

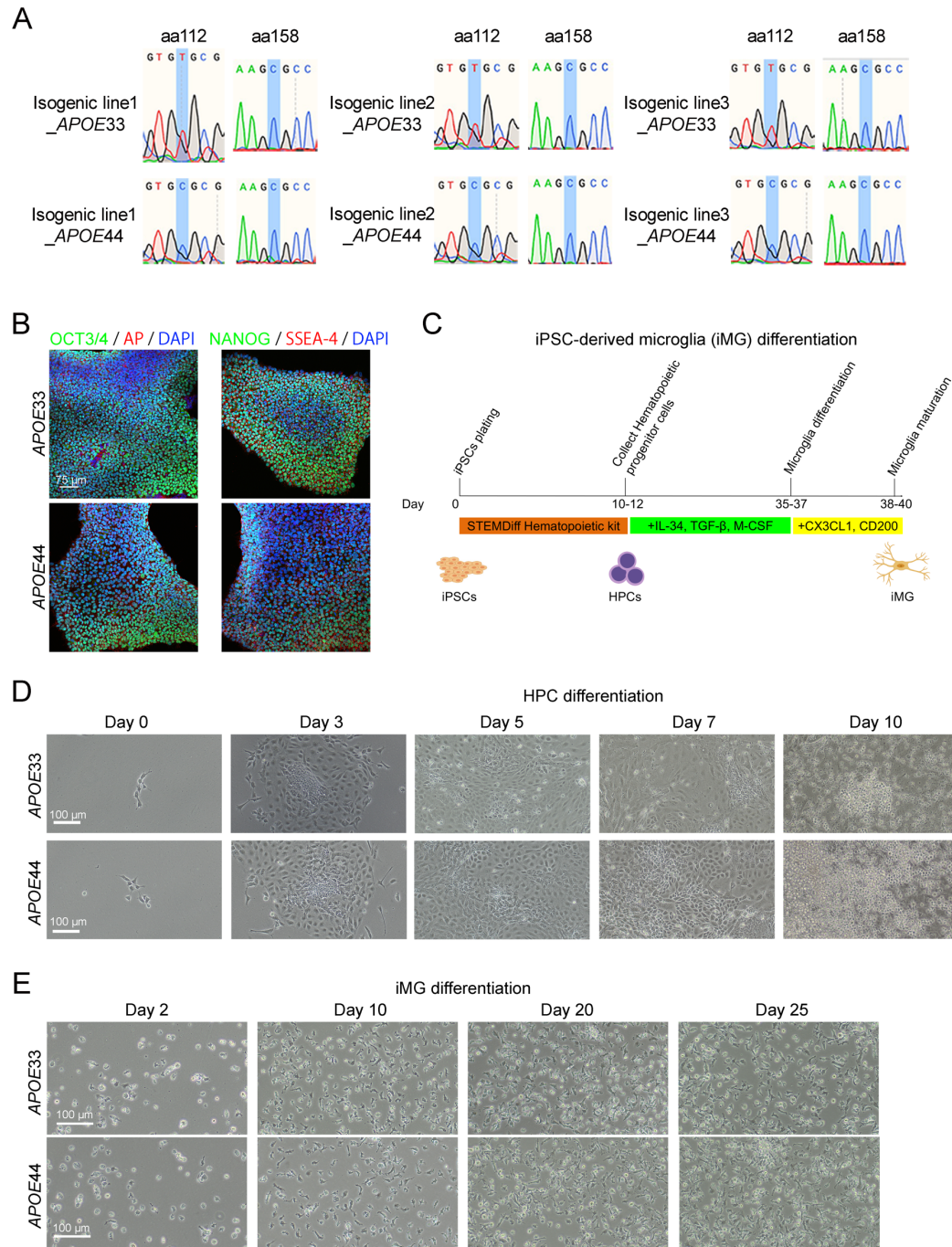
Supplementary Information

Human iPSC models reveal *APOE4*-induced microglial remodeling and extracellular vesicle dysregulation in AD pathogenesis

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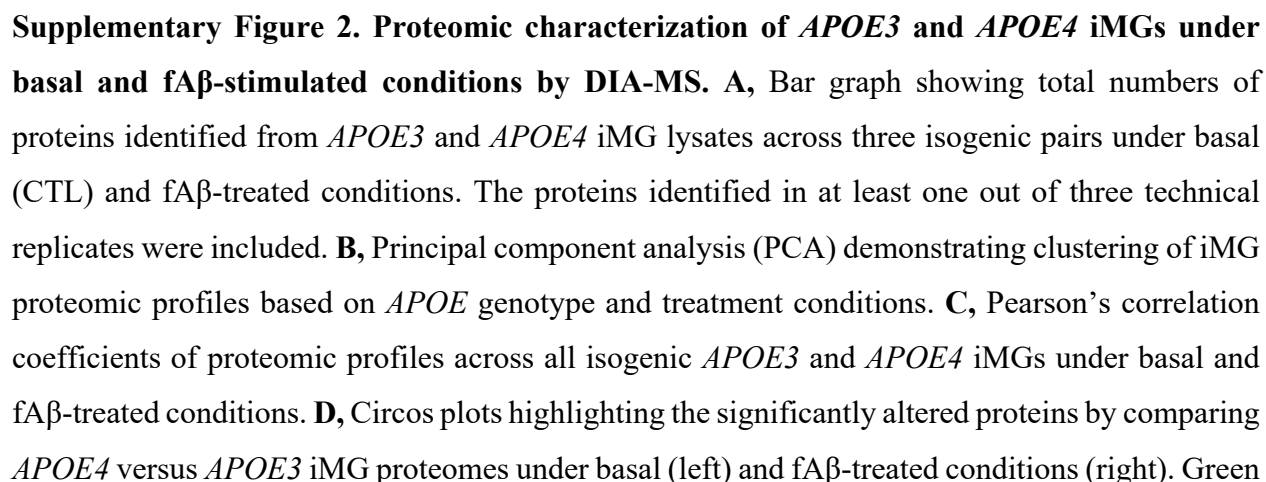
Supplementary Figures 1-10

Supplementary Tables 1-14



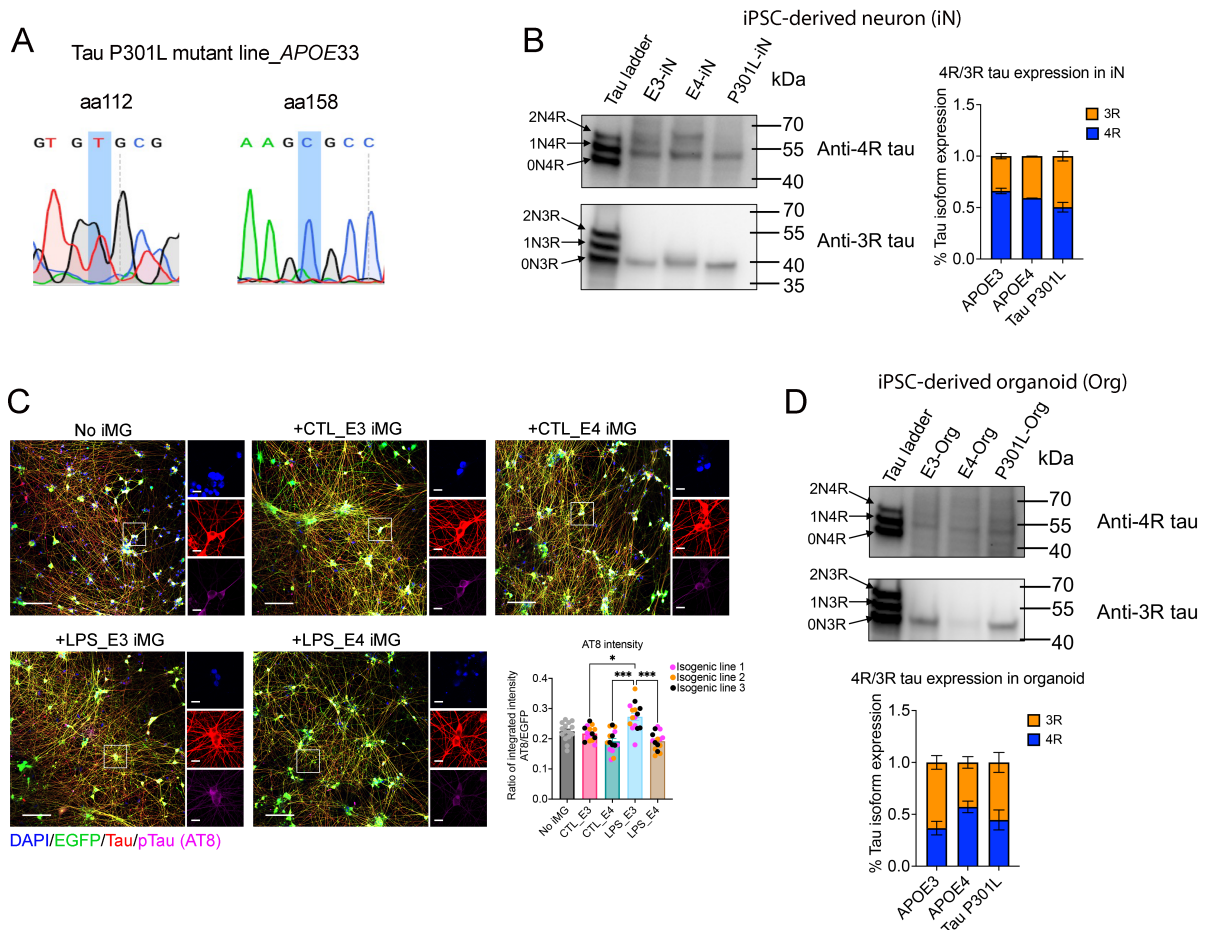
Supplementary Figure 1. Generation and differentiation of isogenic *APOE3* and *APOE4* iPSC lines into iPSC-derived microglia. **A**, Sanger sequencing validation of three isogenic pairs of human iPSC lines homozygous for either *APOE3/3* or *APOE4/4* alleles. Sequencing at codons 112 and 158 confirms the presence of *APOE3* (Cys112, Cys158) or *APOE4* (Arg112, Arg158) genotypes. **B**, Representative images of *APOE3* and *APOE4* iPSCs showing pluripotency markers

OCT3/4, NANOG, alkaline phosphatase (AP) and SSEA4 by immunofluorescence staining. **C**, Schematic overview of iPSC-derived microglia differentiation protocol. iPSCs are first differentiated into hematopoietic progenitor cells (HPCs) using a hematopoietic induction kit, followed by microglial differentiation and maturation by adding IL-34, TGF- β , M-CSF, CX3CL1, and CD200. **D**, Phase-contrast images showing the differentiation of *APOE3* and *APOE4* iPSCs into HPCs over 12 days. **E**, Phase-contrast images of *APOE3* and *APOE4* iMGs differentiation from HPCs over 25 days.



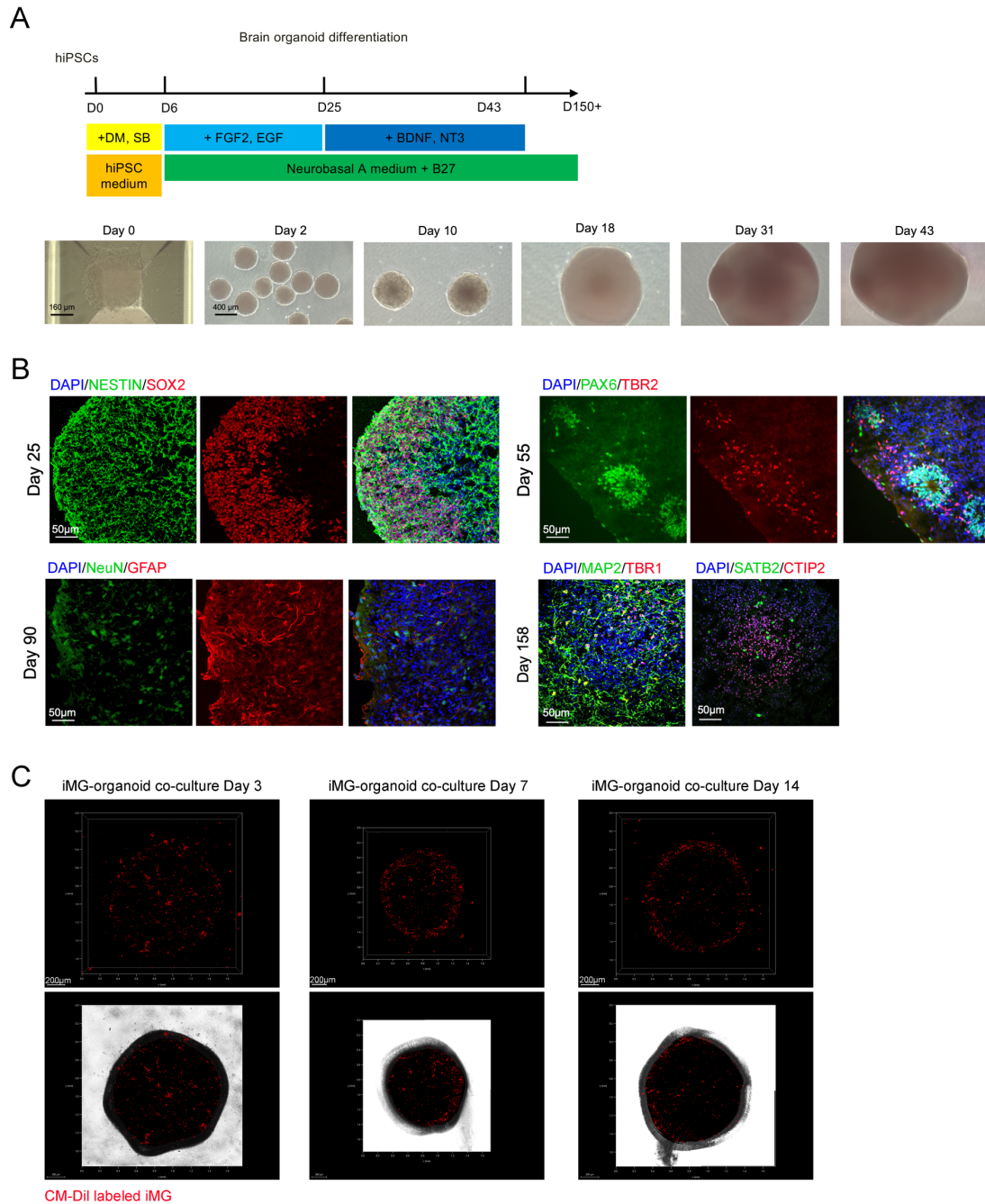
Supplementary Figure 2. Proteomic characterization of *APOE3* and *APOE4* iMGs under basal and fA β -stimulated conditions by DIA-MS. **A**, Bar graph showing total numbers of proteins identified from *APOE3* and *APOE4* iMG lysates across three isogenic pairs under basal (CTL) and fA β -treated conditions. The proteins identified in at least one out of three technical replicates were included. **B**, Principal component analysis (PCA) demonstrating clustering of iMG proteomic profiles based on *APOE* genotype and treatment conditions. **C**, Pearson's correlation coefficients of proteomic profiles across all isogenic *APOE3* and *APOE4* iMGs under basal and fA β -treated conditions. **D**, Circos plots highlighting the significantly altered proteins by comparing *APOE4* versus *APOE3* iMG proteomes under basal (left) and fA β -treated conditions (right). Green

color indicates upregulated proteins in *APOE4* iMGs compared to *APOE3* iMGs, while blue color indicates downregulated proteins. (E-F) Gene ontology and KEGG enrichment analyses highlighting significantly enriched biological processes (BP), molecular functions (MF), cellular components (CC), and pathways of proteins differentially expressed following fA β treatment in *APOE3* (E) and *APOE4* (F) iMGs.



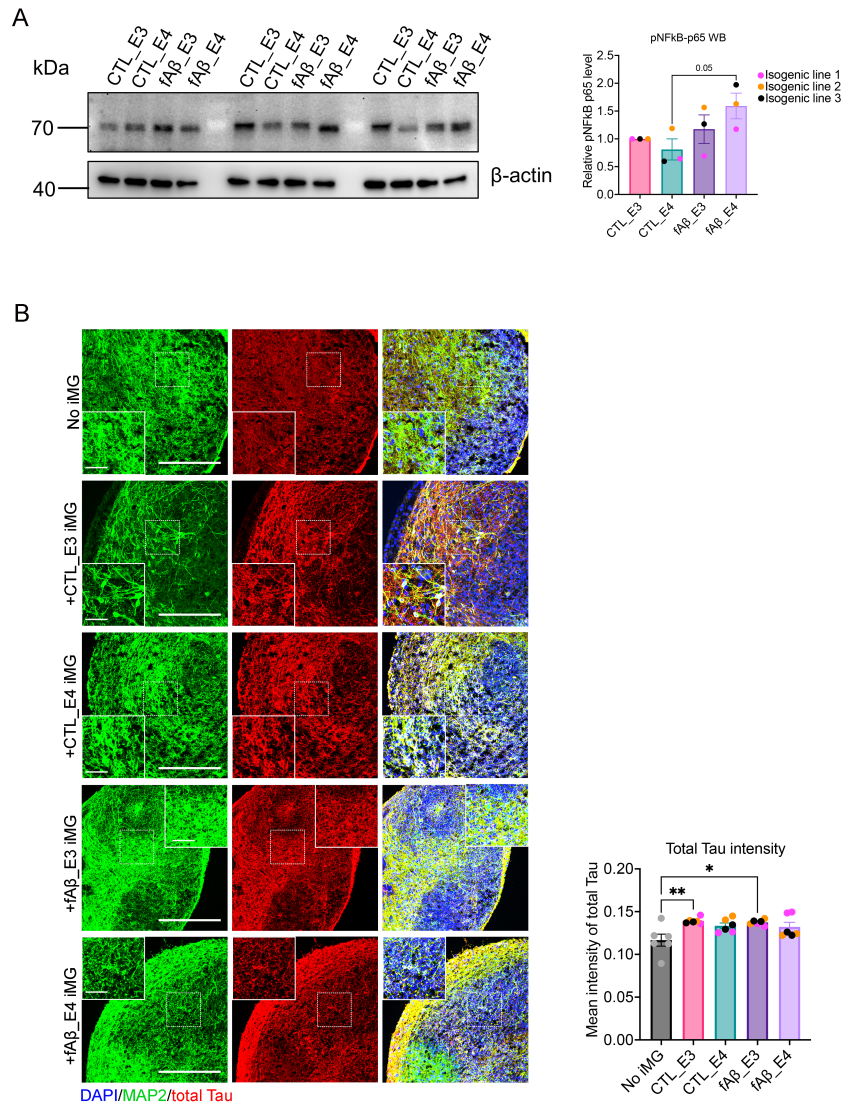
Supplementary Figure 3. *APOE* genotyping and tau isoform characterization of MAPT P301L neuronal models, and differential effects of LPS-stimulated *APOE3* and *APOE4* iMGs on neuronal tau phosphorylation. **A**, Sanger sequencing validation of *APOE* genotype for MAPT P301L iPSC line. Sequencing at codons 112 and 158 confirms the presence of *APOE3* (Cys112, Cys158) genotype. **B**, Immunoblotting showing 4R- and 3R-tau isoform expression in *APOE3*, *APOE4*, and MAPT P301L iPSC-derived neurons (iNs). A recombinant tau isoform ladder was included to indicate the migration of 2N4R, 1N4R, 0N4R, 2N3R, 1N3R, and 0N3R tau isoforms. The stacked bar graph shows the relative abundance of 4R and 3R tau, calculated as the signal for each isoform class divided by the combined 3R- plus 4R-tau signal. Data are presented as mean $\pm$ SEM from two independent batches. **C**, Representative immunofluorescence images

and quantification of tau phosphorylation in MAPT P301L iNs cultured alone or co-cultured with *APOE3* or *APOE4* iMGs under basal conditions or following LPS stimulation. Neuronal AT8 immunoreactivity was quantified as integrated AT8 intensity normalized to the EGFP-positive neuronal signal. N = 15 images from iMG/iN co-culture with three pairs of isogenic lines were quantified for each group. Scale bar, 100 μm and 10 μm . Data are presented as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$. Kruskal-Wallis test with Dunn's multiple comparisons test. **D**, Immunoblotting showing 4R- and 3R-tau isoform expression in *APOE3*, *APOE4*, and MAPT P301L iPSC-derived brain organoids. Data are presented as mean $\pm$ SEM from two independent batches.



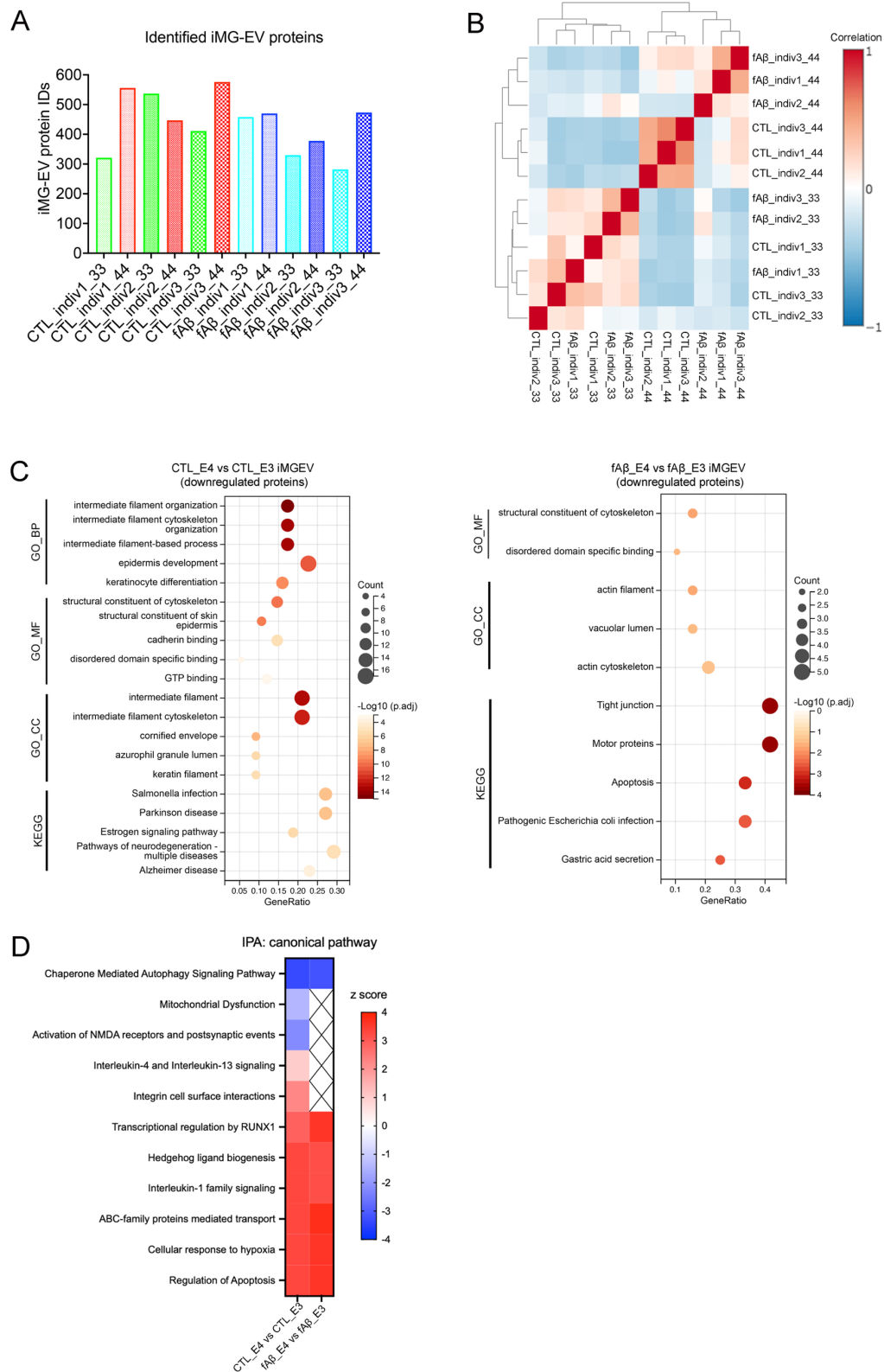
Supplementary Figure 4. Brain organoid differentiation and development of iMG-organoid co-culture system. **A**, Schematic timeline of brain organoid differentiation from human iPSCs. Dual SMAD inhibition (DM, SB) is applied at day 0-6, followed by sequential addition of growth factors (FGF2, EGF, BDNF, and NT3) to promote neuroepithelial and cortical development. Representative bright-field images show growth and expansion of organoids at key time points from day 0 to day 43. Scale bars, 100 μ m and 400 μ m. **B**, Immunofluorescence characterization of

brain organoids at multiple differentiation stages. Day 25 organoids exhibit expression of early neural markers NESTIN and SOX2. Day 55 organoids show the expression of cortical progenitor markers PAX6 and TBR2. At day 90, mature neuronal (NeuN) and astrocytic (GFAP) populations are observed. At day 158, neuron dendritic marker MAP2 as well as upper and deep cortical layer markers TBR1, SATB2, and CTIP2 were observed. Scale bar, 50 μm . **C**, Development of the iMG-organoid co-culture model. 100K iMGs labeled with CM-DiI dye (red) were added to brain organoids and monitored for integration and distribution at day 3, day 7, and day 14 post co-culture. Confocal imaging confirms progressive infiltration and integration of iMGs within the 3D organoid structure over a two-week timeframe. Scale bar, 200 μm .



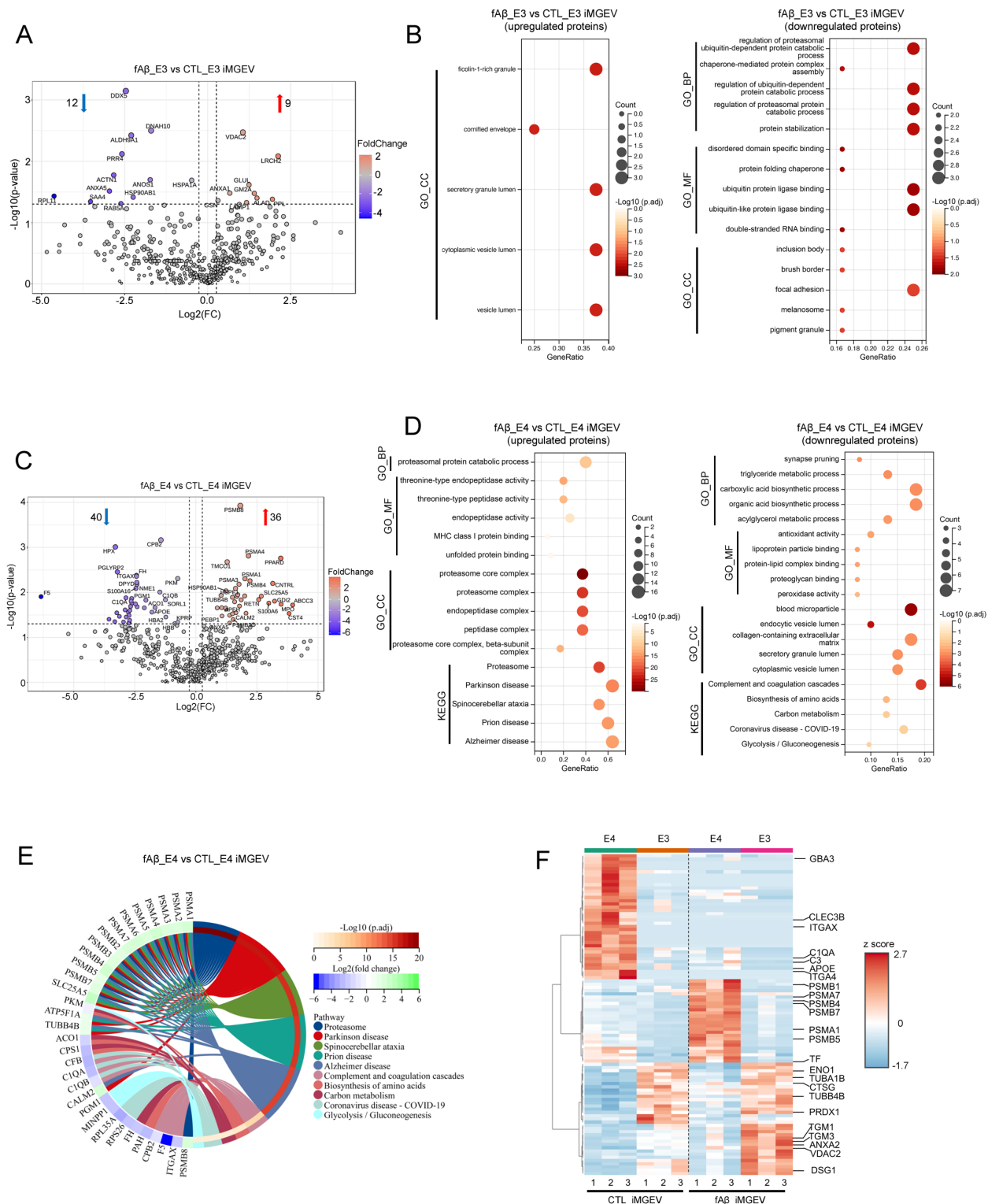
Supplementary Figure 5. Phosphorylated NF- κ B p65 levels in *APOE3* and *APOE4* iMGs and total tau abundance in iMG-organoid co-cultures. **A**, Immunoblotting and quantification of phosphorylated NF- κ B p65 in iMGs from three independent *APOE* isogenic pairs under CTL or fA β -treated conditions. pNF- κ B p65 level was normalized to β -actin, and relative protein levels to CTL_E3 group were calculated. Each point represents an individual isogenic line. Data represent mean $\pm$ SEM. $p = 0.05$. RM one-way ANOVA with Tukey's multiple comparisons test. **B**, Quantification of total tau intensity in organoids. *APOE4* iMGs significantly elevated tau phosphorylation in P301L Tau mutant organoids, while MAP2 levels remained unchanged. $N = 6$ organoids/group. Two iMG-organoid co-cultures for each line were quantified. Data are shown as

mean $\pm$ SEM. *p < 0.05, **p < 0.01. Ordinary one-way ANOVA with Tukey's multiple comparisons test. Scale bar, 250 μ m and 50 μ m.



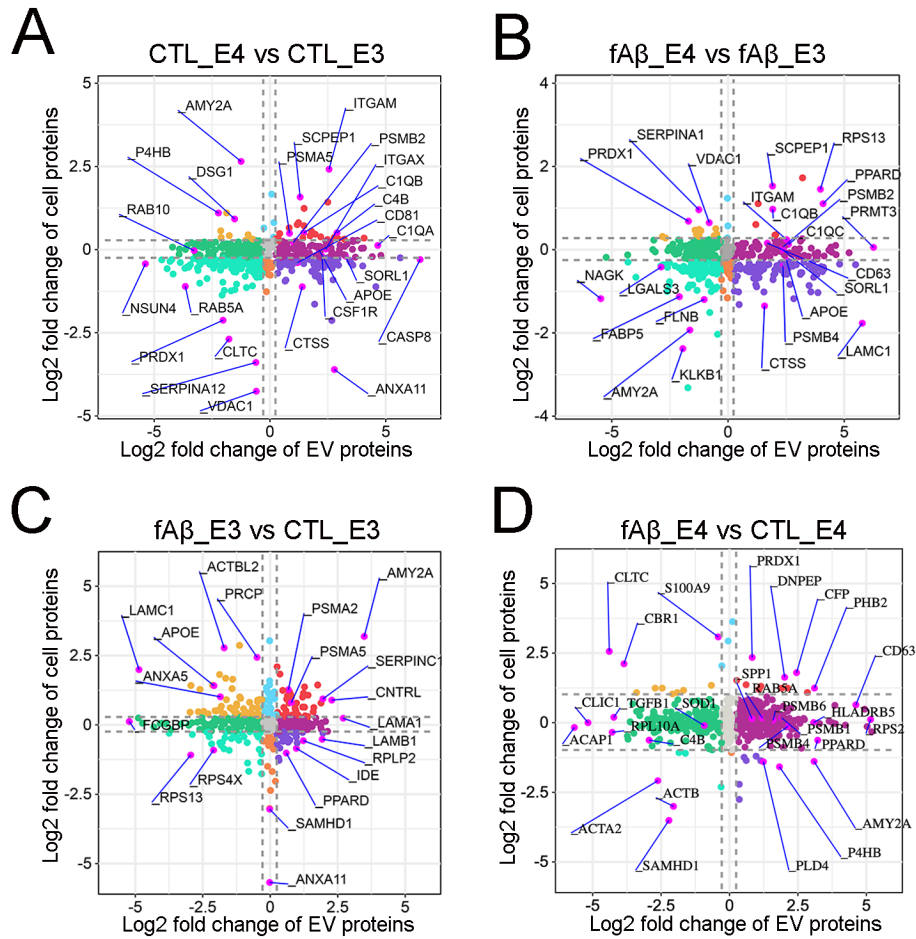
Supplementary Figure 6. Proteomic analysis of iMG-derived EVs across *APOE* genotypes and treatment conditions. A, Bar graph showing the number of proteins identified in iMG-EVs

from three isogenic *APOE3* and *APOE4* iMG lines under control and fA β -treated conditions. The proteins identified in at least one out of three technical replicates were included. **B**, Pearson's correlation coefficients of proteomic profiles across all isogenic *APOE3* and *APOE4* iMGs under basal and fA β -treated conditions. **C**, Gene ontology and KEGG enrichment analyses of downregulated proteins in *APOE4* versus *APOE3* iMGs under basal and fA β -treated conditions. **D**, IPA canonical pathways revealed differential activation and inactivation in *APOE4* versus *APOE3* iMGs under both basal and fA β -treated conditions.

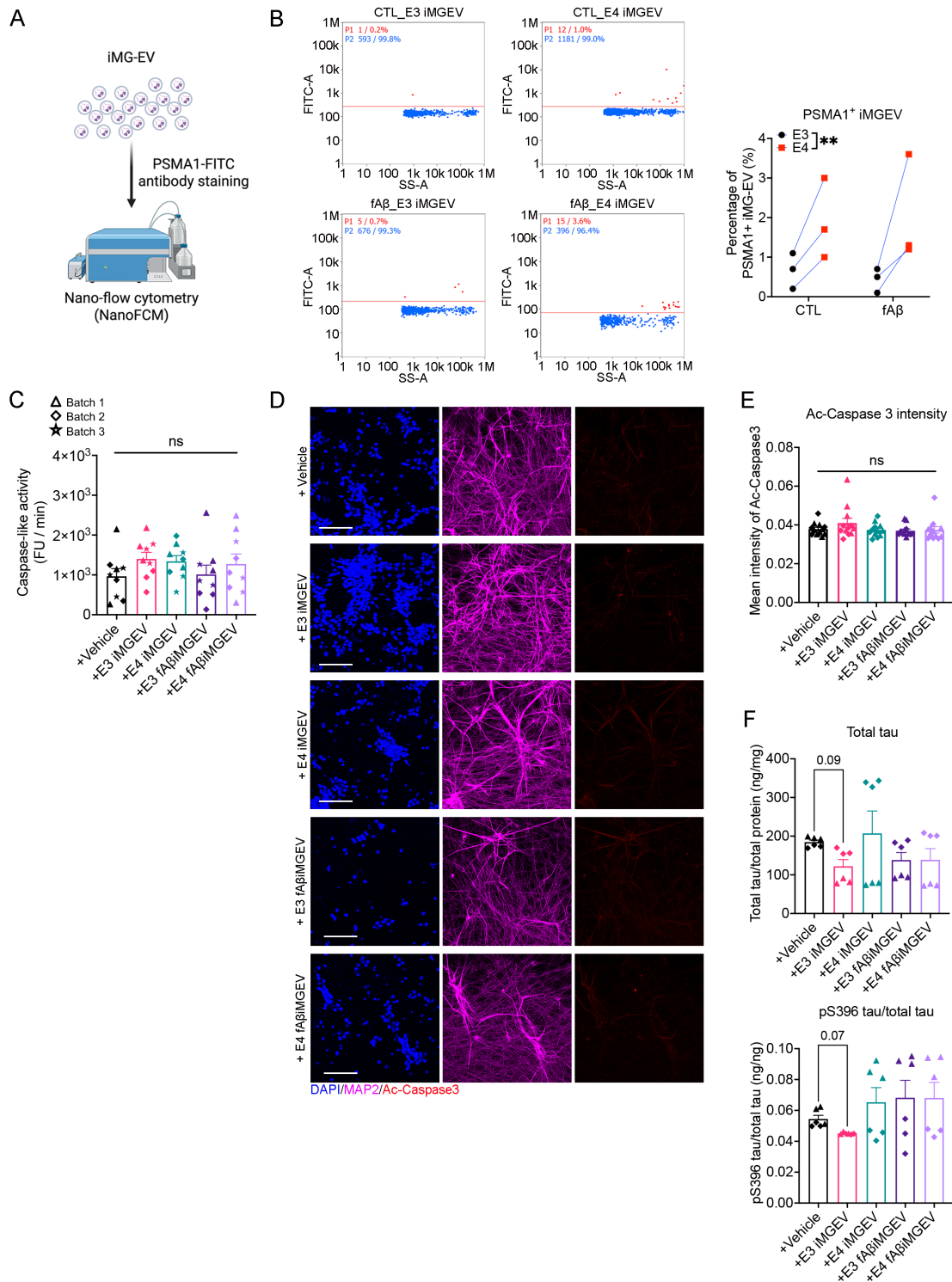


Supplementary Figure 7. Comparative analysis of iMGEVs proteome in response to fAβ stimulation within *APOE* genotypes. **A**, Volcano plot showing differentially expressed proteins in fAβ-treated versus basal *APOE3* iMGEVs (fAβ_E3 vs. CTL_E3). Proteins significantly

upregulated or downregulated are highlighted in red and blue, respectively. **B**, GO enrichment analysis of upregulated (left) and downregulated (right) proteins in *APOE3* iMGEVs following fA β stimulation. **C**, Volcano plot of differentially expressed proteins in fA β -treated versus basal *APOE4* iMGEVs (fA β _E4 vs. CTL_E4). Proteins significantly upregulated or downregulated are highlighted in red and blue, respectively. **D**, GO and KEGG pathway enrichment analysis of upregulated (left) and downregulated (right) proteins in *APOE4* iMGEVs after fA β exposure. **E**, Circos plot visualizing the top dysregulated pathways and associated proteins in fA β -treated *APOE4* iMGEVs compared to basal *APOE4* iMGEVs. **F**, Heatmap of protein expression profiles across *APOE3* and *APOE4* iMGEVs under basal and fA β -treated conditions. Proteins related to integrins signaling, proteasome function, complements, and glycogenesis are shown.

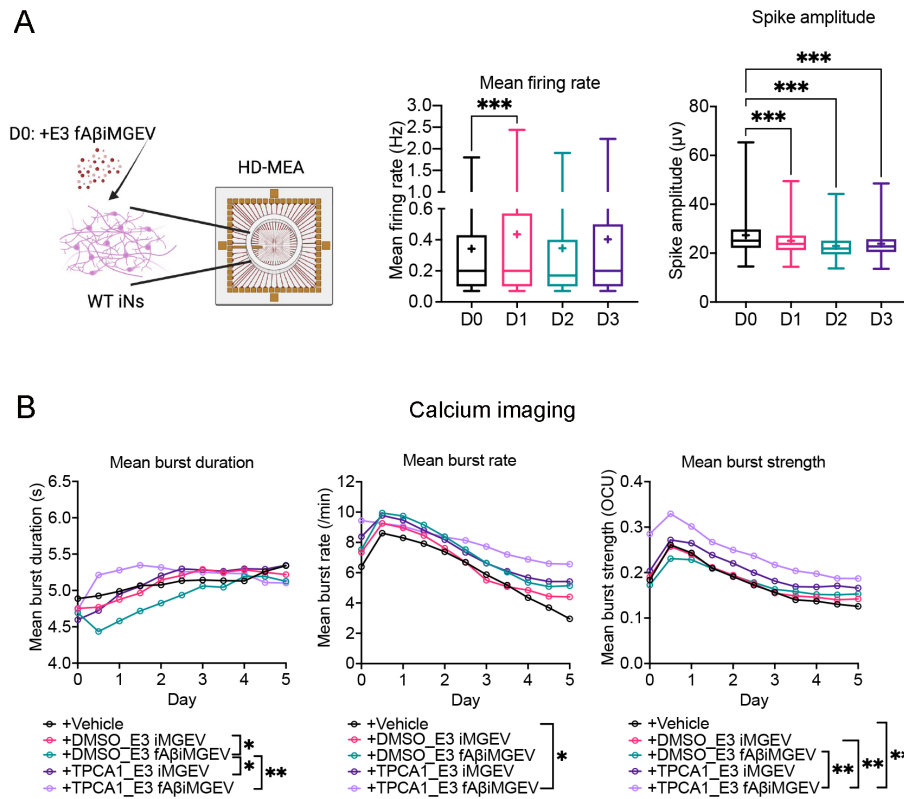


Supplementary Figure 8. Integrated proteomic analysis of *APOE3* and *APOE4* iMGs and their derived EVs under basal and fAβ-stimulated conditions. (A-D) Scatter plots of log₂ fold changes of differentially expressed proteins in iMG cells (y-axis) versus iMG-derived EVs (x-axis) across indicated comparisons. Each point represents a protein identified in both cellular and EV proteomes. Selected representative proteins are annotated in each panel, highlighting both concordant and discordant changes across cellular and EV compartments.



Supplementary Figure 9. Nano-flow cytometry analysis of proteasomal protein expression in iMG-EVs and assessment of caspase-like proteasomal activity and neurotoxicity in recipient neurons. **A**, Schematic showing the detection of PSMA1⁺ EV particles from *APOE3* and *APOE4*

iMG under basal and fA β -treated conditions by nano-flow cytometry. **B**, Quantification of FITC-conjugated PSMA1⁺ EVs population in *APOE3* and *APOE4* iMGEVs under basal and fA β -treated conditions by nano-flow cytometry. Two-way ANOVA ** $p < 0.01$. **C**, Caspase-like proteasomal activity measured in iPSC-derived neurons after treatment of vehicle or fA β -treated *APOE3* or *APOE4* iMGEVs for 7 days. FU, fluorescence unit. N = 9 per group. Shapes indicate three independent experimental batches. Individual points represent technical replicate wells from the indicated batch. Data were shown as mean $\pm$ SEM. ns, no significance. RM one-way ANOVA with Holm-Sidak's multiple comparisons test. **D**, Representative immunofluorescence images showing neuronal apoptosis (activated caspase-3, red) in P301L Tau neurons (MAP2, magenta) after adding iMGEVs derived from basal or fA β -treated isogenic *APOE3* and *APOE4* iMGs. Scale bar, 100 μ m. **E**, Quantification of activated caspase-3 in P301L Tau neurons after iMGEVs treatment. N = 12 images from two independent batches were quantified. Data shown as mean $\pm$ SEM. ns, no significance. Ordinary one-way ANOVA with Tukey's multiple comparisons test. **F**, ELISA of total tau and pS396-tau levels in P301L Tau induced neurons following 7-day iMGEV treatment. N = 6 per group. Shapes indicate two independent experimental batches. Individual points represent technical replicate wells from the indicated batch. Data were shown as mean $\pm$ SEM. Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test.



Supplementary Figure 10. Effects of *APOE3* iMGEVs and NF-κB inhibition in donor iMGs on neuronal electrophysiological activity. **A**, High-density multielectrode array (HD-MEA) recordings of mean firing rate in D25 WT iNs following exposure to fAβ-treated E3iMGEVs at D0 with longitudinal follow-up for 3 days (D1-D3). Data were shown with the Box and whiskers plot (Min to Max). The cross line indicates mean value. *** $p < 0.001$. Kruskal-Wallis test with Dunn's multiple comparisons test was used. **B**, Time-course analysis of neuronal network activity metrics (mean burst duration, burst rate, and burst strength) over 5 days following treatment with APOE3 iMGEVs generated under DMSO or TPCA1 conditions. N = 4 technical replicates per batch from two independent experiments. Data were shown as mean value. * $p < 0.05$, ** $p < 0.01$. Receiver Operating Characteristic (ROC) analysis was used.