

Electroacupuncture alleviate cognitive impairment via activation of TREM2-positive microglia in APP/PS1 mice

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Abstract

Background

Microglial activation-mediated neuroinflammation and impaired A β phagocytosis are critical contributors to Alzheimer's disease (AD) pathogenesis. Triggering receptor expressed on myeloid cells 2 (TREM2), a key microglial receptor, is closely implicated in AD by promoting A β clearance and limiting tau spreading. Electroacupuncture (EA) has been shown to alleviate microglial activation-induced neuroinflammation and restore impaired microglial phagocytosis in AD, yet whether it ameliorates cognitive deficits via TREM2 + microglial functional modulation remains elusive.

Methods

APP/PS1 mice received EA treatment, with or without intracerebroventricular injection of AAV-shTREM2 to knock down TREM2. Cognitive function was evaluated using the Morris Water Maze and Novel Object Recognition tests. A β deposition, neuronal injury, microglial phenotypic polarization, and TREM2 expression were detected via immunofluorescence, Western blotting (WB), and enzyme-linked immunosorbent assay (ELISA). Recruitment of TREM2⁺ microglia to A β plaques and their phagocytic activity (marked by CD68) were evaluated using triple immunofluorescence staining. Single-cell RNA sequencing (scRNA-seq) was conducted to characterize microglial heterogeneity and intercellular communication networks.

Results

EA ameliorated cognitive performance in APP/PS1 mice, attenuated neuronal damage and A β deposition, and upregulated the expression of TREM2 and DAP12. EA induced a shift of microglia from a pro-inflammatory toward a repair-associated phenotype, promoted recruitment of TREM2⁺ microglia to A β plaques, and enhanced their phagocytic activity. scRNA-seq analysis revealed that EA enriched a subpopulation of disease-associated microglia (DAM) and upregulated genes related to phagocytosis and lipid metabolism in this subpopulation. EA also inhibited pro-inflammatory intercellular signaling and potentiated protective intercellular communication.

Conclusions

EA ameliorated cognitive impairment in APP/PS1 mice by enhancing TREM2⁺ microglia-mediated A β clearance and suppressing neuroinflammation. scRNA-seq further revealed that EA reshaped microglial transcriptional states and intercellular communication networks in a TREM2-dependent manner. These

Introduction

Dementia is one of the most pressing global health challenges in recent decades. According to the WHO, approximately 55.2 million people worldwide were affected by dementia in 2022 [1]. Of these, Alzheimer's disease (AD) is the most prevalent form of dementia, accounting for 60–70% of all cases [2]. As a progressive neurodegenerative disorder, AD leads to insidious impairment of memory and cognition, which worsens over time.[3]. Its early clinical manifestations often include depression, anxiety, social withdrawal, and sleep disorders. With disease progression, patients present with severe memory loss, neuropsychiatric symptoms including hallucinations and delusions, and progressive behavioral abnormalities [4, 5]. Despite decades of extensive research, the exact molecular mechanisms underlying AD pathogenesis remain poorly understood. Currently, the two widely recognized pathological hallmarks of AD are extracellular amyloid- β (A β) plaques and intracellular neurofibrillary tangles formed by hyperphosphorylated tau.[6]. Among the existing pathogenic hypotheses, the A β cascade hypothesis remains the prevailing theory regarding AD etiology, which posits that A β accumulation serves as the primary initiating event in AD. [7].

This pathological cascade inevitably activates microglia, the resident innate immune cells of the central nervous system (CNS). Under physiological conditions, microglia constantly survey the neural parenchyma and mount rapid responses to brain injury or infection [8]. Microglia in AD were traditionally thought to undergo a phenotypic transition from a neuroprotective to a pro-inflammatory state with disease progression, thereby exacerbating neuronal injury. However, recent evidence indicates that microglial responses are highly heterogeneous[9]. Triggering receptor expressed on myeloid cells 2 (TREM2), a transmembrane receptor predominantly expressed by microglia, is a key regulator of these responses and contributes to this heterogeneity. It modulates key cellular functions, including phagocytosis, inflammatory responses, and cell migration[10]. One key example of this heterogeneity is disease-associated microglia (DAM), whose transition from homeostatic microglia requires TREM2 signaling[11]. Meanwhile, TREM2 mutations have been identified as genetic risk factors for AD [12]. The R47H variant, the most common TREM2 mutation, confers a 2- to 3-fold increased risk of AD development [13]. TREM2 deficiency impairs microglial recruitment to A β plaques and exacerbates neuronal loss in AD mouse models. [14, 15]. Over the past five years, significant progress has been achieved in the development of TREM2-targeted therapeutic strategies, especially for AD [16–18], However, the clinical translation of these novel agents is limited by the lack for extensive validation and concerns about potential toxicity.

Acupuncture, a non-pharmacological intervention with a favorable safety profile, has been demonstrated to ameliorate cognitive impairment in AD. Our previous studies have demonstrated that electroacupuncture (EA) effectively ameliorates cognitive deficits in AD mouse models [19, 20]. However, whether these beneficial effects are mediated by TREM2-positive microglia remains unclear. Our study investigated the role of TREM2 in mediating EA-induced improvements in microglial phagocytic activity, inflammatory polarization, and transcriptional profiles using viral-mediated TREM2 knockdown combined with single-cell RNA sequencing.

Materials and Methods

Animals

A total of 40 male, 6-month-old APP/PS1 (APP^{swe}/PS1^{dE9}) double-transgenic mice were purchased from Jiangsu Huachuang Xinuo Pharmaceutical Technology Co., Ltd (Jiangsu, China). Eight age-matched wild-type (APP/PS1-negative) C57BL/6J littermates were used as the control group. All animals were housed in an independent ventilation cage (IVC) system at the Experimental Animal Center of Hubei University of Chinese Medicine under a specific pathogen-free (SPF) condition, with the room temperature maintained at $23 \pm 2^{\circ}\text{C}$ and relative humidity at 50–60%. The mice were maintained on a 12 h light/dark cycle with free access to sterile water and standard pellet chow. The bedding was changed regularly. All animals were allowed to acclimate to the environment for at least one week prior to the experimental procedures.

Animal Grouping and EA Treatment

The APP/PS1 mice were randomly assigned to five groups: Model, EA, AAV-shTREM2 (TREM2 knockdown), AAV-Ctrl (empty vector), and EA + AAV-shTREM2. Age-matched wild-type C57BL/6J mice served as the Control group (Fig. 1A).

For EA treatment, the mice were gently restrained in a custom-made cloth bag, and sterile acupuncture needles were inserted into the Baihui (GV20) and bilateral Shenshu (BL23) acupoints. The bilateral Shenshu (BL23) acupoints were connected to a HANS-100A EA stimulator (2 Hz, 1 mA, continuous wave) for 15 min per day. The EA treatment was administered for 10 consecutive days per course. A total of four courses conducted and a 1-day interval between consecutive courses.

For TREM2 knockdown, adeno-associated virus (AAV) carrying shRNA targeting TREM2 (AAV-U6-shRNA(TREM2)-CMV-EGFP-pA) or a scrambled control shRNA (AAV-U6-shRNA(scramble)-CMV-EGFP-pA) was purchased from Wuhan BrainVTA Biotechnology Co., Ltd (Wuhan, China). The viral vectors were stereotactically injected into the lateral ventricles (0.3 mm posterior to bregma, 1.0 mm lateral, 2.5 mm in depth; 2 μL /side at a rate of 0.2 μL /min). Mice in the EA + AAV-shTREM2 group received the viral vector injection first, followed by EA treatment as described above.

Cognitive Behavioral Tests

Subsequent to the EA treatment, all mice were subjected to a series of cognitive behavioral tests.

Morris Water Maze (MWM) Test

The MWM test was conducted to evaluate spatial learning and memory ability. The test procedure consisted of three phases: a visible platform session (day 1), a hidden platform acquisition phase (days 2–5), and a probe trial (day 6). During the hidden platform acquisition phase, the escape latency to

locate the submerged platform was recorded daily for each mouse. In the probe trial, the hidden platform was removed, and the number of target platform crossings and swimming path length were quantified and analyzed.

Novel Object Recognition (NOR) Test

The NOR test was used to evaluate the recognition memory ability of the mice. The assay comprised two consecutive sessions: a training session and a test session. On the training day, the mice were placed in the open-field apparatus containing two identical objects (Object A and Object B) and allowed to explore the apparatus freely for a predetermined period. On the test day, one of the familiar object (Object A) was replaced with a novel object (Object C), and the mice were again allowed to explore the apparatus again under the same conditions. The time spent exploring each object was recorded by a blinded observer, and the recognition index (RI) was calculated using the following formula: $RI = (\text{Time spent exploring the novel object} / \text{Total exploration time}) \times 100\%$.

Open Field (OF) Test

The OF test was conducted to evaluate spontaneous locomotor activity and exploratory behavior of the mice. The mice were gently placed in the central area of the open-field apparatus and allowed to move freely for 5 min. The total travel distance and the number of entries into the central zone of the apparatus were automatically recorded. The open-field apparatus was thoroughly cleaned with 70% ethanol solution between individual trials to eliminate residual olfactory cues.

Histological and Immunofluorescence Staining

The mice were deeply anesthetized and subjected to transcardial perfusion with ice-cold normal saline followed by 4% paraformaldehyde (PFA) fixative solution. The whole brains were rapidly harvested, post-fixed in 4% PFA, dehydrated in a series of graded sucrose solutions, and embedded in optimal cutting temperature (OCT) compound. Serial coronal brain sections were cut using a cryostat and stored at -20°C for subsequent staining experiments.

Nissl Staining

The brain sections were air-dried at room temperature for 15–30 min, fixed in 95% ethanol for 5–10 min, and rehydrated through a graded ethanol series (100%, 95%, 70%) to distilled water. The sections were then stained with pre-warmed 0.5% toluidine blue solution at 50–60°C for 10 min, briefly rinsed briefly with distilled water, and differentiated in 95% ethanol for approximately 10 s. Following dehydration through absolute ethanol and clearing in xylene, the sections were mounted with neutral balsam and sealed with coverslips. Representative images were captured under a light microscope after 1–2 days of air-drying at room temperature.

Congo Red Staining

Following the same fixation, drying, and rehydration procedures as described above, sections were stained with pre-warmed 1% Congo Red solution at 50°C for 15 min, then rinsed rapidly with distilled water to remove excess staining solution. The cell nuclei were counterstained with Mayer's hematoxylin for 1–2 min and then blued under running tap water for 5 min. The sections were differentiated in an alkaline differentiation solution for approximately 15 s, immediately transferred to 95% ethanol to terminate the differentiation reaction, and subsequently dehydrated through absolute ethanol. The clearing and mounting procedures were performed in the same manner as described for Nissl staining.

Immunofluorescence Staining

For immunofluorescence staining, the brain sections were incubated with the respective primary antibodies overnight at 4°C, followed by incubation with the corresponding fluorescent secondary antibodies for 1 h at room temperature (RT). The following primary antibodies were used in this study: mouse anti-TREM2 (Proteintech, China), sheep anti-TREM2 (R&D Systems, USA), rabbit anti-IBA1 (Huabio, China), rabbit anti-NeuN (Huabio, China), rabbit anti-GFAP (Servicebio, China), rabbit anti-iNOS (Santa Cruz, USA), mouse anti-Arg1 (Santa Cruz, USA), rabbit anti-A β 1–42 (Huabio, China), and rabbit anti-CD68 (Abcam, UK). The secondary antibodies included Alexa Fluor 555-conjugated anti-mouse IgG, Alexa Fluor 647-conjugated anti-rabbit IgG, and species-specific HRP-conjugated secondary antibodies for TSA amplification. DAPI was used for nuclear counterstaining to label cell nuclei.

For triple immunofluorescence staining of TREM2 combined with A β and IBA1 or TREM2 with CD68 and IBA1, a tyramide signal amplification (TSA) multiplex immunofluorescence staining method was employed. Sequential staining was performed with antibody stripping conducted between each staining round via microwave treatment in 0.01 M citrate buffer (pH 6.0). The staining order was as follows: (1) sheep anti-TREM2 detected with CY3-TSA, (2) mouse anti-A β 1–42 or mouse anti-CD68 detected with 700-TSA, and (3) rabbit anti-IBA1 detected with CY5-TSA.

Representative double immunofluorescence images were captured using a confocal laser scanning microscope (CLSM) (AXNIS-Elements 6.0, Nikon, Japan). Representative triple immunofluorescence images were acquired using a digital slide scanner (Pannoramic SCAN II, Jinan Denger Electronics, China).

Western Blotting (WB) Analysis

Total protein was extracted from hippocampal and cortical tissues using RIPA lysis buffer supplemented with protease and phosphatase inhibitors, and protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein (50 μ g per lane) were separated by SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature (RT), and then incubated overnight at 4°C with anti-TREM2 (Proteintech, China), anti-DAP12 (Abcam, UK), anti-TNF- α (Abcam, UK), anti-IL-10 (Proteintech, China), and anti- β -actin (Proteintech, China) primary antibodies. After washing, the membranes were incubated with HRP-

conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were visualized using an ECL detection kit and quantified by densitometry with ImageJ software. β -actin served as the internal loading control.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from hippocampal and cortical tissues of the mice using TRIzol® Reagent (Invitrogen, USA). Reverse transcription of total RNA into complementary DNA (cDNA) was performed using a PrimeScript™ RT Reagent Kit (TaKaRa, Japan). qRT-PCR was performed using SYBR® Green Premix Ex Taq™ Master Mix on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). The following gene-specific primer sequences were used in this study (GenBank Accession No.): β -actin (NM_007393.5): forward CTGAGAGGGAAATCGTGCGT, reverse CCACAGGATTCCATACCCAAGA; TREM2 (NM_031254): forward GAGGGTGTCATGTACTTATGACG, reverse GTTCTTCAGAGTGATGGTGACG; DAP12 (NM_011662): forward CTGACTGTGGGAGGATTAAGTCC, reverse CTGATAAGGCGACTCAGTCTCAG. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to the endogenous reference gene β -actin.

Enzyme-Linked Immunosorbent Assay (ELISA)

Peripheral blood samples were collected from the orbital venous plexus of deeply anesthetized mice, allowed to clot at room temperature for 30 min, and then centrifuged at $3,000 \times g$ for 15 min at 4°C to obtain the serum supernatant. The levels of soluble full-length TREM2 (sTREM2) and TREM2 in the serum were quantified using commercial ELISA kits (Jianglai Biotechnology, China; Yousi Biotechnology, China) in strict accordance with the manufacturers' instructions.

Single-Cell RNA Sequencing (scRNA-seq) Analysis

Subsequent to the cognitive behavioral tests, two mice from each experimental group were randomly selected and euthanized by cervical dislocation under deep anesthesia. The mouse brain tissues were rapidly dissected and collected and immediately placed in ice-cold tissue preservation solution. Following low-speed centrifugation, the tissue preservation solution was discarded and the brain tissues were washed twice with ice-cold Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA). The brain tissue samples were minced into 2–3 mm pieces and digested in a pre-warmed enzymatic digestion solution at 37°C with gentle pipetting every 15–20 min. The digestion process was monitored via trypan blue staining and terminated by the addition of complete DMEM medium when no visible tissue fragments were observed. The resulting single-cell suspension was filtered through a $40 \mu\text{m}$ sterile cell strainer, washed twice with pre-cooled complete DMEM medium, and then centrifuged at $300 \times g$ for 5 min at 4°C . Red blood cells (RBCs) in the cell pellet were lysed using ice-cold erythrocyte lysis buffer for 2–5 min at room temperature with gentle mixing, followed by the addition of an equal volume of pre-cooled PBS containing 0.04% BSA to terminate the lysis reaction. Following a second centrifugation at $300 \times g$ for 5 min at 4°C , the supernatant was carefully discarded, and the resulting cell pellet was retained. Only the single-cell samples with cell viability of more than 80% (assessed via trypan blue exclusion assay) were used for subsequent library construction.

The qualified single-cell suspensions were processed using the 10x Genomics Chromium Next GEM Single Cell 3' Reagent Kit v3.1 platform. Gel beads in emulsion (GEMs) were generated on the Chromium Controller system, followed by reverse transcription of the captured mRNA into cDNA within the GEMs. Following GEM disruption and cDNA purification, the scRNA-seq libraries were constructed via a series of steps including cDNA fragmentation, end repair, A-tailing, adapter ligation, and PCR amplification. Quality control (QC) was performed on the final constructed scRNA-seq libraries to assess library quality and concentration. Single-strand cDNA circularization was conducted, and the non-circularized linear DNA fragments were digested with specific nucleases. DNA nanoballs (DNBs) were generated via rolling circle amplification (RCA) and then sequenced on a DNBSE™ sequencing platform (BGI, Shenzhen, China) using the combinatorial probe-anchor synthesis (cPAS) technology. The entire scRNA-seq library construction and high-throughput sequencing were performed by BGI Genomics Co., Ltd (Shenzhen, China).

Statistical Analysis

All experimental data were analyzed using GraphPad Prism 10.1.2 and ImageJ software and presented as the mean $\pm$ standard error of the mean (SEM). Normality and homogeneity of variance were assessed by Shapiro–Wilk and Levene's tests, respectively. For Morris water maze acquisition (days 2–5), escape latency was analyzed by two-way repeated-measures ANOVA (group $\times$ day) with Bonferroni's post hoc test. For all other single-time-point measurements, group comparisons were performed using one-way ANOVA with Tukey's post hoc test for parametric data, or Kruskal–Wallis H test for non-parametric data. Statistical significance was set at $P < 0.05$. Behavioral tests were scored blinded to group allocation.

All single-cell RNA-seq bioinformatics analyses were performed using R software (v4.5.1) with the Seurat package (v5.0.0). Data preprocessing and integration. QC was conducted by filtering out cells with fewer than 200 detected genes or with total gene counts exceeding the 90th percentile. Cells with a mitochondrial read percentage in the top 15% were also excluded from downstream analysis. Doublets were identified and removed using DoubletDetection package. Cell cycle effects were evaluated using the CellCycleScoring function in Seurat package. Gene expression matrices were normalized and scaled, and the top 2000 highly variable genes (HVGs) were selected for principal component analysis (PCA, $n = 15$). Dimensionality reduction and unsupervised cell clustering were performed using uniform manifold approximation and projection (UMAP). Marker genes for each cluster were identified using FindAllMarkers function ($\log_{2}FC > 0.25$, $\text{min.pct} > 0.1$, $\text{padj} \leq 0.05$), and cell types were annotated using the SCSA automated annotation method. Differentially expressed genes (DEGs) between experimental groups were identified using FindMarkers with the identical statistical thresholds.

Microglial subpopulation analysis. Microglial cells were extracted and re-clustered using SCTransform normalization, PCA, UMAP, and clustering (resolution = 0.5). Microglial subclusters were defined based on canonical cell-type marker genes reported in previous studies, and differentially expressed genes among subclusters were identified as described above.

Functional enrichment analysis. Lists of significant DEGs were submitted to the Metascape online bioinformatics platform (<https://metascape.org>). Enrichment analysis was performed for Gene Ontology (GO) biological processes (BP), molecular functions (MF), cellular components (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the following parameters: species = *Mus musculus*, P-value < 0.01, minimum count = 3, enrichment factor > 1.5. Functional enrichment results and interactive visualizations were generated and exported from Metascape platform.

Cell–cell communication analysis. Intercellular communication networks were inferred and quantified using the CellChat package (v1.6.1) based on its built-in mouse ligand–receptor interaction database. Communication probabilities were calculated, differentially active signaling pathways were identified, and core signaling pathways were visualized for quantitative and qualitative comparisons among experimental groups.

Results

EA ameliorates cognitive deficits in APP/PS1 mice

To evaluate the cognitive effects of EA, APP/PS1 mice were subjected to the MWM, NOR, and OF tests following EA treatment (Fig. 1).

During the spatial acquisition training period of MWM, the Model group exhibited significantly longer escape latency compared to the Control group from day 4 onward ($P < 0.05$, Fig. 1A, C). EA treatment significantly reduced escape latency compared to the Model group ($P < 0.05$), an effect that was abolished by TREM2 knockdown (Fig. 1A, C). In the probe trial, the Model group showed reduced platform crossing frequency and shorter time in the target quadrant compared to the Control group ($P < 0.01$), whereas EA-treated mice exhibited increased platform crossings ($P < 0.05$), an effect reversed by TREM2 knockdown (Fig. 1B, F).

In the NOR test, the discrimination index (DI) was significantly lower in the Model group than in Control group ($P < 0.01$). EA treatment significantly increased the DI compared with the Model group ($P < 0.05$), whereas TREM2 knockdown abrogated this effect (Fig. 1D, G).

In the OF test, total travel distance was comparable across all groups, indicating that locomotor activity was not impaired in any group (Fig. 1E, H). However, the time spent in the central zone was significantly reduced in Model group mice compared with Control group ($P < 0.01$). This reduction was attenuated by EA treatment ($P < 0.05$ vs. Model group), an effect that was abrogated following TREM2 knockdown (Fig. 1F).

These results demonstrate that EA mitigates cognitive deficits and exploratory behavioral abnormalities in APP/PS1 mice via a TREM2-dependent mechanism.

EA attenuates neuronal injury and A β plaque deposition

Nissl staining was performed to assess neuronal morphological integrity in the hippocampus and cortex (Fig. 2A). Consistent with our previous findings, the Model group exhibited significant neuronal loss, Nissl body dissolution, and pallid Nissl staining compared with the Control group. The AAV-Ctrl and AAV-shTREM2 groups exhibited similar neuronal pathological changes to those in the Model group. EA treatment markedly attenuated these neuronal abnormalities, as evidenced by the improved neuronal morphological integrity in the EA group. However, the neuroprotective effect of EA was significantly abrogated by TREM2 knockdown, with the EA + AAV-shTREM2 group exhibiting no significant improvement in neuronal morphology compared with the EA group.

Congo Red staining was performed to quantify A β plaque deposition in the hippocampus and cortex (Fig. 2B). Both the AAV-Ctrl and AAV-shTREM2 groups displayed typical orange-red A β plaques with irregular or diffuse morphological characteristics, which were consistent with those in the Model group. Notably, the AAV-shTREM2 group exhibited a significantly greater number of A β plaques with a more diffuse distribution throughout the brain. Although the EA + AAV-shTREM2 group exhibited a mild reduction in A β plaque burden compared with the AAV-shTREM2 group, A β plaque deposition remained significantly higher in the EA + AAV-shTREM2 group than in the EA group. These results demonstrate that EA attenuates neuronal injury and A β plaque pathology in APP/PS1 mice, and that these neuroprotective effects are predominantly dependent on TREM2.

EA upregulates TREM2/DAP12 expression in the brain and periphery

qPCR and western blot analyses were performed to examine TREM2 and DAP12 expression in the hippocampus and cortex (Fig. 3A-F). Compared with Control group, the Model group exhibited significantly increased protein levels of both TREM2 and DAP12 in the hippocampus and cortex ($P < 0.05$). EA treatment further elevated TREM2 and DAP12 protein expression compared to the Model group ($P < 0.05$), consistent with our initial findings. TREM2 knockdown effectively reduced TREM2 and DAP12 protein levels, as evidenced by significantly lower expression in the AAV-shTREM2 group compared with the AAV-Ctrl group ($P < 0.05$). Similarly, the EA + AAV-shTREM2 group exhibited significantly reduced TREM2 and DAP12 protein levels compared to the EA group alone ($P < 0.05$). No significant differences were observed between the Model and AAV-Ctrl groups or between the AAV-shTREM2 and EA + AAV-shTREM2 groups. mRNA expression levels of TREM2 and DAP12 in the cortex followed a similar pattern to their protein levels.

ELISA was performed to measure TREM2 and sTREM2 levels in serum (Fig. 3I, J). Consistent with the tissue findings, serum TREM2 and sTREM2 levels were significantly lower in the EA + AAV-shTREM2 group compared to the EA group ($P < 0.05$). No significant differences were detected among the Model, AAV-Ctrl, AAV-shTREM2, and EA + AAV-shTREM2 groups.

These results demonstrate that EA upregulates TREM2 and DAP12 expression both in the brain and periphery, and that this upregulation is effectively blocked by TREM2 knockdown.

EA modulates microglial neuroinflammation via TREM2

Immunofluorescence double staining was performed to assess microglial phenotypes using iNOS (pro-inflammatory) and Arg1 (repair-associated) in combination with Iba1 (Fig. 4). Quantitative analysis revealed that, compared with the Control group, the Model group exhibited a significantly higher proportion of iNOS⁺/Iba1⁺ microglia and a lower proportion of Arg1⁺/Iba1⁺ microglia in both cortex and hippocampus (all $P < 0.05$). EA treatment significantly reversed this imbalance, reducing the pro-inflammatory population and increasing the repair-associated population relative to the Model group ($P < 0.05$). These effects were largely abolished by TREM2 knockdown, as the EA + AAV-shTREM2 group showed a significantly higher pro-inflammatory percentage and lower repair-associated percentage compared with the EA group alone ($P < 0.05$).

WB analysis of inflammatory cytokines showed that IL-10 protein levels were significantly decreased, whereas TNF- α levels were significantly increased, in the hippocampus and cortex of Model mice compared with those in the Control group (both $P < 0.05$, Fig. 4). EA treatment significantly elevated IL-10 and reduced TNF- α expression relative to the Model group (both $P < 0.05$). However, these anti-inflammatory effects of EA were markedly attenuated by TREM2 knockdown, with the EA + AAV-shTREM2 group showing significantly lower IL-10 and higher TNF- α levels compared to the EA group (both $P < 0.05$).

These results demonstrate that EA promotes a shift from a pro-inflammatory toward a repair-associated microglial phenotype and modulates cytokine balance in a TREM2-dependent manner.

Essential role of TREM2 in EA-enhanced microglial recruitment and phagocytosis at A β plaques

Triple immunofluorescence staining for A β , TREM2, and IBA1 was performed to examine the spatial relationship between TREM2-positive microglia and A β plaques (Fig. 5A,B). A β deposition was observed in all groups except Control group. Compared with the EA group, the Model, AAV-Ctrl, AAV-shTREM2, and EA + AAV-shTREM2 groups displayed a greater number of A β plaques. Notably, the AAV-shTREM2 and EA + AAV-shTREM2 groups exhibited more diffuse plaque morphology and reduced microglial clustering around plaques. Quantitative analysis of TREM2-positive microglia within 50 μ m of A β plaques revealed significant accumulation in the Model, EA, and AAV-Ctrl groups. Notably, the EA group showed significantly more plaque-associated TREM2-positive microglia than the Model and AAV-Ctrl groups ($P < 0.05$), suggesting that EA enhances microglial recruitment to A β plaques.

To assess microglial phagocytic function, triple immunofluorescence staining for TREM2, IBA1, and CD68 was performed (Fig. 5C, D). In the Control group, microglia exhibited a ramified, resting morphology with low expression of TREM2 and CD68. In the Model group, some microglia showed activated morphology with increased TREM2 and CD68 signals. The EA group displayed robust TREM2 and CD68 co-localization in microglia, with cells forming ring-like clusters around plaques and abundant

CD68-positive intracellular vesicles, indicating active phagocytosis. In contrast, CD68 signal was markedly reduced in the AAV-shTREM2 and EA + AAV-shTREM2 groups despite persistent microglial activation. Quantitative analysis confirmed that the proportion of CD68⁺/TREM2⁺/IBA1⁺ microglia was significantly higher in the EA group than in the Model group ($P < 0.05$).

These results demonstrate that EA promotes both the recruitment of TREM2-positive microglia to A β plaques and their phagocytic activation, and that these effects are dependent on TREM2.

Single-cell transcriptomics reveals TREM2-dependent reprogramming of microglial heterogeneity and intercellular communication by EA

To elucidate the cellular and molecular mechanisms by which EA exerts its TREM2-dependent neuroprotective effects, we performed scRNA-seq on brain tissue from four groups of mice (Control, Model, EA, and EA + AAV-shTREM2). Following stringent quality control, a total of 68,298 high-quality cells were retained for analysis. Unsupervised clustering identified 26 distinct cell clusters (Fig. 6A), which were annotated into eight major cell types based on canonical lineage markers (Fig. 6B): microglia, astrocytes, endothelial cells, oligodendrocytes, pericytes, macrophages, T cells, and B cells. The cellular composition was comparable across groups (Control: 17,217 cells; Model: 16,575 cells; EA: 16,930 cells; EA + AAV-shTREM2: 17,576 cells).

EA alters cellular composition in a TREM2-dependent manner

Analysis of cellular composition revealed that the proportion of microglia was increased in the Model group compared with Control group, indicating neuroimmune activation under AD pathological conditions (Fig. 6C). This microglial proportion was further elevated by EA. Notably, this effect was partially reversed by TREM2 knockdown in the EA + AAV-shTREM2 group, which concomitantly showed a relative increase in astrocyte proportions. DEG analysis across groups showed that the most pronounced transcriptional changes occurred in endothelial cells and microglia (Fig. 6D). Specifically, comparison between the EA and Model groups showed that microglial clusters 1, 2, and 7 exhibited the highest number of DEGs, suggesting that EA may specifically modulate distinct microglial subpopulations.

EA drives TREM2-dependent protective microglial reprogramming

To investigate the effect of EA on microglial heterogeneity, we performed a subclustering analysis of all microglia. This analysis identified five distinct subpopulations (Fig. 7A), which were annotated based on established gene signatures: homeostatic microglia (HM) [21, 22], disease-associated microglia (DAM) [11], cycling/proliferating microglia (CPM) [23], MHC class II-expressing microglia (MHCII) [23], and

interferon-responsive microglia (IRM) [22, 23]. The molecular identity of each cluster was confirmed by the expression of their signature genes (Fig. 14B). Consistent with previous reports [11], TREM2 expression was predominantly restricted to the DAM subpopulation (Fig. 7B, C)

Analysis of the distribution of cells from each experimental group within these microglial subpopulations revealed that EA specifically altered microglial phenotypic polarization (Fig. 7D). Notably, within the DAM subpopulation, the proportion of cells originating from the EA group was markedly higher than that from the Model group (EA: 44.4% vs. Model: 38.2%), indicating that EA promotes adoption of this protective microglial state. Crucially, this effect was completely abrogated by TREM2 knockdown, with the EA + AAV-shTREM2 group constituting only 15.8% of the DAM cluster. This demonstrates that TREM2 is an essential molecular target for EA-induced microglial reprogramming toward a DAM phenotype.

To further delineate the functional consequences of EA on DAM, we performed differential expression analysis between groups. Compared with the Model group, EA upregulated genes in DAM associated with phagocytosis (e.g., *Ms4a7*, *Adgre5*), antioxidant responses (e.g., *Gpx1*, *Gpx3*), and lipid metabolism and transport (e.g., *Apoe*, *Lpl*), whereas downregulating genes linked to stress and inflammatory signaling (e.g., *Btg1*, *Hsp90aa1*) (Fig. 7E). This suggests EA promotes a DAM phenotype with enhanced phagocytic and protective capabilities.

GO enrichment analysis of these DEGs showed that EA broadly activated biological processes in DAM, including immune cell activation/migration, response to oxidative stress, and lipid metabolism (Fig. 7H). To identify which of these processes are specifically TREM2-dependent, we compared the EA and EA + AAV-shTREM2 groups. TREM2 knockdown significantly attenuated the EA-induced protective gene expression program. Genes upregulated by EA (e.g., *Apoe*, *Gpx3*) were no longer induced, while pro-inflammatory genes (e.g., *Ifi44*, *Cst7*) were upregulated in the EA + AAV-shTREM2 group. Correspondingly, functional enrichment analysis revealed that TREM2 was specifically required for EA-mediated regulation of processes such as positive regulation of mononuclear cell migration, cellular import, phospholipid efflux, and regulation of apoptotic cell clearance (Fig. 7I). These findings establish TREM2 as a core mediator of a specific subset of functional effects of EA on DAM, particularly those related to migration, endocytosis, and phagocytosis-associated lipid metabolism.

EA rewires intercellular communication networks through TREM2

We next employed CellChat to analyze how EA and TREM2 shape the intercellular communication landscape. Overall, the communication network was more complex in all AD groups compared with the Control group, with DAM and HM acting as central hubs (Fig. 8A). Quantitative analysis confirmed that DAM and HM in the EA group were the most active in both sending and receiving signals (Fig. 8B, C).

Chord diagrams illustrating specific ligand-receptor interactions between HM and DAM revealed that AD pathology (Model group) was characterized by pro-inflammatory signaling (e.g., *Csf1-Csf1r*, *Tnf-Tnfrsf1a*)

(Fig. 8D). EA intervention suppressed these pro-inflammatory signals while enhancing protective ones, such as Cxcl16-Cxcr6 signaling (Fig. 8E). Crucially, this EA-induced protective signaling pattern was largely lost upon TREM2 knockdown, with concomitant resurgence of pro-inflammatory interactions (Fig. 8F).

Differential interaction analysis further corroborated these observations. The AD brain (Model vs. Control) showed enhanced signaling from DAM to vascular cells (endothelial cells and pericytes), suggesting involvement of blood-brain barrier dysfunction (Fig. 2S). EA treatment (EA vs. Model) specifically attenuated this pathological DAM-vascular communication while strengthening immunomodulatory interactions, such as those between T cells and HM (Fig. 2S). This EA-mediated network remodeling was again highly dependent on TREM2, as its knockdown reinstated pathological DAM-vascular signaling and diminished protective T cell-HM communication (Fig. 2S). Analysis of specific ligand-receptor pairs confirmed that the effect of EA was a targeted correction of key pathological signals (e.g., Spp1-Cd44, Hbegf-ErbB4) and activation of protective checkpoints (e.g., Cd80/86-Ctla4), a pattern that was profoundly reversed by TREM2 deficiency. Collectively, these data demonstrate that TREM2 is indispensable for EA-mediated reprogramming of microglial function and re-establish of a protective multicellular communication network in the AD brain.

Discussion

AD is a progressive neurodegenerative disorder characterized by irreversible cognitive decline, for which currently approved disease-modifying therapies remain limited[24–26]. In this study, we combined behavioral assessments, histological analysis, molecular biology, and scRNA-seq to demonstrate that EA ameliorates cognitive deficits in APP/PS1 mice by upregulating TREM2 to reprogram microglial phenotypic and transcriptional profiles, enhance A β clearance, and remodel intercellular communication networks in the AD brain. Additionally, our findings identify TREM2 as a critical mediator of the microglial functional benefits conferred by EA, and highlight the TREM2-driven DAM as a potential mechanistic target for EA in AD treatment.

A core finding of this study is that EA exerts cognitive-protective effects in APP/PS1 mice in a strictly TREM2-dependent manner. Behavioral tests (Morris Water Maze, Novel Object Recognition, and Open Field Test) confirmed that EA significantly improved spatial learning and memory, recognition memory, and exploratory behavior in AD mice, whereas intracerebroventricular injection of AAV-shTREM2 completely abrogated these benefits. Histological analyses further revealed that EA attenuated neuronal loss and reduced A β plaque deposition. Neuronal loss was assessed via Nissl staining, and A β plaque deposition was assessed via Congo Red staining. Notably, these neuroprotective effects were abolished by TREM2 knockdown. Consistent with these phenotypic alterations, EA upregulated the mRNA and protein expression of TREM2 and its signaling adaptor DAP12 in the hippocampus and cortex, which are key brain regions vulnerable to AD pathology. Additionally, EA increased the serum levels of circulating TREM2 and soluble TREM2 (sTREM2). These results extend previous studies on the neuroprotective effects of EA in AD[20, 27] by identifying TREM2 as an indispensable molecular target: EA does not

merely alleviate AD-related pathologies but modulates a core innate immune receptor critical for microglial functional homeostasis in AD. Notably, the upregulation of TREM2 by EA was observed in both the CNS and periphery, suggesting a systemic modulation of TREM2 signaling. Given that sTREM2 is a promising AD biomarker[28], the EA-induced increase in peripheral sTREM2 also highlights the potential translational value of EA in regulating AD-related peripheral immune responses.

Microglial activation-state remodeling is a key process in AD-related neuroinflammation, in which pro-inflammatory programs exacerbate neuronal damage while homeostatic or tissue-reparative programs promote tissue repair and A β clearance[29–31]. Our study demonstrated that EA attenuated AD-associated pro-inflammatory microglial activation and promoted reparative microglial states in the hippocampus and cortex, as reflected by a reduced proportion of iNOS⁺/Iba1⁺ cells and an increased proportion of Arg1⁺/Iba1⁺ cells, accompanied by decreased TNF- α and increased IL-10. TREM2 knockdown abrogated this immunomodulatory effect, restoring the pro-inflammatory microglial phenotype. Beyond phenotypic polarization, EA specifically promoted the recruitment of TREM2⁺ microglia to A β plaques and enhanced their phagocytic activity, as evidenced by triple immunofluorescence staining which revealed increased TREM2⁺/Iba1⁺ microglial clustering around A β plaques and elevated CD68⁺/TREM2⁺/Iba1⁺ triple-positive cells in EA-treated mice. TREM2 deficiency not only reduced microglial recruitment to A β plaques but also induced a morphological shift in plaques from compact to diffuse, a pathological feature associated with increased A β neurotoxicity [15]. These results indicate that EA modulates microglial function at two key levels— inflammatory polarization and A β -directed phagocytosis—with both processes being dependent on TREM2. This finding aligns with the known role of TREM2 in regulating microglial chemotaxis and phagocytosis[10, 32] and highlights that EA harnesses TREM2 to shift microglia from a pathological pro-inflammatory state to a protective state capable of efficient A β clearance, thereby breaking the vicious cycle of A β accumulation and neuroinflammation in AD.

To dissect the TREM2-dependent effects of EA on microglial heterogeneity at single-cell resolution, we performed scRNA-seq on brain tissue from Control, Model, EA, and EA + AAV-shTREM2 mice. Bulk tissue analysis cannot capture the cellular diversity of the AD brain, and scRNA-seq has emerged as a powerful tool to unravel microglial subpopulation dynamics in neurodegenerative diseases[21, 33]. Our scRNA-seq analysis identified eight major cell types in the mouse brain, with microglia exhibiting the most pronounced transcriptional changes in response to EA and TREM2 knockdown. Subclustering of microglia identified five distinct subpopulations: homeostatic microglia (HM), disease-associated microglia (DAM), cycling/proliferating microglia (CPM), MHC class II-expressing microglia (MHCII), and interferon-responsive microglia (IRM). This classification is consistent with previous studies on microglial heterogeneity in AD [23, 24]. Notably, TREM2 expression was predominantly restricted to the DAM subpopulation, confirming DAM as the primary TREM2-expressing microglial subset in AD[11, 34]. EA significantly increased the proportion of DAM in APP/PS1 mice, and this effect was completely abolished by TREM2 knockdown, demonstrating that EA promotes DAM expansion in a TREM2-dependent manner. More importantly, EA did not simply increase DAM numbers but reprogrammed their transcriptional profile: DAM from EA-treated mice exhibited upregulation of genes associated with

phagocytosis (Ms4a7, Adgre5), lipid metabolism and transport (Apoe, Lpl), and antioxidant responses (Gpx1, Gpx3), while downregulating pro-inflammatory and stress-related genes (Btg1, Hsp90aa1). Lipid metabolism is a critical process for microglial phagocytosis of A β , as A β clearance is accompanied by extensive lipid remodeling [23, 35], and Apoe—a key lipid transport gene upregulated by EA—is a well-established AD risk gene that modulates microglial A β phagocytosis[28]. The EA-induced upregulation of antioxidant genes also suggests a role for TREM2-dependent modulation of oxidative stress, a secondary pathological event in AD that exacerbates neuronal damage[6]. TREM2 knockdown reversed these transcriptional changes in DAM, restoring a pro-inflammatory and anti-phagocytic profile, indicating that TREM2 is a core regulator of EA-induced DAM functional maturation. These results extend the current understanding of DAM in AD. While AD pathology alone induces partial DAM activation[11], EA via TREM2 drives the full maturation of DAM into a protective phagocytic and antioxidant phenotype, a state critical for effective A β clearance and neuroinflammation resolution.

The CNS is a sophisticated cellular network, and AD pathogenesis involves disrupted intercellular communication among microglia, neurons, astrocytes, and vascular cells[36, 37]. CellChat analysis revealed that AD pathology in the model group triggered a pro-inflammatory communication pattern dominated by DAM. This pattern was accompanied by elevated pro-inflammatory signaling such as Csf1-Csf1r and Tnf-Tnfrsf1a pathways, as well as abnormal DAM-vascular cell crosstalk involving endothelial cells and pericytes. These observations align with previous studies connecting DAM activation to BBB dysfunction in AD[37, 38]. EA treatment selectively inhibited these pathological signaling pathways and activated protective intercellular communication, including the Cxcl16-Cxcr6 axis between microglia and other immune cells. This axis has been reported to mitigate AD pathology by regulating immune cell infiltration and neuroinflammation in the CNS[39]. The EA-mediated remodeling of cellular communication was strongly dependent on TREM2. TREM2 knockdown restored pro-inflammatory DAM signaling and pathological DAM-vascular interactions, and disrupted protective immune crosstalk such as T cell-HM interactions. Notably, EA did not globally alter the entire communication network. Instead, it induced targeted and moderate rebalancing of key signaling nodes, suppressing pathological pathways without excessive immune activation. This represents a major advantage over conventional pharmacological interventions, which frequently cause off-target effects via systemic immune stimulation. Our findings indicate that TREM2 acts not only as a regulator of microglial cell-autonomous function but also as a central hub for EA-driven remodeling of the CNS cellular network. By modulating TREM2-dependent DAM activity, EA restores homeostatic intercellular communication and re-establishes immune balance in the AD brain.

This study has several strengths that enhance the robustness of our findings. First, we employed a TREM2-knockdown model to determine whether the neuroprotective effects of EA are TREM2-dependent, thereby overcoming the limitations of observational studies and establishing a causal role for TREM2 in EA-induced neuroprotection. Second, we integrated multi-scale analyses (behavioral, histological, molecular, and single-cell transcriptomic) to elucidate the mechanisms of EA from phenotypic changes to cellular and molecular regulation, providing a comprehensive mechanistic framework. Third, our scRNA-seq and CellChat analyses unraveled the TREM2-dependent effects of EA

on microglial heterogeneity and intercellular communication at an unprecedented resolution, filling the gap in understanding the single-cell mechanisms of EA in AD.

Nonetheless, several limitations should be acknowledged. To begin with, this study was conducted exclusively in APP/PS1 mice, which primarily model amyloid pathology; validation in tauopathy or humanized models is warranted. Additionally, while our TREM2 knockdown approach established causality, we did not distinguish between central and peripheral TREM2 contributions, nor include a sham-EA control group to exclude non-specific handling effects. Furthermore, the fixed EA parameters used here require further optimization through dose–response studies. Fourth, the scRNA-seq analysis was based on two mice per group, and although we obtained sufficient cell numbers with rigorous quality control, larger-scale sequencing is needed to confirm the cell population dynamics. Moreover, this is a preclinical study, and clinical translation remains to be evaluated in future trials.

Conclusion

Altogether, these findings suggest that EA improves cognitive function in APP/PS1 mice. Mechanistically, EA upregulates TREM2, which shifts microglia from a pro-inflammatory toward a repair-associated phenotype. This phenotypic shift subsequently enhances A β phagocytosis and promotes protective DAM maturation. Moreover, our results support TREM2-dependent microglial reprogramming as an important mechanism underlying the neuroprotective effects of EA. Furthermore, they suggest that EA holds potential as a potentially safe, non-pharmacological intervention for AD, with TREM2 serving as a candidate efficacy biomarker.

Declarations

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics approval

This study was approved by the Institutional Animal Care and Use Committee of Hubei University of Chinese Medicine (Approval Number: HUCMS 74452727). All procedures were performed in accordance with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Author Contribution

Qian Tan and Anqi Xie contributed equally to this work as co-first authors. Qian Tan: Investigation (lead), Writing – original draft (equal), Writing – review & editing (equal), Visualization (lead). Anqi Xie: Investigation (lead), Writing – original draft (equal), Writing – review & editing (equal), Visualization (equal). Jiangmin Chen: Investigation (supporting), Formal analysis (supporting), Data curation (supporting). Sican Wei: Investigation (supporting), Formal analysis (supporting), Data curation (supporting). Yu Guo: Investigation (supporting), Formal analysis (supporting), Data curation (supporting). Guangya Liu: Investigation (supporting), Formal analysis (supporting), Data curation (supporting). Li Wang: Methodology (supporting), Investigation (supporting), Validation (supporting). Xinyuan Liu: Methodology (supporting), Investigation (supporting), Validation (supporting). Yanjun Du: Conceptualization (lead), Funding acquisition (lead), Project administration (lead), Supervision (lead), Writing – review & editing (lead). The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to the large size of the sequencing data, but are available from the corresponding author on reasonable request. The scRNA-seq data will be deposited in the Gene Expression Omnibus (GEO) upon acceptance.

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Figures

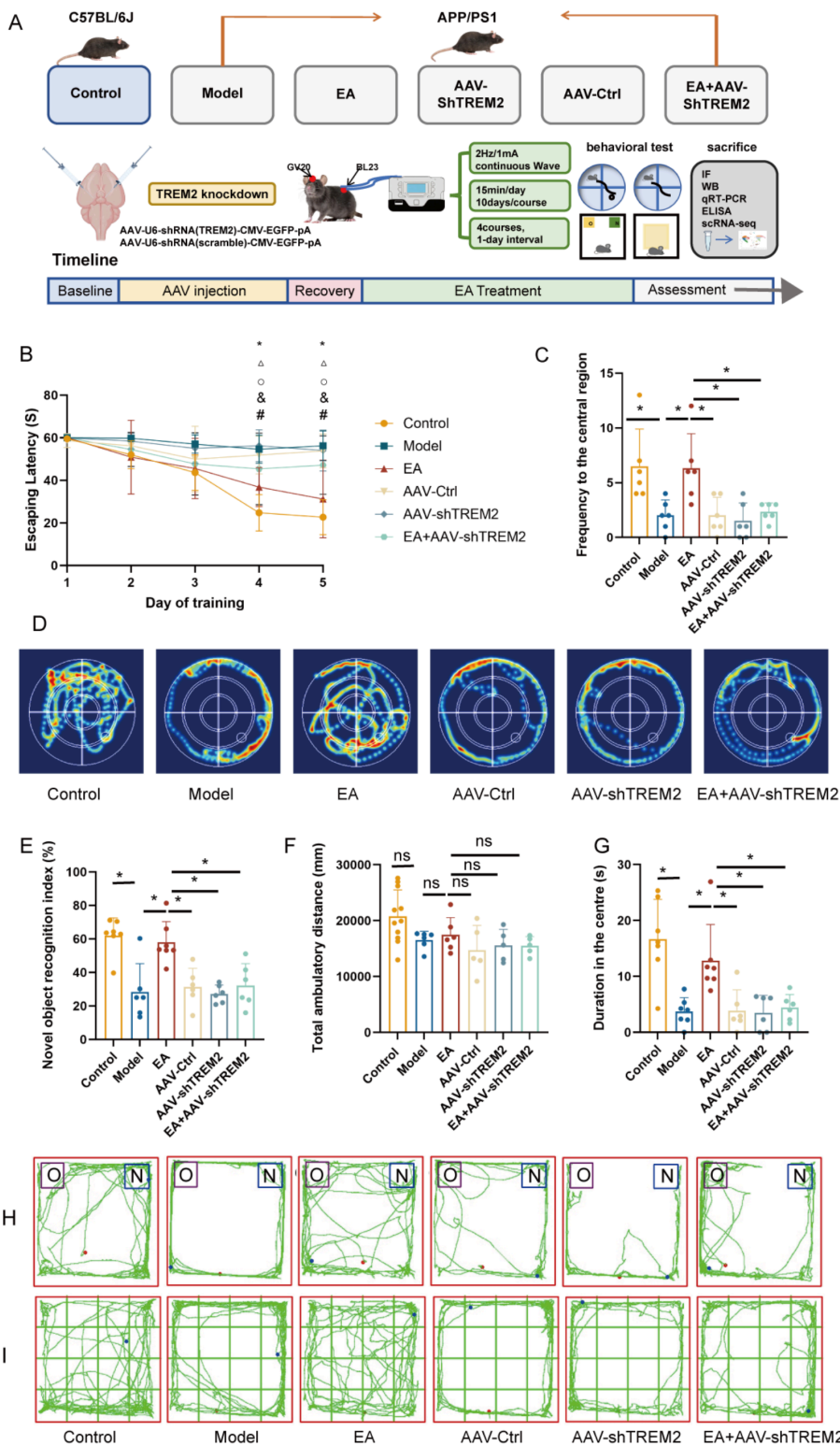


Figure 1

Behavioral performance of mice in each group. (A) Workflow of TREM2 knockdown and EA treatment. (B) Escape latency to the hidden platform in the MWM test. (C) Number of platform crossings in the MWM probe trial. (D) Representative swimming paths during the MWM probe trial. (E) DI in the NOR test, calculated as: Time exploring the novel object / (Time exploring the novel object + familiar object) × 100%. (F) Total distance traveled in the open field test (OFT). (G) Time spent in the center zone of the

OFT arena. (H) Representative exploration paths in the NOR test. (I) Representative movement paths in the OFT. Data are presented as mean $\pm$ SEM (or SD). * $P < 0.05$, ** $P < 0.01$ vs. Control group; $\Delta P < 0.05$ vs. Model group; $P < 0.05$ vs. AAV-Ctrl group; # $P < 0.05$ vs. AAV-shTREM2 group; & $P < 0.05$ vs. EA+AAV-shTREM2 group.

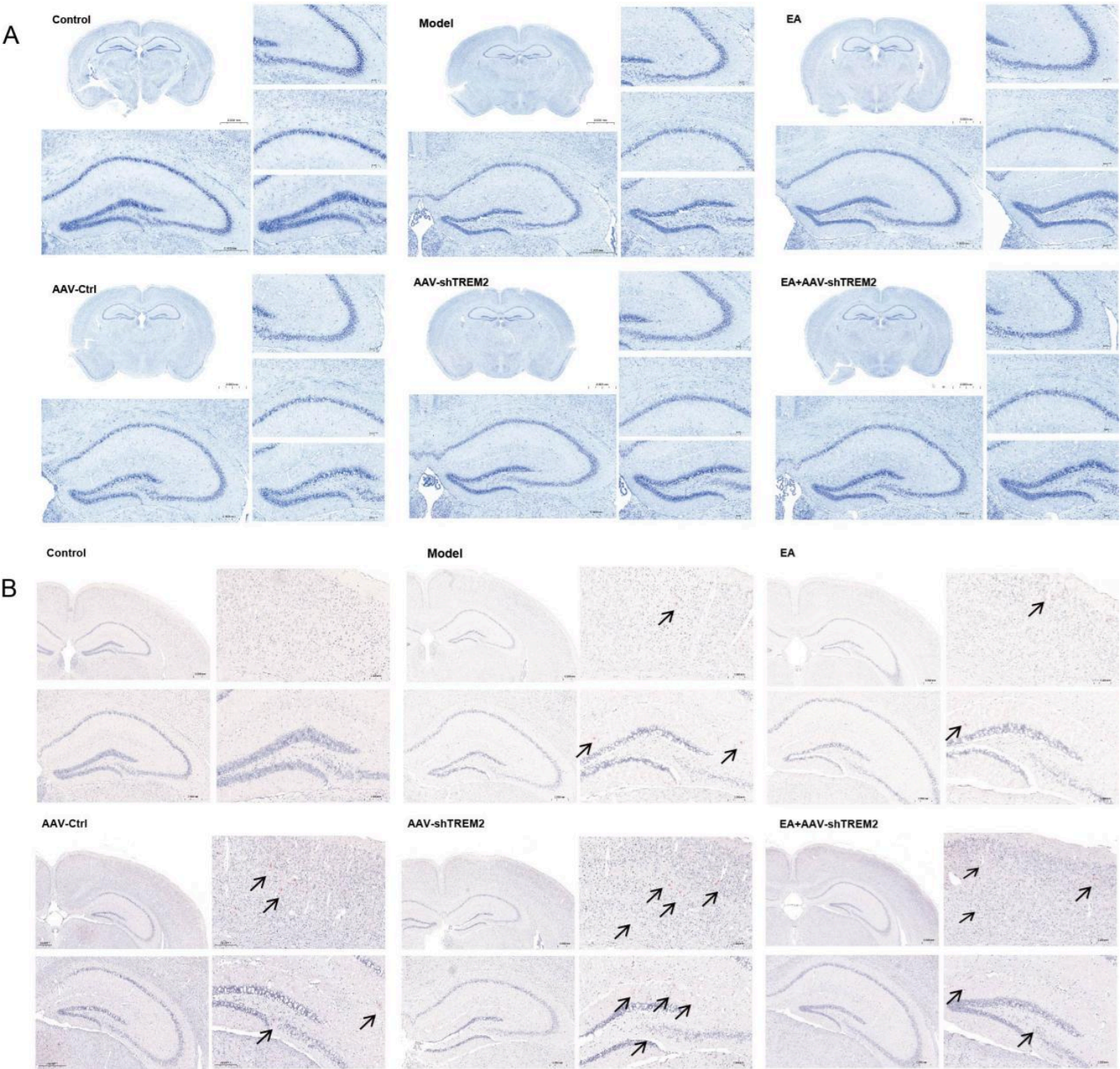


Figure 2

Morphological results of brain tissue in each group. (A) Representative images of Nissl staining in the brain tissue of mice from each group. (B) Representative images of Congo Red staining in the brain tissue of mice from each group.

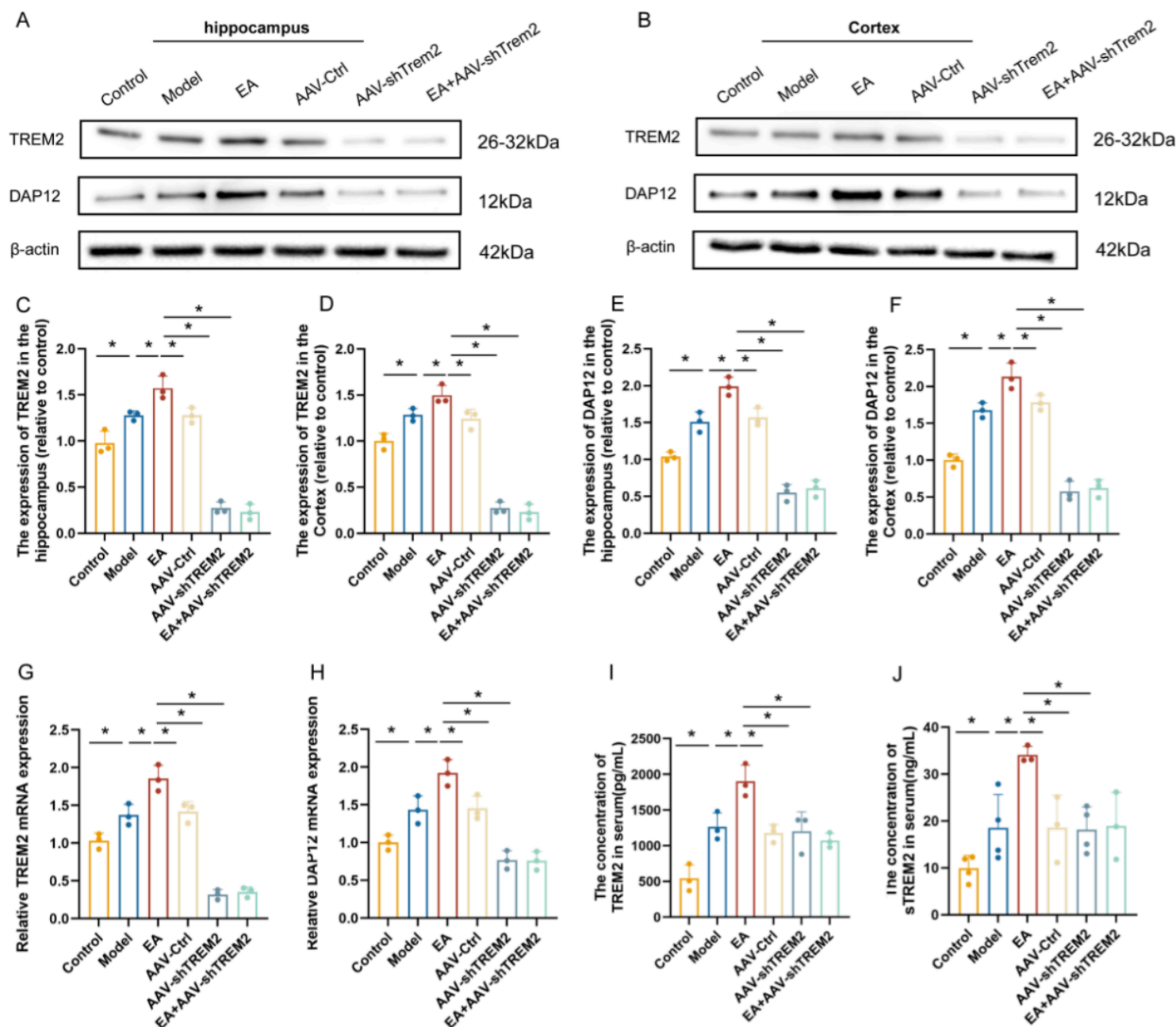


Figure 3

TREM2 and DAP12 protein and mRNA expression in brain tissue and serum ELISA results of mice in each group. (A) Representative western blot bands of TREM2 and DAP12 proteins in the hippocampus. β-actin was used as a loading control. (B) Representative western blot bands of TREM2 and DAP12 proteins in the cortex. β-actin was used as a loading control. (C, E) Quantitative analysis of relative TREM2 protein expression in the hippocampus and cortex, respectively. (D, F) Quantitative analysis of relative DAP12 protein expression in the hippocampus and cortex, respectively. (G) Relative TREM2 mRNA expression in the cortex. (H) Relative DAP12 mRNA expression in the cortex. (I, J) Protein expression levels of TREM2 and sTREM2 in serum measured by ELISA. Data are presented as mean ± SEM. * $P < 0.05$.

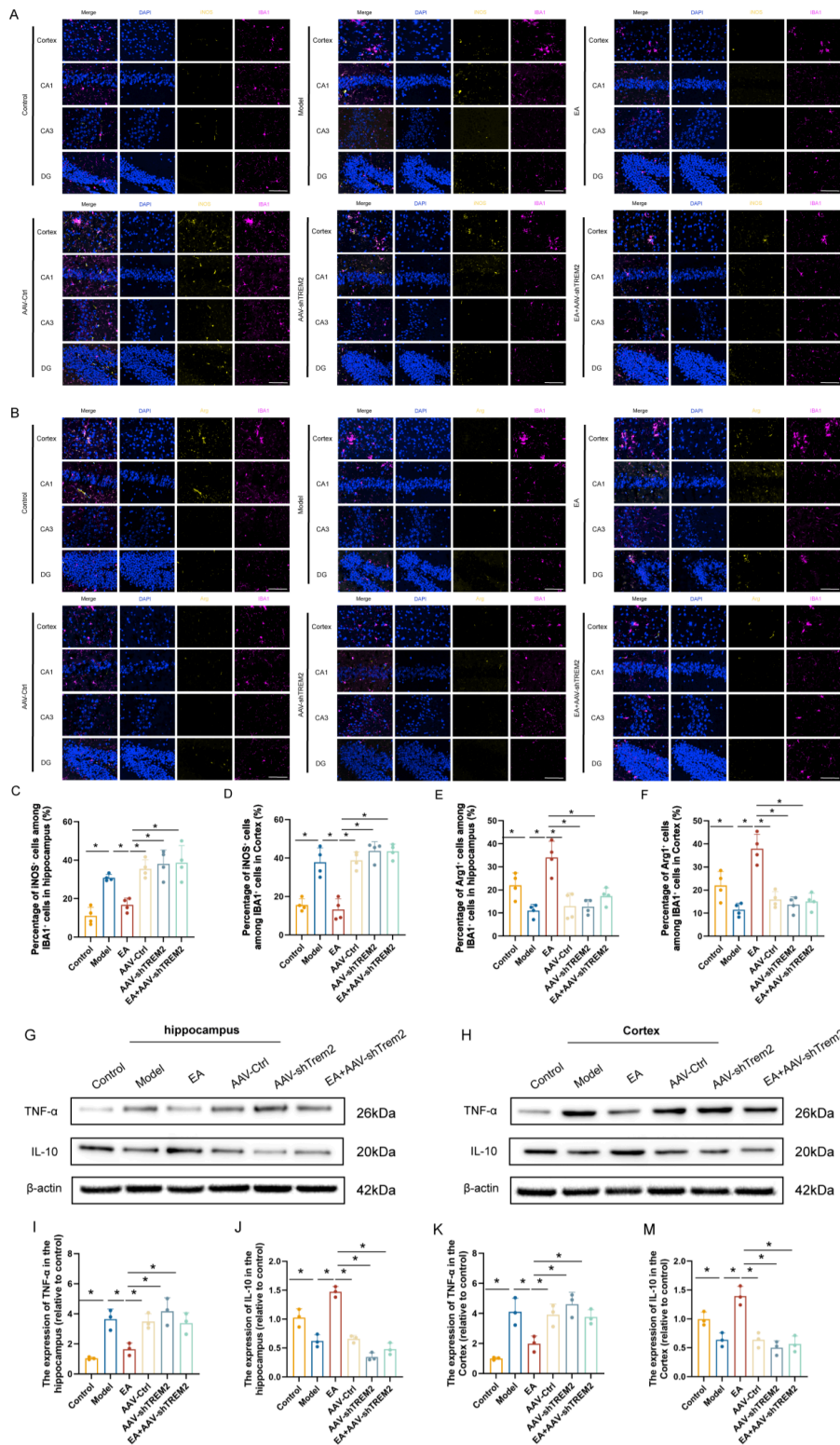


Figure 4

Electroacupuncture modulates microglial pro-inflammatory and repair-associated phenotypes and inflammatory cytokine expression in the hippocampus and cortex. (A) Representative images of pro-inflammatory microglia (iNOS⁺/Iba1⁺) in the cortex and hippocampal CA1, CA3, and DG regions. (B) Representative images of repair-associated microglia (Arg1⁺/Iba1⁺) in the same regions. Scale bar = 50 μ m. (C–F) Quantification of microglial proportions in the cortex and hippocampus. Data are presented

as mean $\pm$ SEM. $P < 0.05$. (G, H) Representative Western blot bands of IL-10 and TNF- α in the hippocampus and cortex, with GAPDH/ β -actin as loading control. (I–L) Quantification of relative protein expression. Data are presented as mean $\pm$ SEM. $P < 0.05$.

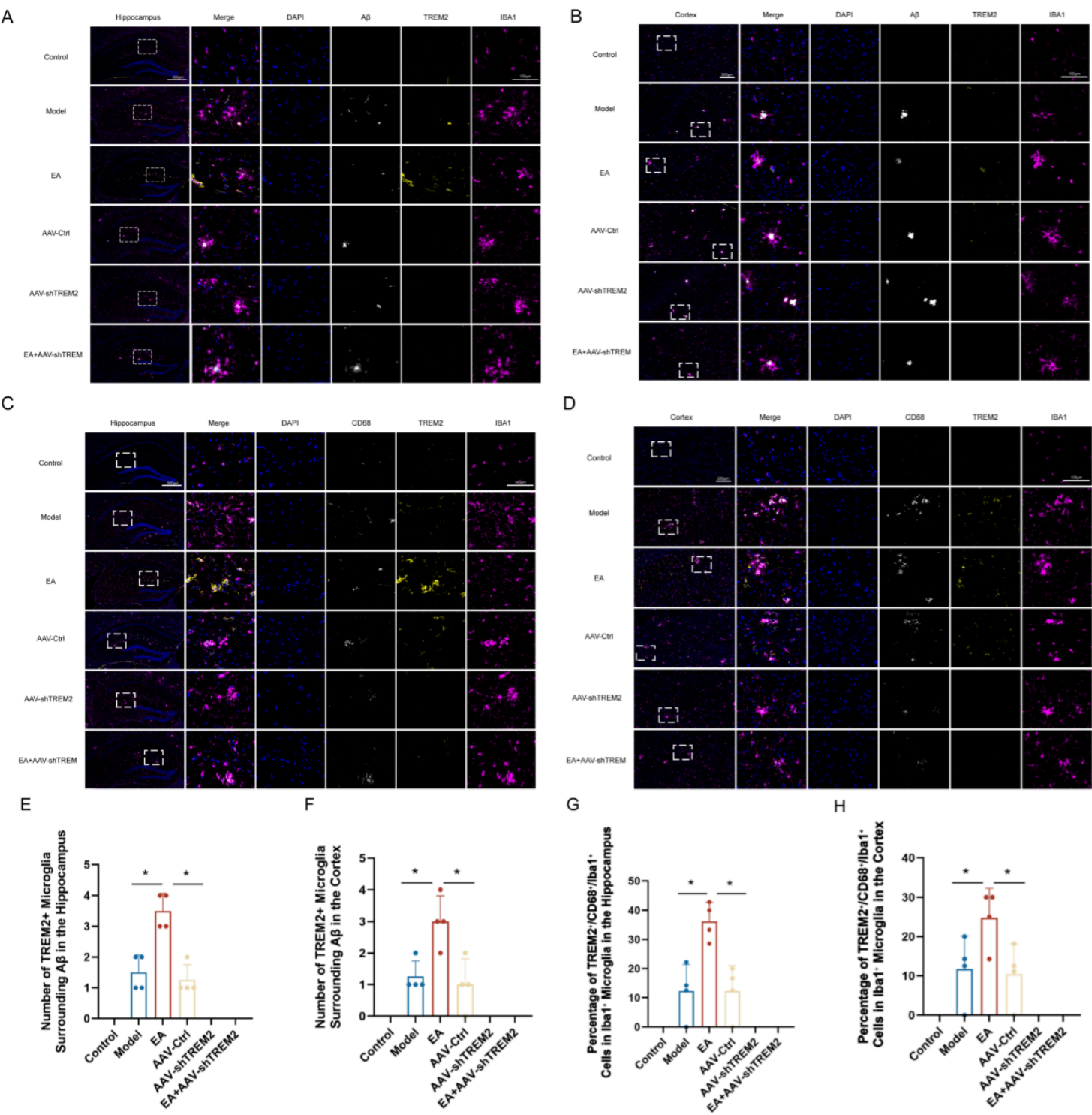


Figure 5

Microglial TREM2 expression around A β plaques and colocalization with CD68 in the hippocampus and cortex of mice from each group. (A) Representative immunofluorescence images of TREM2

(yellow)/IBA1 (magenta)/A β (white) triple staining in the hippocampus. (B) Representative images of TREM2 (yellow)/IBA1 (magenta)/A β (white) triple staining in the cortex. (C) Quantitative analysis of the number of TREM2⁺ microglia within a 50 μ m radius around A β plaques in the hippocampus. (D) Quantitative analysis of the number of TREM2⁺ microglia within a 50 μ m radius around A β plaques in the cortex. (E) Representative immunofluorescence images of TREM2 (yellow)/IBA1 (magenta)/CD68 (white) triple staining in the hippocampus. Scale bar = 50 μ m. (F) Representative images of TREM2 (yellow)/IBA1 (magenta)/CD68 (white) triple staining in the cortex. Scale bar = 50 μ m. (G) Percentage of TREM2⁺/CD68⁺/IBA1⁺ triple-positive cells among total IBA1⁺ cells in the hippocampus. (H) Percentage of TREM2⁺/CD68⁺/IBA1⁺ triple-positive cells among total IBA1⁺ cells in the cortex. Data are presented as mean $\pm$ SD, **P* < 0.05.

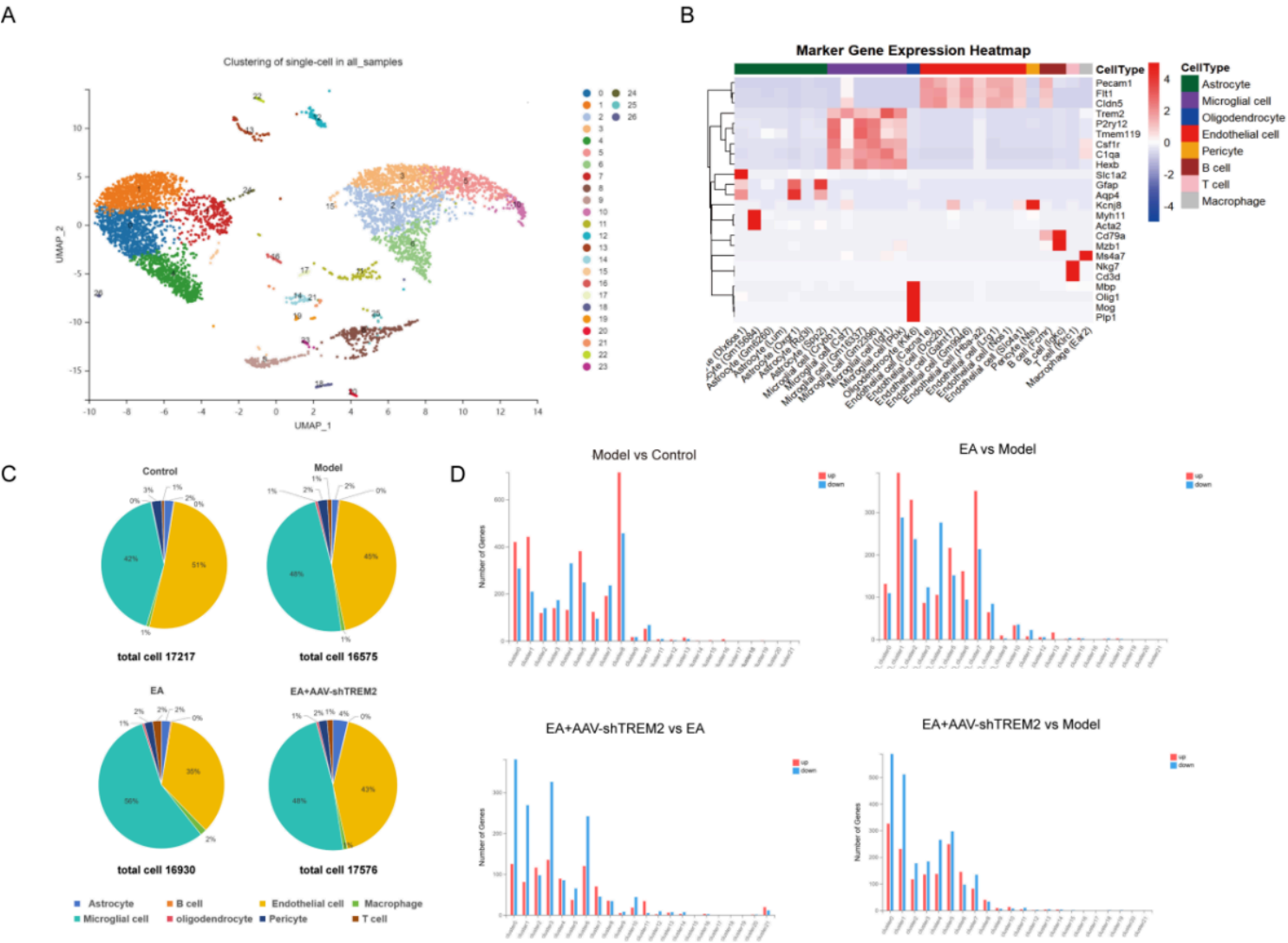


Figure 6

Cell clustering and compositional differences analysis of multi-group single-cell sequencing data. (A) UMAP dimensionality reduction plot of all cells. Single-cell data from Control, Model, EA, and EA+AAV-shTREM2 groups were integrated and dimensionally reduced. The UMAP plot shows unsupervised clustering results, with different colors representing distinct cell clusters. (B) Heatmap of cell type-specific marker gene expression. The heatmap displays the expression patterns of core marker genes for each annotated cell type, with color gradients corresponding to normalized expression levels, visualizing the molecular characteristics of cell types. (C) Pie charts of cell type distribution across groups. Relative proportions of each cell type in Control, Model, EA, and EA+AAV-shTREM2 groups. (D) Bar plot showing the number of DEGs between groups.

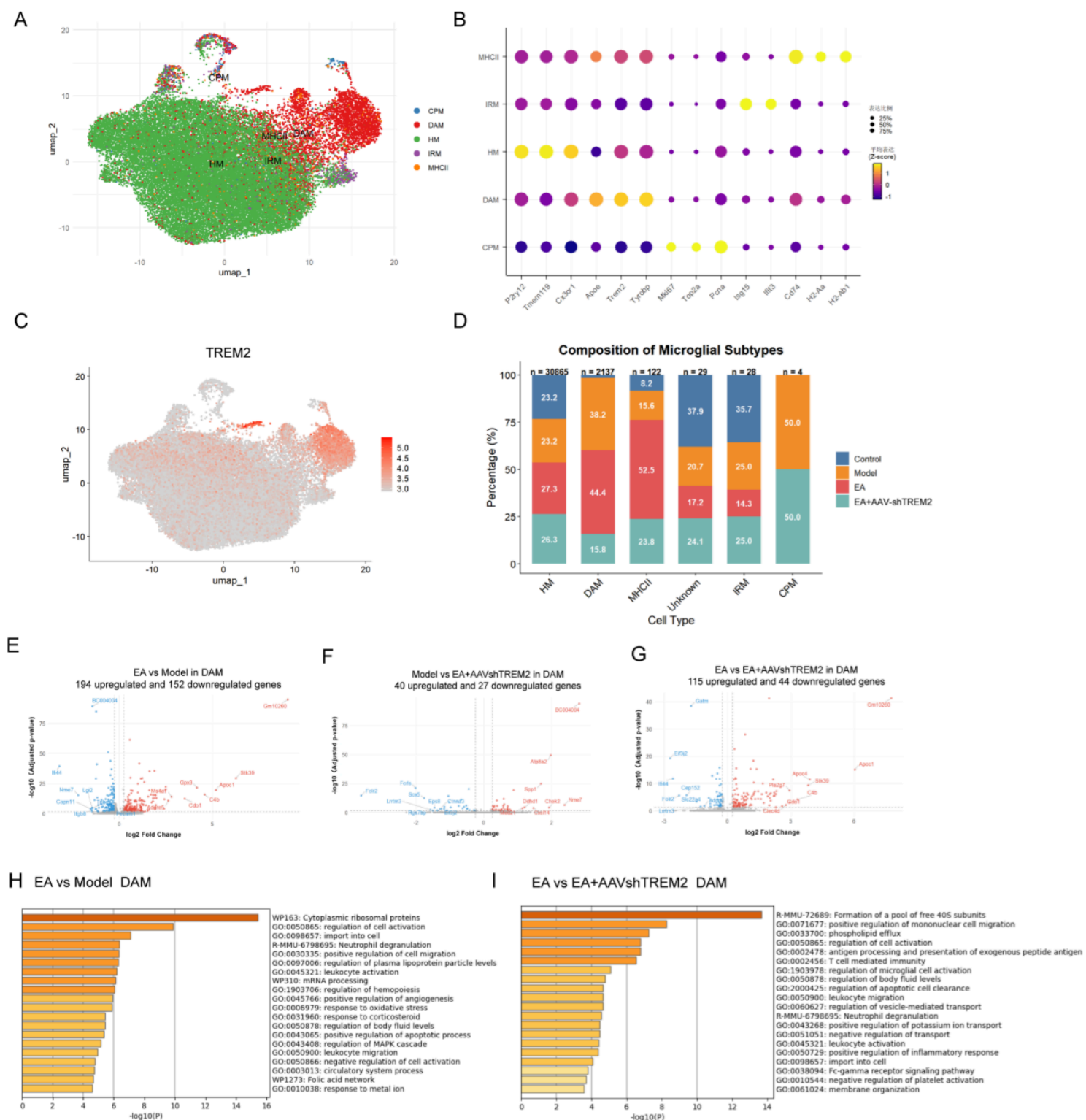


Figure 7

Microglial heterogeneity and DAM subset analysis. (A) UMAP plot of microglial subclusters. (B) Bubble plot of marker gene expression across microglial subclusters. (C) UMAP plot showing TREM2 expression distribution. (D) Bar plot of microglial subcluster proportions in each group. (E–H) Volcano plots of DEGs in DAM subsets for Model vs Control (E), EA vs Model (F), Model vs EA+AAV-shTREM2 (G), and EA vs EA+AAV-shTREM2 (H). Red: up-regulated; blue: down-regulated. (I, J) Bar plots of functional

enrichment for DEGs in DAM subsets in EA vs Model (I) and EA vs EA+AAV-shTREM2 (J). Bar length indicates $-\log_{10}P$.

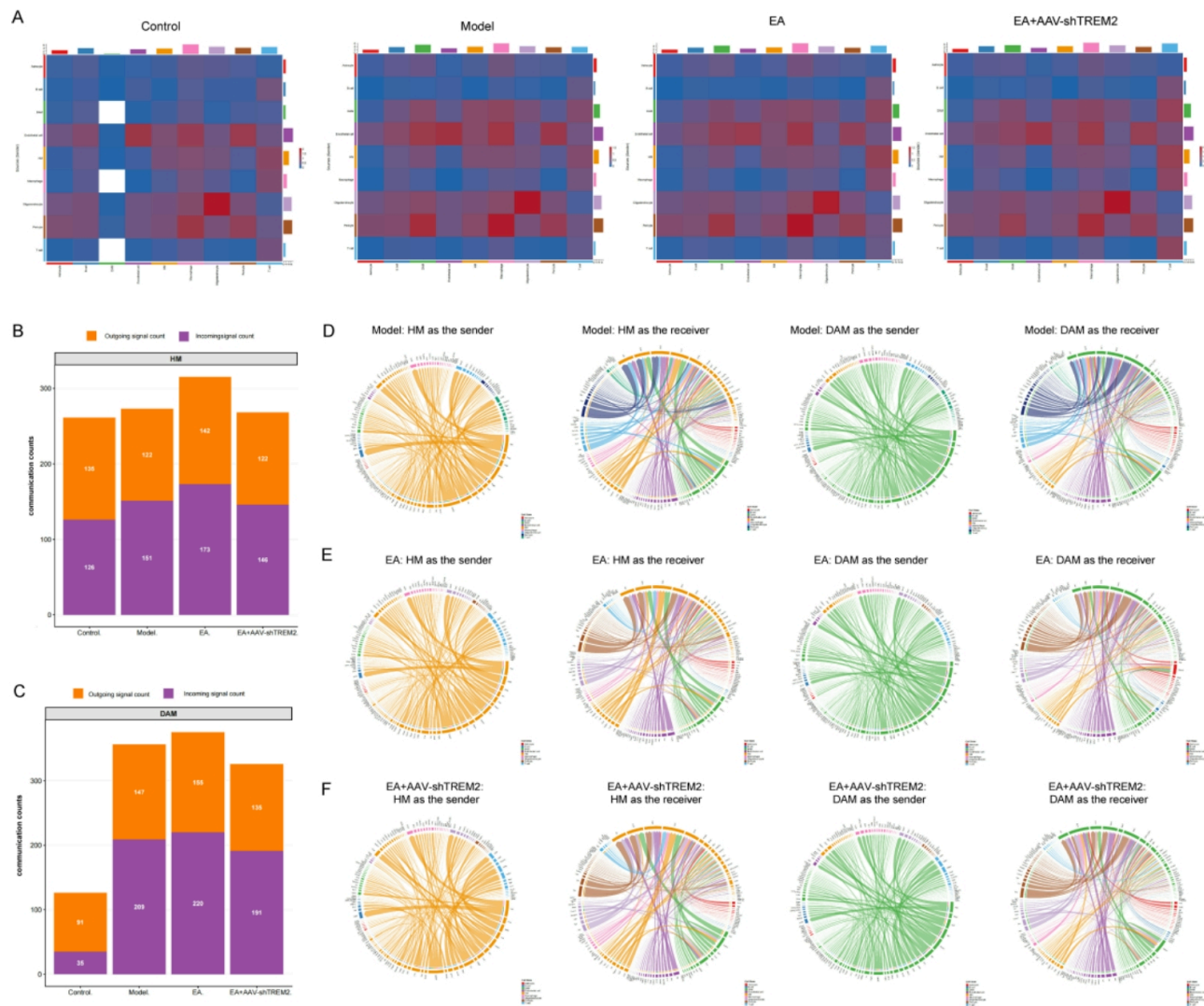


Figure 8

Cell communication patterns of microglial subsets.(A) Heatmap showing communication strength between cell types across groups. Rows: sender cells; columns: receiver cells.Color intensity reflects communication strength.(B, C)Number of incoming (purple) and outgoing (orange) signals for HM (B) and DAM (C). (D–F)Circle plots depicting intercellular communication networks of microglial subsets in Model (D), EA (E), and EA+AAV-shTREM2 (F) groups.HM (left) and DAM (right) are shown as senders/receivers; line thickness represents communication strength.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [fulluncroppedGelsandBlotsimage.docx](#)
- [S2.docx](#)
- [floatimage1.png](#)