Supplementary Information

Engineering microglia exosome-mediated MicroRNA-124-3p delivery for Alzheimer's disease combinational therapy

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Experimental Materials

Interleukin-4 (IL-4; Dalian Meilun Biotechnology Co., LTD, CHN), A$\beta_{1\text{-}42}$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were purchased from Sigma-Aldrich (Shanghai, China). Anti-CD206, Anti-CD16/CD32, Anti-Bcl-2, Anti-BCL2L11 and Anti-Cleaved Caspase-3 antibody were purchased from Abcam (UK). Anti-β-actin antibody was purchased from Proteintech (Wuhan, China). Oligomer A11 Polyclonal Antibody was purchased from Thermo Fisher Scientific Co., Ltd. (USA). Mouse TNF-α and IL-1β ELISA kit were purchased from Absin Biotechnology Co., Ltd. (Shanghai, China).

Cytotoxicity assay

The biocompatibility of exosomes was evaluated by MTT assay. Generally, the PC-12 cells were seeded on a 96-well plate at a density of 8×10^3 cells/well in 200 μL culture medium and allowed to attach overnight. Then, the culture medium was replaced by a serum-free medium containing different exosomes for 24 h. After that, 20 μL MTT solution (5 mg/mL in PBS) was added to the wells and incubated at 37 °C in 5% CO_2 for another 4 h. Then the solution in the well was aspirated gently and 200 μL DMSO was added into each well to dissolve the formazan crystal. The absorbance was measured at a wavelength of 570 nm. The relative cell growth (%) related to control cells cultured in the medium without the polymer was calculated by the following formula: Cell viability (%) = $(OD_{sample})/(OD_{control}) \times 100\%$, where OD_{sample} and $OD_{control}$ represent the OD values of treatment group, and control group, respectively.

Cellular uptake of exosomes

Exosomes were labeled with the green fluorescent probe PKH67 (MIDI67-1KT, Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 20 mg exosomes were diluted in the kit buffer (diluent C) 1:1, and then PKH67 in diluent C (1:75) was mixed with the diluted sample. Subsequently, samples were incubated at room temperature for 3 min, following purification by ultracentrifugation to obtain PKH67-Exo, PKH67-Exo-NC and PKH67-Exo-124-3p. Then, PC-12 cells were seeded on 24-well plates at 10×10^4 cells/well for 24 h. The medium was then removed and replaced with fresh culture medium containing PKH67-Exo, PKH67-Exo-NC and PKH67-Exo-124-3p. After 2 h and 6 h incubation, followed by staining with Hoechst for 5 min. Cellular uptake was observed with laser scanning confocal microscopy (IX81-FV1000, Olympus, Japan). The mean fluorescence intensity was analyzed with ImageJ software.

Quantified fluorescence intensity

PC-12 cells were seeded at a density of 20×10^4 cells/well in 6-well plates and cultured at 37 °C for 24 h. After treated with PKH67-Exo, PKH67-Exo-NC and PKH67-Exo-124-3p for 6 h, the cells were washed three times with PBS. The single-cell suspension was prepared and quantified by flow cytometry (Cytoflex, Beckman Coulter, USA).

Cell lines

The bEnd.3 and PC-12 cell lines were obtained from the Chinese Academy of Sciences

Cell Bank (Shanghai, China). Mouse microglia BV2 cells was purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China). Cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were placed in an incubator at 37 °C with 5% CO_2 and 99% humidity.

Apoptosis assay

The Annexin V-FITC/PI apoptosis detection kit (Dalian Meilun Biotech Co., China.) was used to evaluate the PC-12 cells apoptosis according to the instructions. Briefly, PC-12 cells were seeded at a density of 5×10^4 cells/well in 12-well plate and incubated overnight. Cells were treated with Exo, Exo-NC and Exo-124-3p for 24 h. Then the single-cell suspension was prepared, and the percentage of apoptosis was determined via flow cytometry (FCM).

In vitro BBB model transcytosis

To establish the *in vitro* BBB models, bEnd.3 cells were seeded in the upper chambers (pore size: 0.4 μm, Corning, USA) at 10×10^4 cells/insert. During the culture for 14 days, the medium was changed every 2 days and the integrity of cell monolayer was verified by measuring the transepithelial electrical resistances (TEER>150 $\Omega\cdot cm^2$) values using a Millicell-ERS volt-ohmmeter (Millipore Co., USA). After that, PC-12 cells were seeded on the lower chamber and co-incubation for 24 h. Then the integrity of the barrier was confirmed by detecting the TEER, and the co-cultured model was established to imitate *in vitro* BBB for further experiments. DiR labeled exosomes were added into upper inserts. After 4 h incubation, PC-12 cells in the lower chamber were rinsed and collected in PBS. Then the cells were assessed with flow cytometry (Cytoflex, Beckman Coulter, USA) to determine the fluorescence intensity of DiR. All samples were tested in triplicates.

Animals

C57BL/6 mice (male, 12-14 weeks) were purchased from the Ziyuan Laboratory Animal Science and Technology Co., LTD (Hangzhou, China). All animal experiments were carried out in accordance with guidelines and all procedures were approved and performed in accordance with national guidelines of the ethical committee of Zhejiang University (ZJU20250124).

In vivo imaging

Exosomes were firstly stained with the liposomal dye according to the manufacturer's instructions to visualize their distribution *in vivo*. Briefly, purified exosomes were incubated with 5 mM DiR for 15 min at 37 ℃, then ultracentrifuged at 120,000×g, 70 min to remove the unbounded dye. After being washed twice in PBS, the labeled exosomes were re-suspended in PBS prior to use. Then, the DiR labeled exosomes (20 μg, protein weight) were administered by tail vein injection. The fluorescence signals were recorded at 1, 2, 4, 8, 12, and 24 h after administration by the Maestro *in vivo* Imaging System (CRI, Woburn, USA) (Ex: 630 nm; Em: 670 nm). At the last time point, the mice were sacrificed and the brains, hearts, livers, spleens, lungs, and kidneys were isolated for observation. The fluorescence signals of DiR signals were recorded

and analyzed.

Bioinformatics analysis and binding sites prediction

The potential miR-124-3p binding sites to 3′-UTR of MEKK3 was predicted by TargetScan (https://www.targetscan.org/vert_72/) to study the potential crossing network among miR-124-3p, MEKK3 and NF-*κ*B. In order to clarify the molecular mechanism, miR-124-3p mimics (sense-5'-AACAUUCAACCUGUCGGUGAGU-3', antisense-5'-UCACCGACAGGUUGAAUGUUUU-3'), MEKK3 siRNA (sense-5'-GUUACGCUUUAUCUUCCGUTT-3', antisense-5'-ACGGAAGAUAAAGCGUAACTT-3') and the negative control siRNA (NC) were purchased from Shanghai GenePharma (Shanghai, China). Then, miR-124-3p mimics, MEKK3 siRNA and NC were transfected according to the manufacturer's instructions.

Supplementary Fig.s

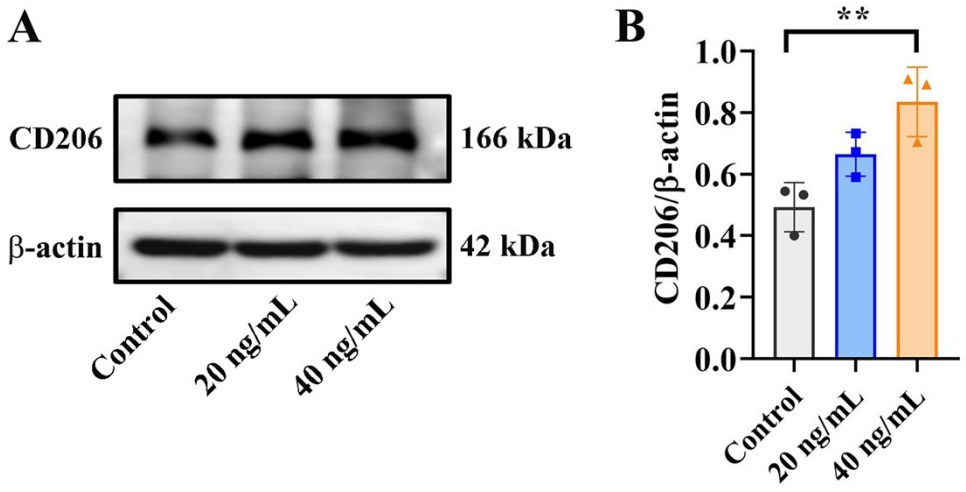


Fig. S1 (A) The expression of M2 phenotype microglia related protein CD206 in BV2 cells cultured with interleukin-4 (B) Quantitative analysis of CD206/*β*-actin in (A). Error bars represent ± SD (n = 3), * p<0.05, ** p<0.01, *** p<0.001.

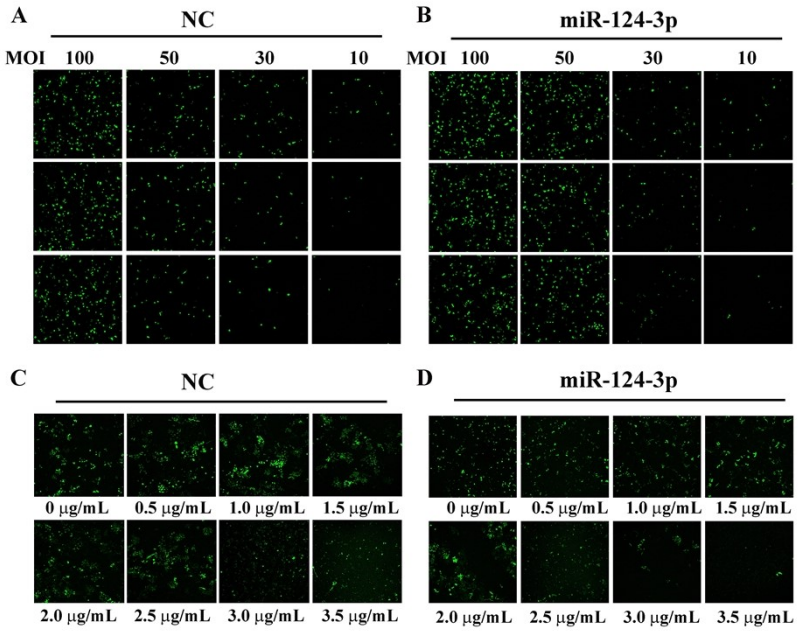


Fig. S2 Fluorescent images of miR-124-3p over-expressed M2BV2 cells establishment. Different multiplicity of infection (MOI) of (A) NC lentivirus and (B) miR-124-3p lentivirus in M2BV2 cells. Different puromycin concentration of (C) NC M2BV2 cells and (D) miR-124-3p over-expressed M2BV2 cells (green, EGFP-tag, n = 3).

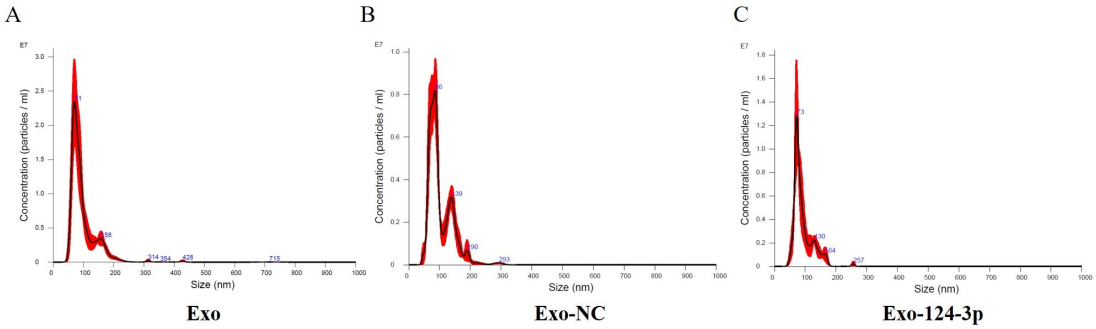


Fig. S3 Averaged FTLA concentration/size for Exo (A), Exo-NC (B) and Exo-124-3p (C) by NTA analysis.

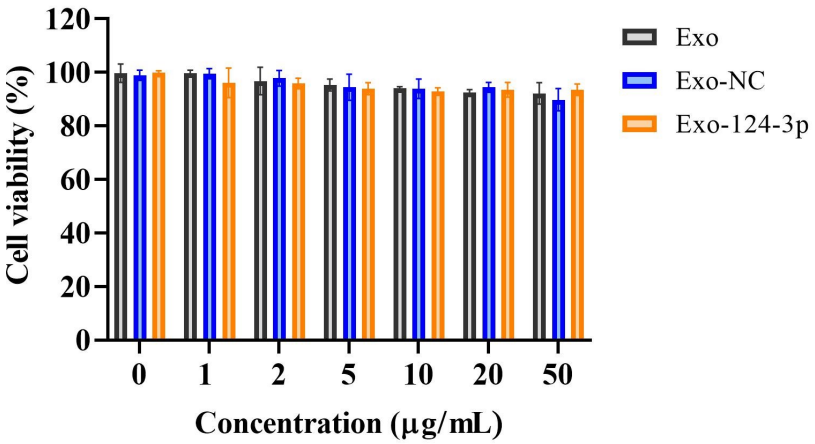


Fig. S4 In vitro cell viability of PC-12 cells treated with Exo, Exo-NC and Exo-124-3p. Error bars represent ± SD (n = 3).

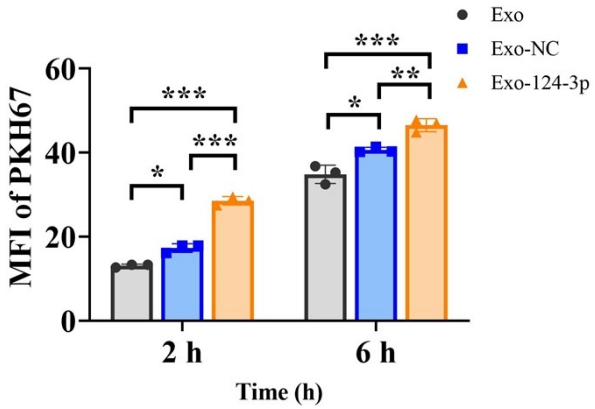


Fig. S5 The mean fluorescence intensity (MFI) of PKH67 in PC-12 cells. Error bars represent ± SD (n = 3), * p<0.05, ** p<0.01, *** p<0.001.

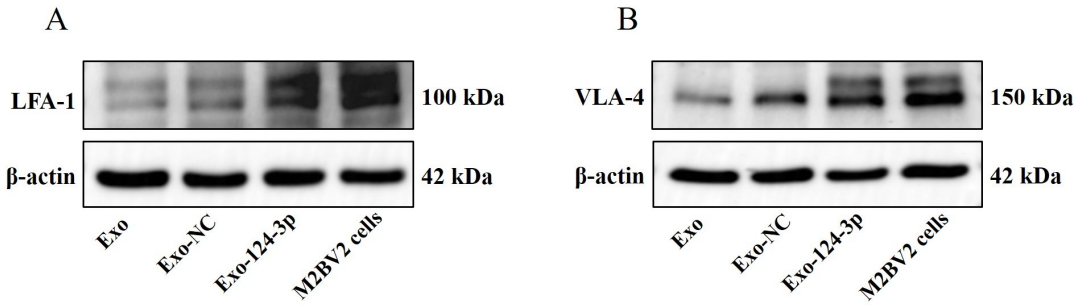


Fig. S6 Western blot assays for LFA-1 (A) and VLA-4 (B). The uncropped blot of were exhibited in Supplementary information.

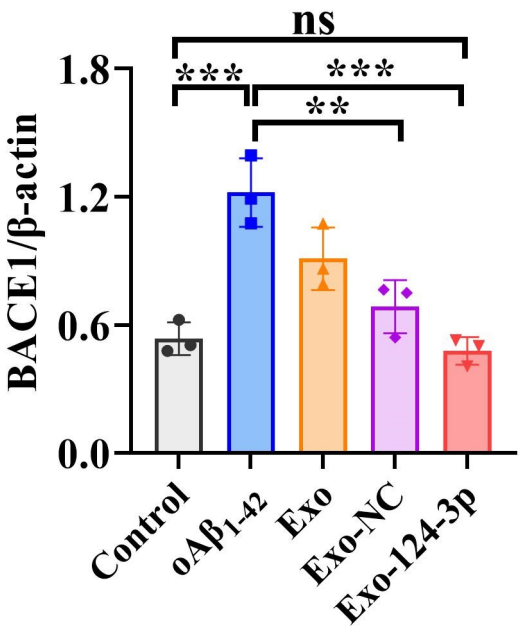


Fig. S7 The BACE1 protein expression level in various groups of PC-12 cells. Error bars represent ± SD (n = 3), * p<0.05, ** p<0.01, *** p<0.001.

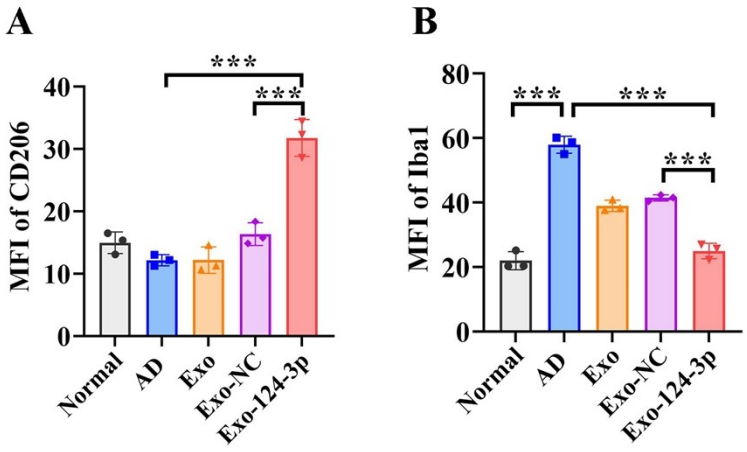


Fig. S8 The mean fluorescence intensity of (A) CD206 and (B) Iba1. Error bars represent ± SD (n = 3). * p<0.05, ** p<0.01, *** p<0.001.

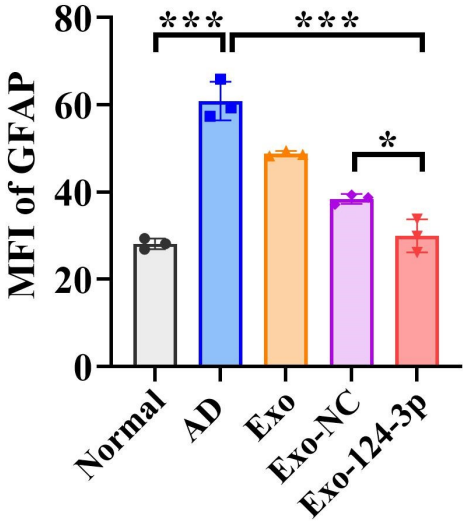


Fig. S9 The mean fluorescence intensity of GFAP in various groups. Error bars represent ± SD (n = 3). * p<0.05, ** p<0.01, *** p< 0.001.

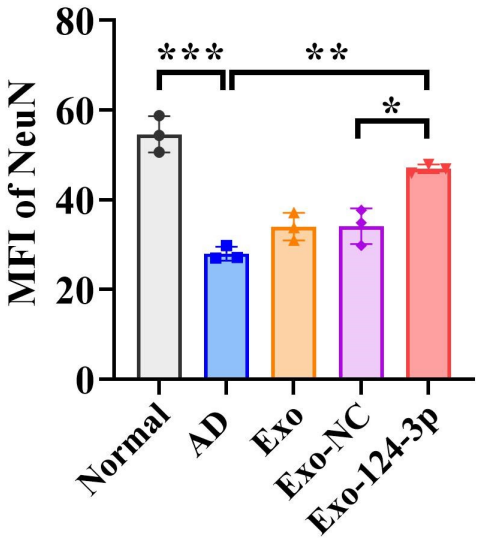


Fig. S10 The mean fluorescence intensity of NeuN in various groups. Error bars represent ± SD (n = 3). * p<0.05, ** p<0.01, *** p<0.001.

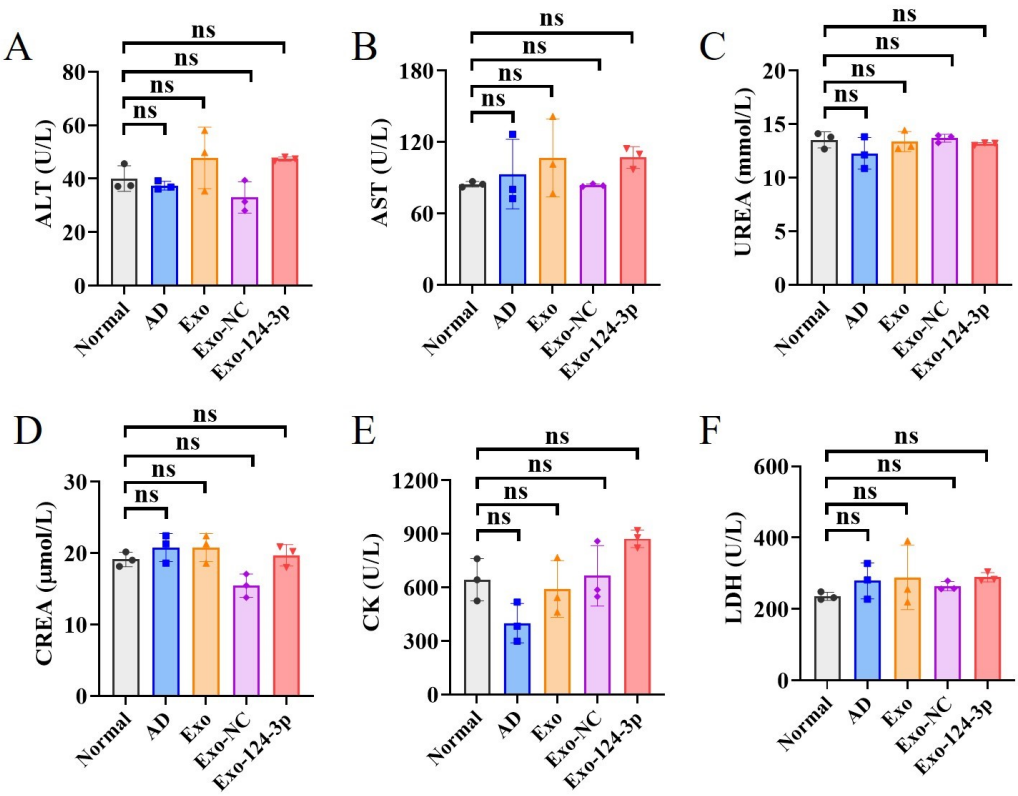


Fig. S11 Analysis of blood biochemical indices in mice. (A-B) Mouse serum ALT (A) and AST (B) levels serve as markers for liver toxicity. (C-D) Serum levels of UREA (C) and UREA (D) are biomarkers of kidney toxicity in mice. (E-F) Mouse Serum CK (E) and LDH (F) Levels as heart toxicity markers. Data are presented as mean ± SD, n

= 3.

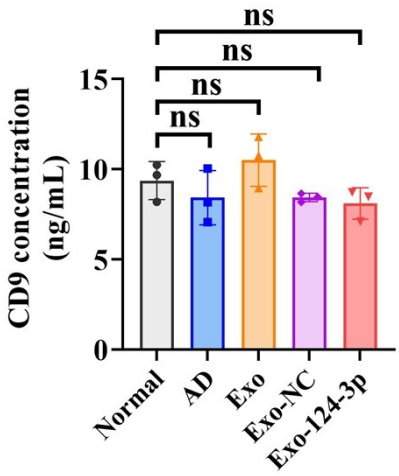


Fig. 12 The concentration of CD9 in serum by ELISA assay.

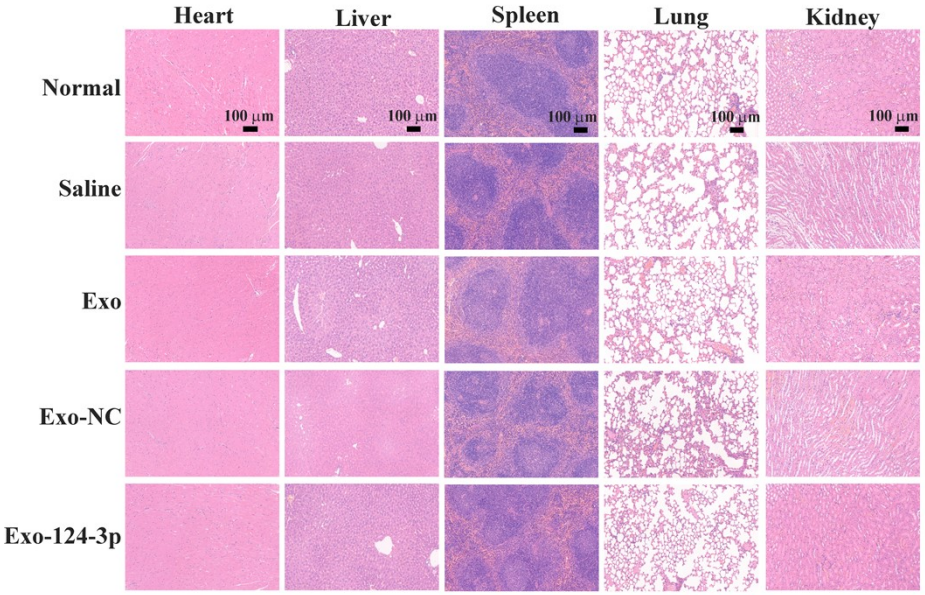
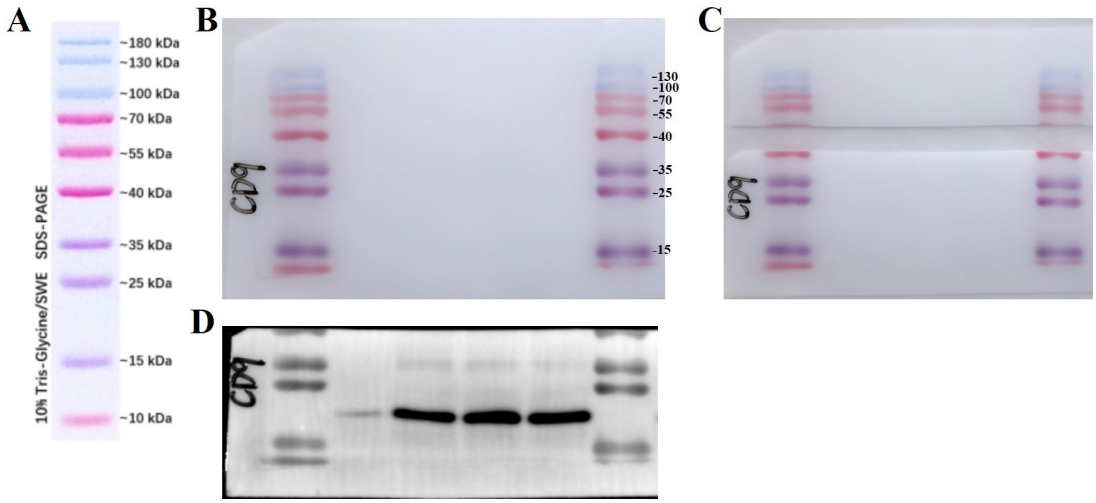
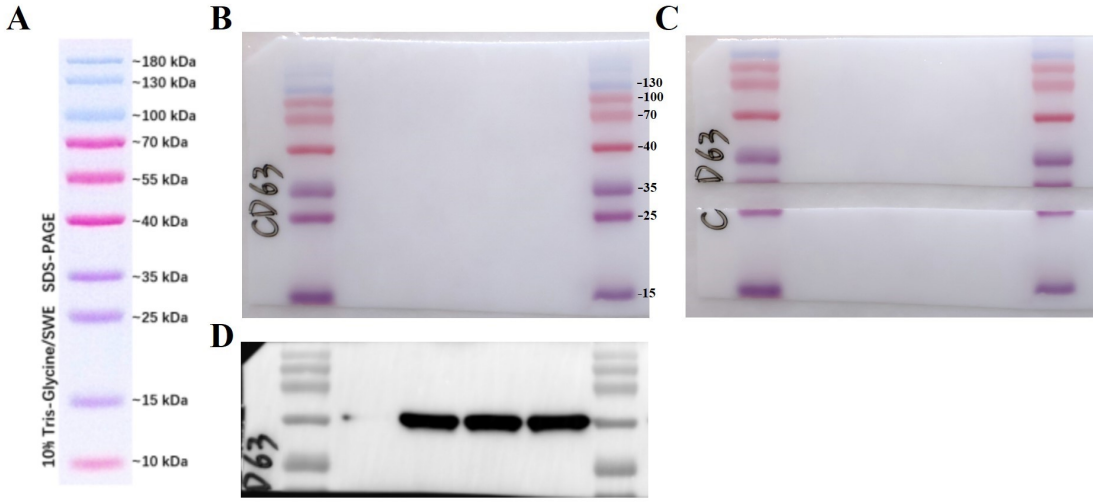


Fig. S13 H&E staining of AD mice after different treatments.

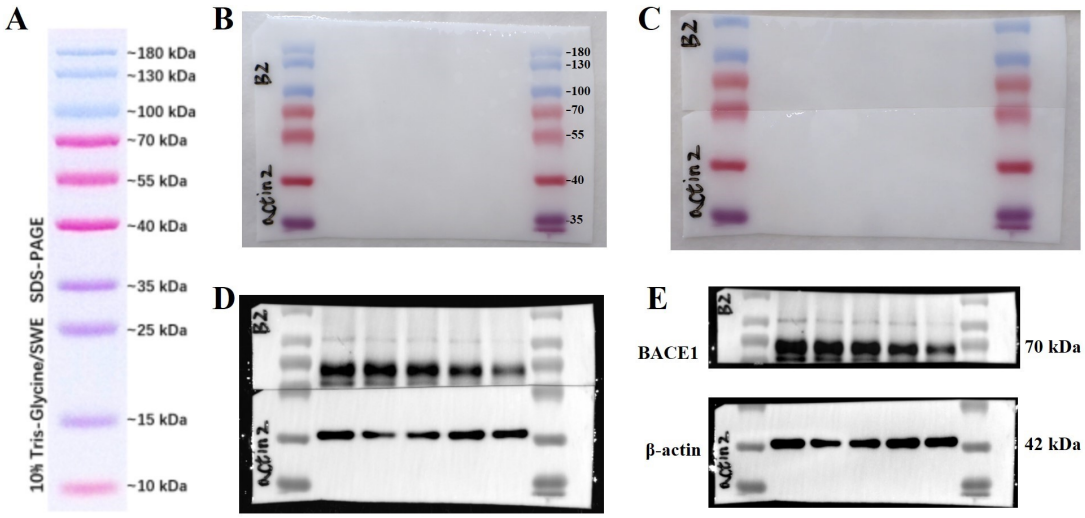
Uncropped blot of CD9 for Fig. 1C



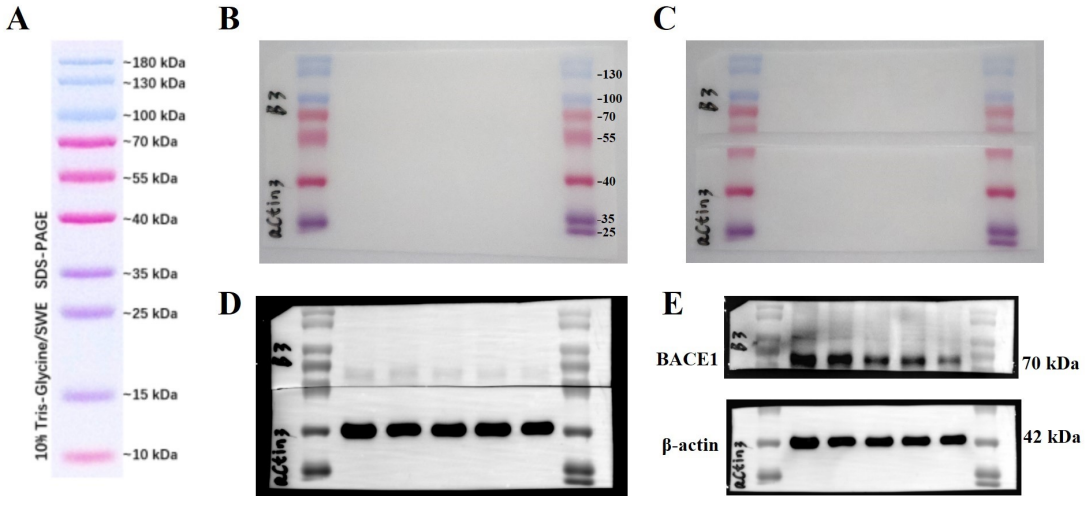
Uncropped blot of CD63 for Fig. 1C



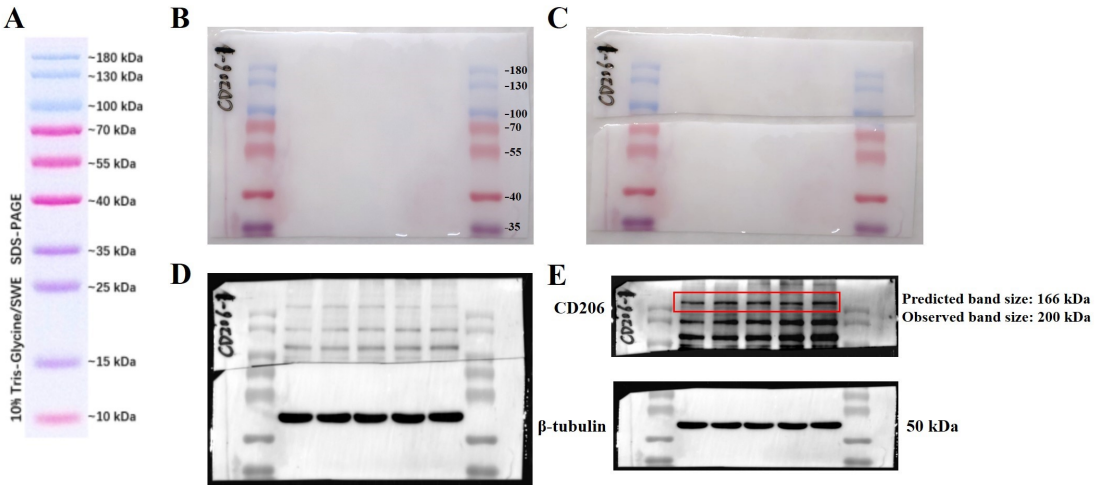
Uncropped blot of BACE1 in PC-12 cells for Fig. 3B



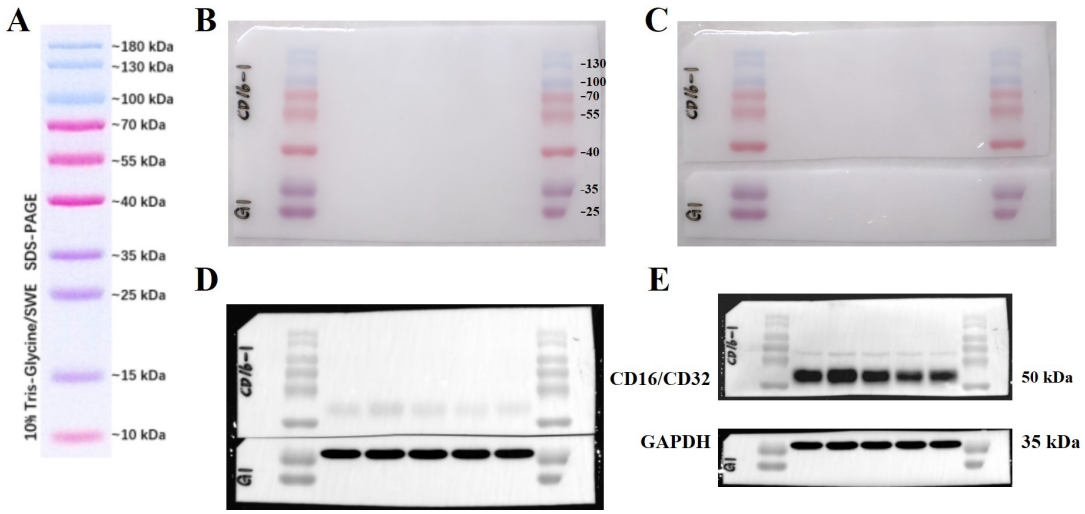
Uncropped blot of BACE1 in AD mice for Fig. 3C



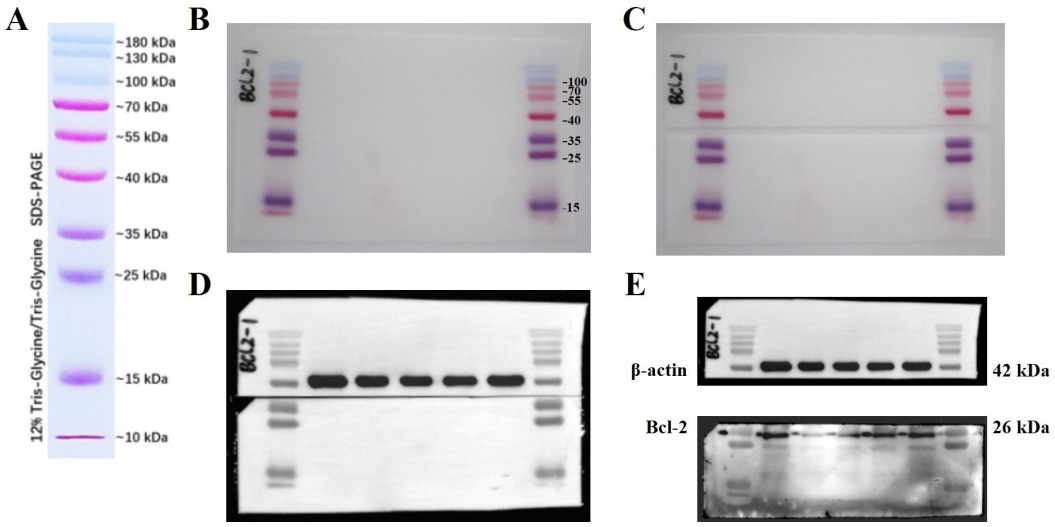
Uncropped blot of CD206 for Fig. 4A



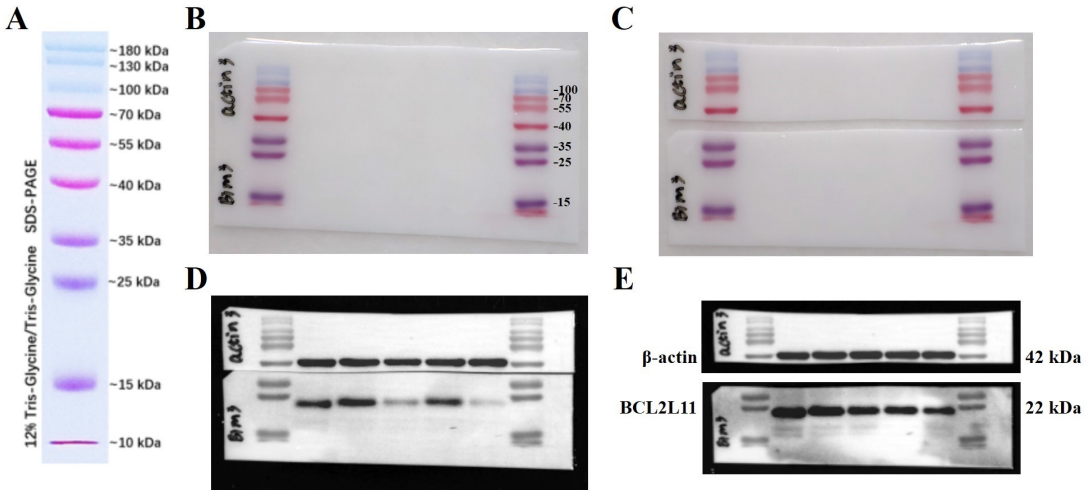
Uncropped blot of CD16/CD32 for Fig. 4A



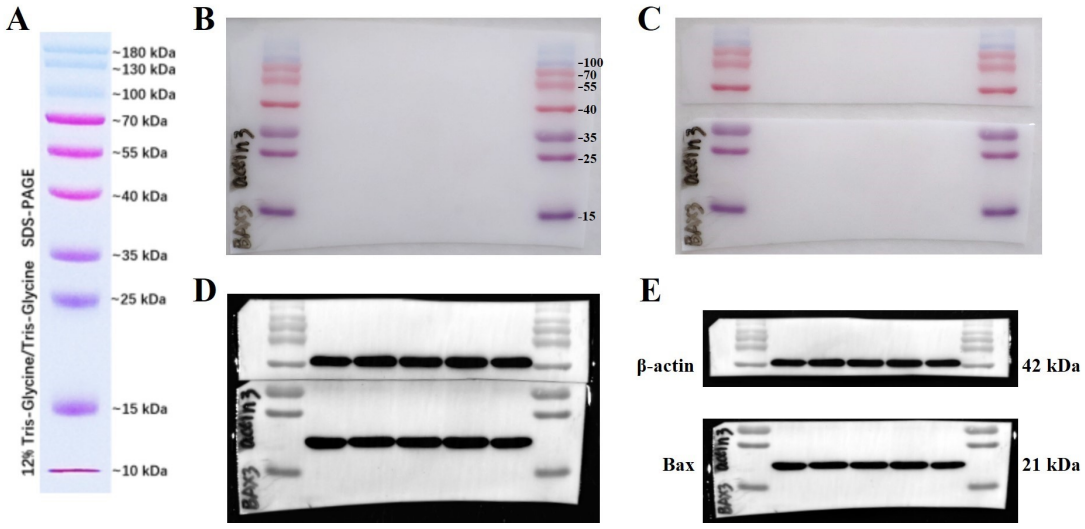
Uncropped blot of Bcl-2 for Fig. 5A



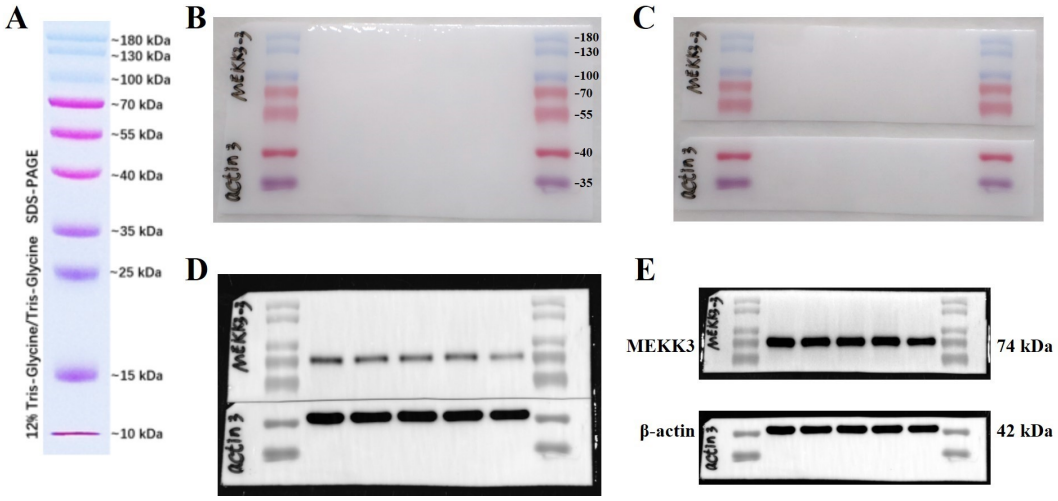
Uncropped blot of BCL2L11 for Fig. 5A



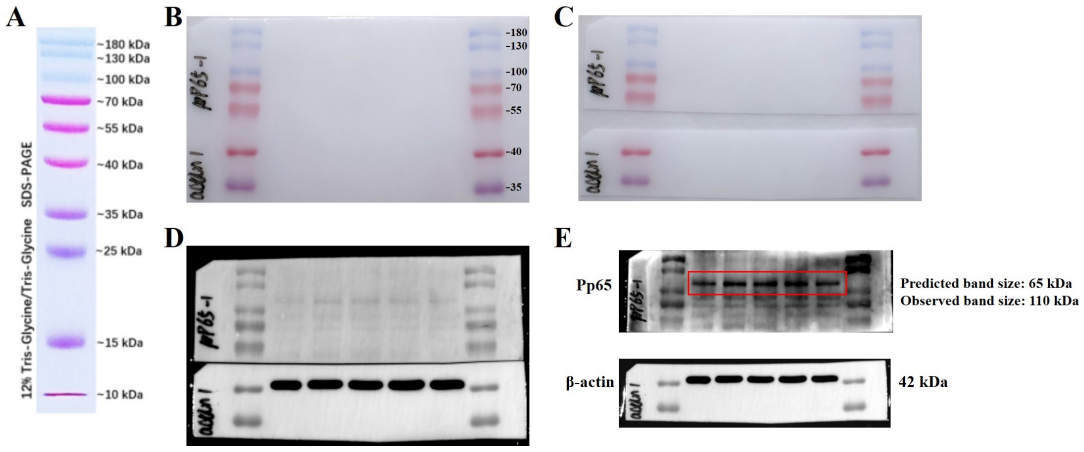
Uncropped blot of Bax for Fig. 5A



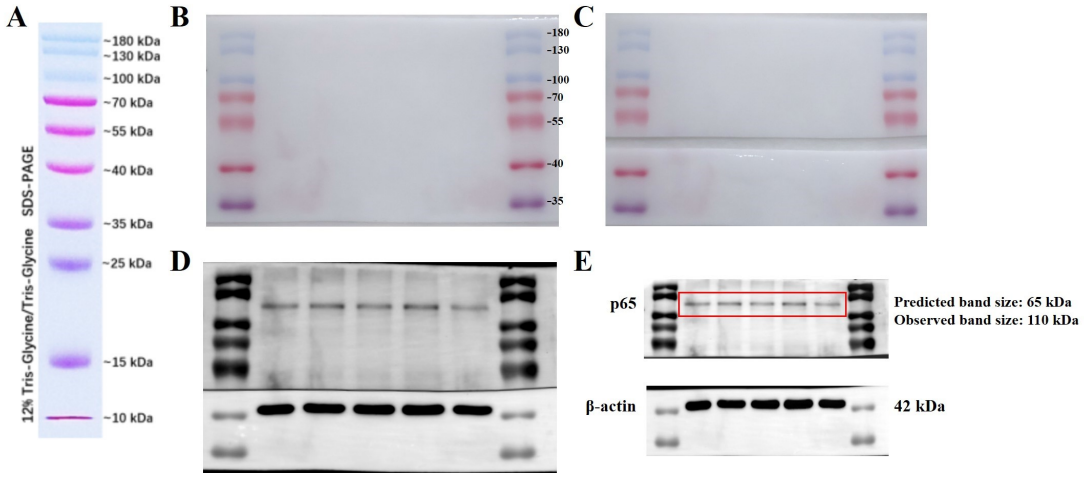
Uncropped blot of MEKK3 for Fig. 7H



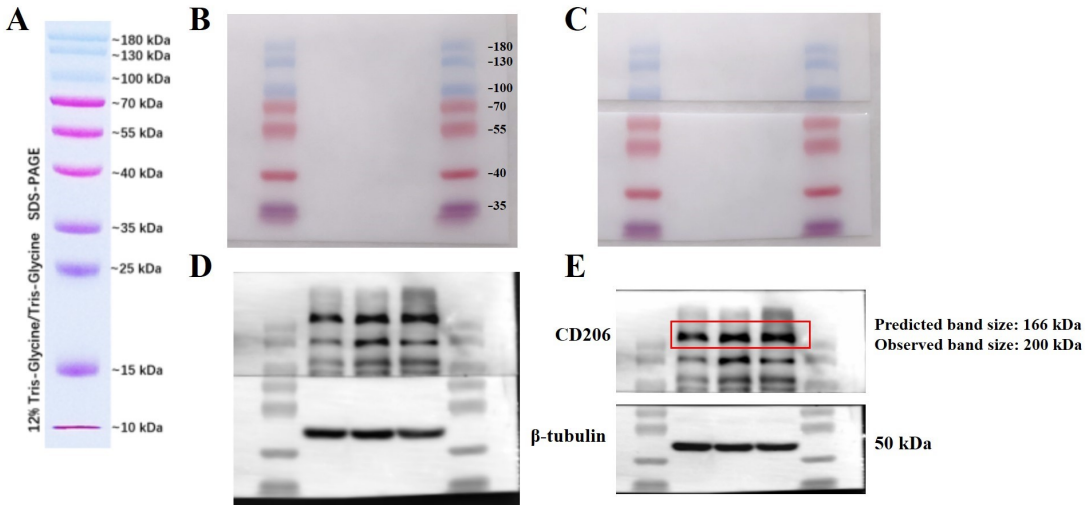
Uncropped blot of Pp65 for Fig. 7H



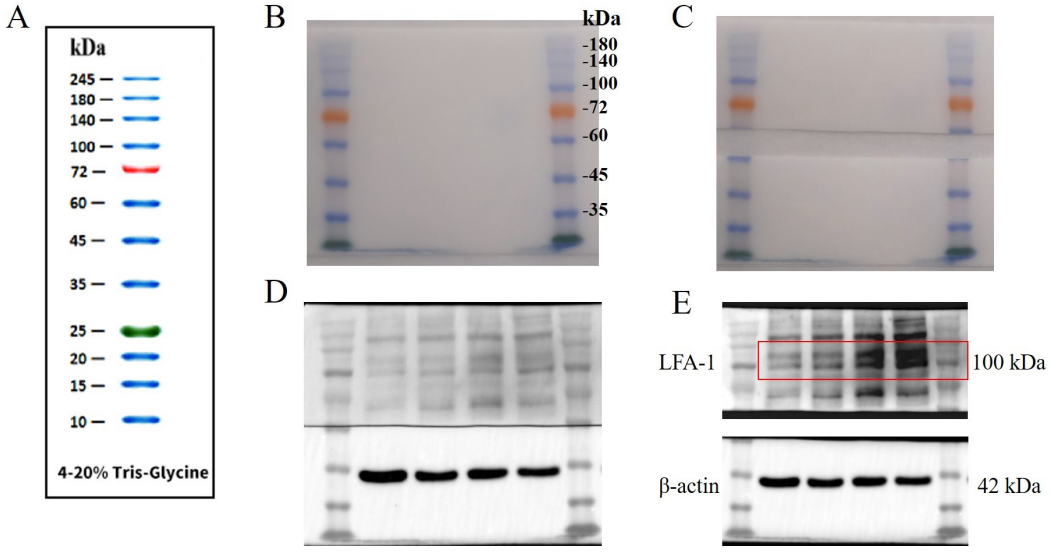
Uncropped blot of p65 for Fig. 7H



Uncropped blot of CD206 for Fig. S1



Uncropped blot of LFA-1 for Fig. S6A



Uncropped blot of VLA-4 for Fig. S6B