik				
	Cspp1				

Appendix Table S2. List of FACS chemical compounds and antibodies.

Antibodies FACS	Brand	Cat No	µl/tube (100 µl)
Hoechst (Bisbenzimide)	Sigma-Aldrich	33342	1 µl
Sytox Red	ThermoFisher	S34859	1 µl
CD16/32	eBioscience	14-0161-85	10 µl
mLy6C PB	Biolegend	128014	0.5 µl
iso IgG2a BV606 400540	Biolegend	400540	5 µl
mCD45 FITC	eBioscience	11-0454-82	1 µl
mCD11b Percp_Cy5.5	eBioscience	550993	5 µl
iso IgG Goat IC108P	R&D	IC1 8P	5 µl
mCD206 APC	Biolegend	141708	2.5 µl
mCD11c APC_Cy7	Biolegend	117324	5 µl
mCD86 APC_Cy7	Biolegend	105029	6 µl
mCD45 FITC	ImmunoTools	22270453	5 µl
mCD11b PE	eBioscience	12011281	1 µl
mCD206 PE_Cy7	Biolegend	141719	2.5 µl
mCD86 BV605	Biolegend	105037	5 µl
mTMEM119	abcam	ab210405	2.5 µl
Alexa Fluor®647 goat anti-rabbit IgG (H+L)	Molecular Probes	A-21244	2.5 µl
mP2RY12 PE	Biolegend	848003	5 µl
mTMEM119	abcam	ab210405	2.5 µl
mCD44 PE_Cy7	Biolegend	103030	1.2 µl
mCD274 APC	Biolegend	124311	2.5 µl
mNOTCH4 PE_Cy7	Biolegend	128407	5 µl

mCCR2 PE	R&D	FAB5538P	5 µl

Appendix Table S3. PCR primer sequences.

Gene	PCR primer sequence (5'-3')
Gapdh	Forward: TGCGACTTCAACAGCAACTC Reverse: CTTGCTCAGTGTCCTTGCTG
Olfml3	Forward: TGTTAGACGGCACCCAGAAC Reverse: CCACTGTTCGGTTTGCCAAG
Fcrls	Forward: TTCTGGTCTTCGCTCCTGTC Reverse: ACCGCGTCTTGCATTCCTAA
Tmem119	Forward: TGCAATGTCGCTGTCACTCT Reverse: AGTTTGTGTTTCCACGGGGT
Siglech	Forward: ATCCAGCTCAATGTCACCTATAAT Reverse: GCTTCTCCCATGGCTACCTG
Gpr34	Forward: GGAAAGCTTCAACTCAGTTCCTG Reverse: TCCATGAGAGGAGCAAAGCC
P2ry12	Forward: GTGCAAGGGGTGGCATCTA Reverse: TGGAACTTGCAGACTGGCAT
Mef2c	Forward: TAACACAGGCGGTCTGATGG Reverse: TTATCCTTTGATTCACTGATGGCA
Itgb5	Forward: GTCCAACTTGACGGTCCTCC Reverse: TACAGGGGGTTTGAGGCCATT
Sall1	Forward: CGTGAGCGGCTGATGTTTGA Reverse: CTTAGTGGGGCGACTTGGTT
Hexb	Forward: CGCAGTCCCCGCGTAG Reverse: AGTTGTAATATCGCCGAAACGC
Tgf-β1	Forward: ATGCTAAAGAGGTCACCCGC Reverse: TGCTTCCCGAATGTCTGACG
Aif1	Forward: CAGGGATTTGCAGGGAGGAAA Reverse: AGTTTGGACGGCAGATCCTC
Cx3cr1	Forward: CCCATCTGCTCAGGACCTCAC Reverse: GGCCTCAGCAGAATCGTCAT

Mertk	Forward: ACATACCAAGAAACAGCTGGCA Reverse: TAAGGGAGTCCTTGAACTTCCAC
Ctss	Forward: CCACGCTGCCATCAGAAGA Reverse: TGCCCACTTGGTAGGTATGC
Tyrobp	Forward: ATGCGACTGTTCTTCCGTGA Reverse: TTGTTTCCGGGTCCCTTCC
Trem2	Forward: CTTGCTGGAACCGTCACCAT Reverse: ACAGGATGAAACCTGCCTGGA
Itgam	Forward: GCTCGACACCATCGCATCTA Reverse: TGGTACTTCCTGTCTGCGTG
Itgax	Forward: TTTGGCTTCCCAGACTTGAAGA Reverse: TGCTGTCACACATGAGGTGC
Gfap	Forward: AGAAAGGTTGAATCGCTGGA Reverse: TCTTGCATGTTACTGGTGGC
Gjb6	Forward: ACCATGCCCTGAAAGAGAGC Reverse: GGGCTCACCTACACTTGACC
Ntsr2	Forward: GGTGAGACACAAGGATGCCA Reverse: CAGTCCATCCATCATCGGGG
Aldh1l1	Forward: ATTCCCAAGGGTGTGGTCAAC Reverse: CATCAGGGTGGTCTGAGAGTCTCT
Mobp	Forward: CAGACCGGCACGGATGAAAA Reverse: CCTCCTCAATCTAGTCTTCTGGC
Mog	Forward: TGCTGACTCTCATCGCACTT Reverse: CTTCGGTGCAGCCAGTTGTA
Cldn1	Forward: TTGCTCTTTCCTCGGGCATT Reverse: GGCTTCCACTGTCGTTGGTA
Tubb3	Forward: TGAGGCCTCCTCTCACAAGTA Reverse: CCGCACGACATCTAGGACTG
Vglut1	Forward: GCCATCTCTGGGTTTAACGTG Reverse: AACACGTACTGCCACTCCTC
NeuN	Forward: ACACACACACTCCATACTGAGG Reverse: GCTCTGGGCTCTCTGTTTGC
Ly6c1	Forward: GTGTGCAGAAAGAGCTCAGG

	Reverse: GAAAGGCACTGACGGGTCTT
Mrc1	Forward: AGTGGCAGGTGGCTTATG Reverse: GGTTCAGGAGTTGTTGTGG
Arg-1	Forward: AGACAGCAGAGGAGGTGAAGAG Reverse: CGAAGCAAGCCAAGGTTAAAGC
Il-1β	Forward: GCTTCAGGCAGGCAGTATC Reverse: AGGATGGGCTCTTCTTCAAAG
Tnf	Forward: GGTTCTGTCCCTTTCACTCAC Reverse: TGCCTCTTCTGCCAGTTCC
Ccl2	Forward: CACTCACCTGCTGCTACTCATTC Reverse: GCTTCTTTGGGACACCTGCTG
Ccr2	Forward: AGCCATACCTGTAAATGC Reverse: GCCGTGGATGAACTGAGGTA
Socs3	Forward: TAGACTTCACGGCTGCCAAC Reverse: CGGGGAGCTAGTCCCGAAG