Supplementary material

The CEBPB–AP-1 (JunB/Fos) Axis Drives Neuroinflammation and Microglial Dysfunction via TNF Signaling in Ischemic Stroke

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#Kun Liang and Shuangshuang Lu contributed equally to this study.

Supplementary Figures

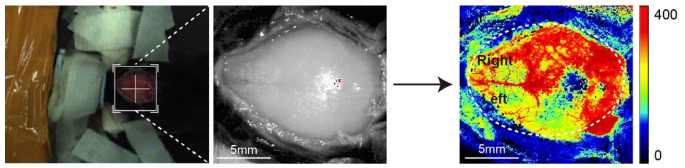

Figure S1. Laser Doppler flowmetry monitoring of cerebral blood flow during transient focal cerebral ischemia (tFCI) surgery in mice. (Left) Surgical setup showing the position of the probe over the exposed skull. During tFCI surgery, insertion of the intraluminal filament into the middle cerebral artery leads to an immediate and sustained drop in regional cerebral blood flow (rCBF) in the left hemisphere, confirming successful occlusion. Subsequent withdrawal of the filament (reperfusion) results in restoration of rCBF, validating the establishment of the transient ischemia-reperfusion model.

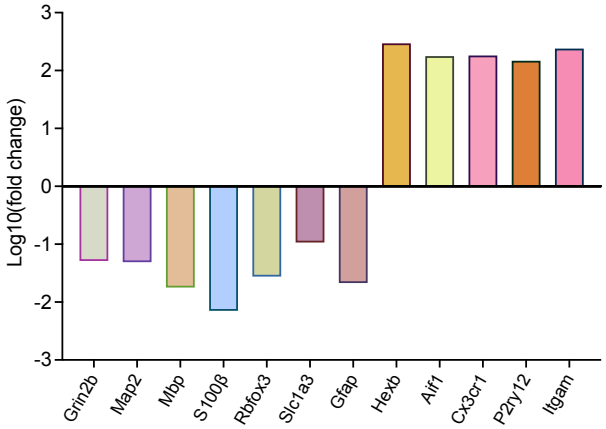

Figure S2. Magnetic bead-based isolation enriches microglia, validated by RT-qPCR for lineage-specific markers. Transcript levels of oligodendrocyte (Mbp), neuronal (Grin2b, Rbfox3, Map2), astrocytic (S100β, Gfap, Slc1a3), and microglial (Aif1, Itgam, Cx3cr1, Hexb, P2ry12) marker genes were quantified. Positive values reflect microglial enrichment, while negative values indicate depletion of other neural lineages after purification.

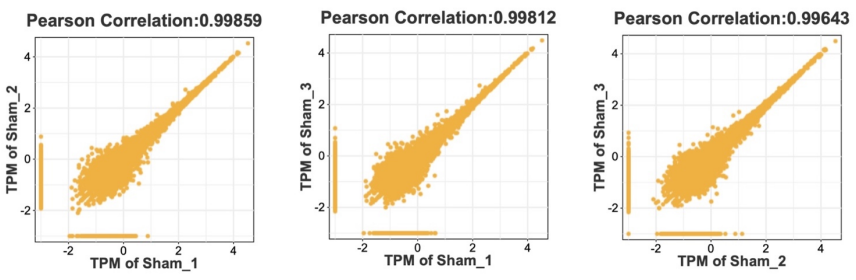

Figure S3. Biological replicates within each group show high transcriptomic reproducibility. Scatter plots display pairwise comparisons of gene expression levels (log_{10}-transformed TPM) for all samples in the

Sham group (Sham-1 vs. Sham-2, Sham-1 vs. Sham-3, Sham-2 vs. Sham-3). Each point represents a gene. Pearson correlation coefficients (R) are indicated in the upper left corner of each plot, demonstrating strong intra-group reproducibility.

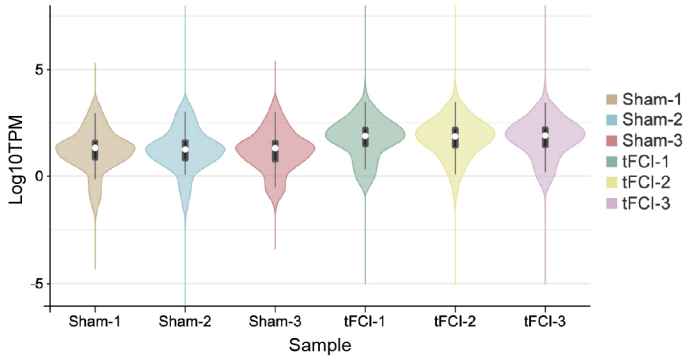

Figure S4. Violin plot of microglial gene expression profiles. Log_{10}-transformed TPM values are shown for six biological replicates (Sham1–3 and tMCAO1–3). Each violin represents the full distribution of expression across all detected genes, with the white dot indicating the median and the thick bar the interquartile range.

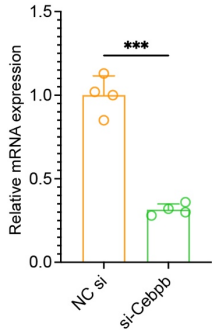

Figure S5. Validation of siRNA-mediated knockdown efficiency of Cebpb in BV2 microglial cells. Quantitative real-time PCR analysis of Cebpb mRNA expression in BV2 microglia transfected with negative control siRNA (NC si) or Cebpb-specific siRNA (si-Cebpb). The relative expression level of Cebpb was significantly reduced in the si-Cebpb group compared with the NC si group, confirming effective knockdown. Data are presented as mean ± SD, n = 4 per group. ***$p < 0.001$.

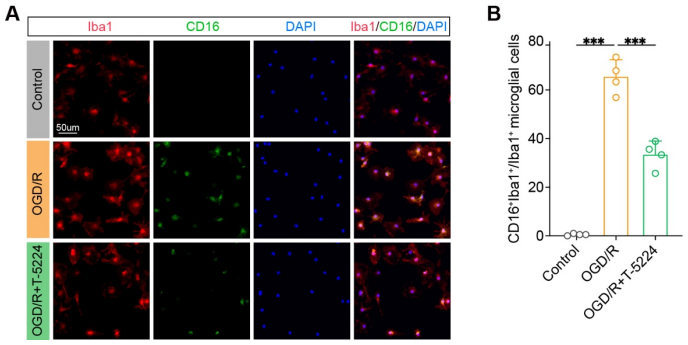

Figure S6. T-5224 inhibits pro-inflammatory polarization of primary microglia under OGD/R conditions. (A) Representative immunofluorescence images of primary microglia stained for the microglial marker Iba1, the pro-inflammatory marker CD16. (B) Quantification of the percentage of $CD16^{+}/Iba1^{+}$ pro-inflammatory microglia. Data are presented as mean ± SD, n = 4 per group. ***$p < 0.001$, one-way ANOVA with Tukey's post-hoc test.

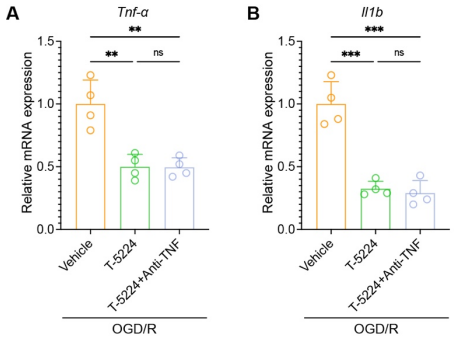

Figure S7. T-5224 inhibits pro-inflammatory gene expression primarily through TNF-α signaling in OGD/R-stimulated microglia. Quantitative real-time PCR analysis of (A) Tnf-α and (B) Il1b mRNA expression in microglia subjected to OGD/R, treated with vehicle, T-5224, or T-5224 plus TNF-α neutralizing antibody (Anti-TNF). Data are presented as mean ± SD, n = 4 per group. **$p < 0.01$, ***$p < 0.001$; ns, not significant, one-way ANOVA with Tukey's post-hoc test.

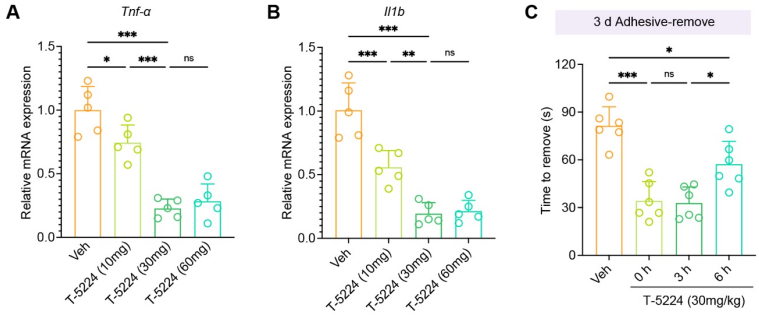

Figure S8. Dose-response and therapeutic time window validation of T-5224 in tFCI mice. (A, B) Quantitative real-time PCR analysis of Tnf-α (A) and Il1b (B) mRNA expression in the ischemic tissue of

tFCI mice treated with vehicle (Veh) or T-5224 at 10, 30, or 60 mg/kg. T-5224 reduced pro-inflammatory cytokine expression in a dose-dependent manner, with the 30 mg/kg dose achieving maximal efficacy, and no further significant reduction observed at 60 mg/kg. (C) Therapeutic time window assessment using the adhesive-removal test at 3 days post-tFCI. Mice received T-5224 (30 mg/kg) at 0, 3, or 6 h after reperfusion. Early intervention (0–3 h) produced the greatest functional improvement, while delayed treatment at 6 h showed reduced but still significant efficacy. Data are presented as mean ± SD, n = 5–6 per group. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant, one-way ANOVA with Tukey's post-hoc test.

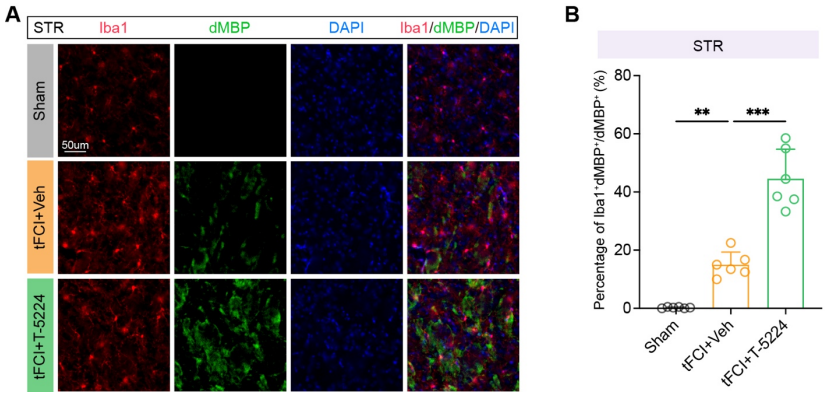

Figure S9. T-5224 enhances myelin debris clearance by microglia in the striatum (STR) of tFCI mice. (A) Representative immunofluorescence images of the striatum stained for the microglial marker Iba1 (red), degraded myelin basic protein (dMBP, green), and the nuclear marker DAPI (blue). (B) Quantification of the percentage of Iba1$^+$ microglia positive for intracellular dMBP. Data are presented as mean ± SD, n = 6 per group. **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey's post-hoc test.

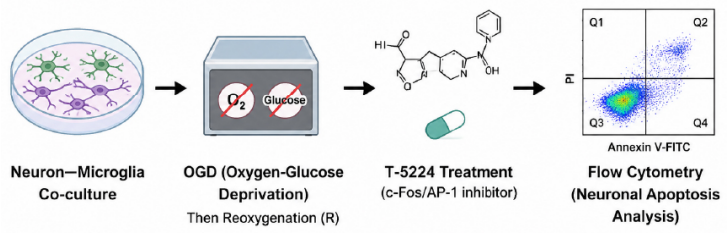

Figure S10. Experimental workflow of the neuron-microglia co-culture system to assess the neuroprotective mechanism of T-5224.