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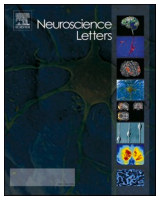

Extracellular vesicles derived from monomeric α-synuclein-treated microglia ameliorate neuroinflammation by delivery of miRNAs targeting PRAK

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ABSTRACT

Parkinson's disease (PD) is characterized by the formation of Lewy body, which mainly contains misfolded α-synuclein. Microglial activation plays a role in neurodegeneration. The pathologically oligomeric α-synuclein promotes inflammatory microglia, while physiologically monomeric α-synuclein induces anti-inflammatory microglia, the relationship between these two forms in activating microglia and the molecular mechanism is essentially unknown. In this study, using in vivo and in vitro models, we challenged primary or BV2 microglia with exogenous stimuli including α-synuclein. We examined microglial activation and the underlying mechanism by Western blot, RT-PCR, ELISA, IF, FCM, miRNA sequencing and bioinformatic analysis. Oligomeric α-synuclein activated microglia via the involvement of the PRAK/MK5 pathway. The specific PRAK inhibitor GLPG0259 could mitigate microglial activation insulted by oligomeric α-synuclein. Monomeric α-synuclein regulated the anti-inflammatory microglia by delivering microglia-derived extracellular vesicles (EVs) in vitro and in vivo. Further sequencing and bioinformatic analysis of microglial EVs-associated miRNAs indicated that most of these miRNAs targeted PRAK. These results suggest that PRAK serves as an intersection in microglial activation when challenged with conformationally different α-synuclein. EVs derived from microglia treated with monomeric α-synuclein promote anti-inflammatory microglia by delivering miRNAs that target PRAK into recipient microglia.

1. Introduction

Microglia serve as the resident immune cells in the CNS and play an important role in maintaining CNS homeostasis. In physiological conditions, microglia facilitate synapse remodeling, nourish neurons, and remove cellular or other debris [1]. Under pathological situations, reactive microglia may induce synaptic dysfunction, or even neuron death by releasing cytokines, chemokines, and ROS [2]. Activation of microglia has been implicated as a pathological contributor to several neurodegenerative diseases. Many of the neurodegenerative diseases are defined pathologically by abnormal accumulation of specific proteins, for example, fibrillary a-synuclein or misfolded a-synuclein are major

Abbreviations: CNS, central nervous system; ROS, active oxygen species; AD, Alzheimer's disease; MSA, multiple system atrophy; ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase type 1; TLR-4, toll-like receptor 4; MAPK, mitogen-activated protein kinase; PRAK/MK5, p38-regulated/activated protein kinase/MAPK-activated protein kinase 5; ERK, extracellular-regulated kinase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; HSP27, heat shock protein 27; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor α; EVs, extracellular vesicles; NTA, Nanoparticle Tracking Analysis; ARG-1, arginase 1; Dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramerthylindocarbocyanine perchlorate; BBB, brain blood barrier; NLPR3, leucine-rich-repeat and pyrin-domain-containing 3; EVs-, EVs from microglia untreated with monomeric α-synuclein; EVs+, EVs from microglia treated with monomeric α-synuclein.

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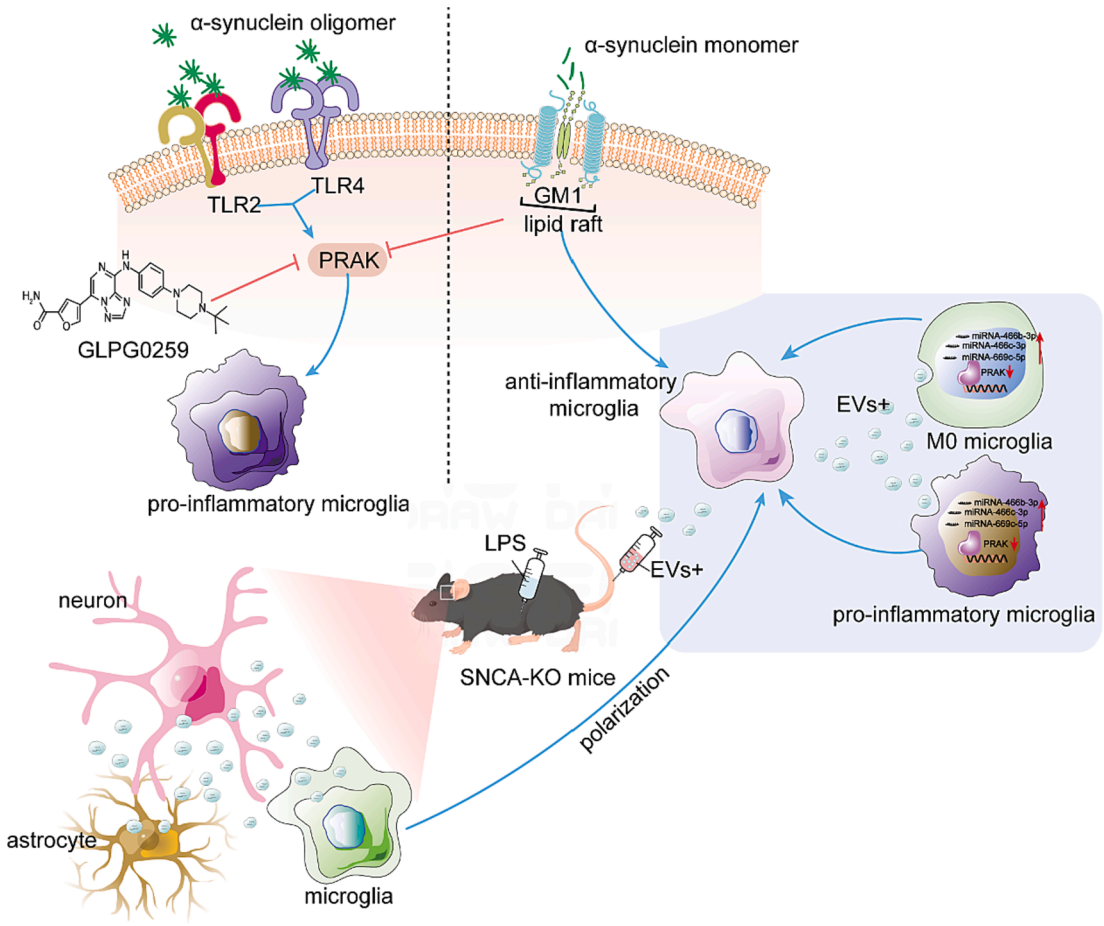

Fig. 1. Graphic illustration shows PRAK as an intersection of conformationally different α-synuclein in microglia activation, and EVs+ **-associated miRNAs targeting PRAK in combination with PRAK inhibitor GLPG0259 may mitigate microglial inflammation.** Oligomeric α-synuclein promotes pro-inflammatory microglia via inducing PRAK, EVs derived from microglia treated with monomeric α-synuclein inhibit PRAK in vitro and promote anti-inflammatory microglia in vivo, the underlying mechanism relates to the delivery of EVs+ -associated miRNAs targeting PRAK in microglia.

components of Lewy bodies or glial cytoplasmic inclusions in PD [2–4] or MSA [2,5], serving as pathological hallmarks, respectively. In addition, filament tau-containing intracellular tangles and beta-amyloid-containing plaques characterize the pathology of AD [6]. Intracellular aggregation of misfolded SOD1 exacerbates neuroinflammation in ALS [7]. The misfolded or aggregated proteins may activate microglia directly or induce neuron damage, which insults microglia. Upon activation, microglia could modulate astrocytes, neurons, or microglia itself in a paracrine or autocrine manner to exacerbate inflammation [8].

The PRAK, a member of the MAPK-activated protein kinase family, is involved in several cellular processes such as cell proliferation, differentiation, apoptosis, motility, embryogenesis, inflammatory process, and neurological process upon phosphorylation by p38MAPK [9,10]. PRAK phosphorylates HSP27 at the sites of Ser-15, Ser-78, and Ser-82, which allows F-actin rearrangement and involves cell migration and metastasis [11]. LPS induces inflammatory response via TLR-4 and p38 MAPK, in which condition MK2 serves as a substrate in microglia [12,13]. TNF-α induces PRAK activation via p38 MAPK in Hela [14]. Monomeric Aβ induces phosphorylation of PRAK in SH-SY5Y neurons via increasing PRAK and RAGE interaction [15]. The role of PRAK in microglial inflammation remains largely unexplored. In this study, we examined the contribution of PRAK in the activation of microglia under pathological protein stimulations. The specific PRAK inhibitor GLPG0259 was used in various cell types, and microglia, GLPG0259 showed potential suppression of microglial inflammation, especially in response to α-synuclein oligomer.

Extracellular vesicles (EVs), based on their origin or size, can be classified as apoptotic bodies (>1000 nm), microvesicles (100–1000 nm), and exosomes (30–150 nm). Upon reaching recipient cells, EVs can induce cell–cell communication via receptor-ligand interaction, or be internalized by endocytosis to deliver their cargo such as proteins, sugars, lipids, and nucleic acids, which can be protected from degradation by the lipid bilayer membrane [16,17]. EVs have been recognized as important messengers among neurons, microglia, astrocytes, and oligodendrocytes both in physiological and inflammatory diseases [18]. Our previous studies have demonstrated that monomeric α-synuclein induces anti-inflammatory microglia through interaction with ERK, upstream of PRAK [19]. Besides, EVs from microglia treated with monomeric α-synuclein protect neurons from MPP^+ toxicity, with the involvement of attenuating mitochondrial fission [20]. In this study, we demonstrated that the component of miRNAs in EVs might facilitate microglial inflammation confronting physiological or pathological α-synuclein and PRAK served as a connection between these conformationally different proteins. Data from this study suggested an important role of PRAK in activating microglia, and that the PRAK inhibitor GLPG0259 in combination with EVs+ -associated miRNAs targeting PRAK showed the potential to mitigate neuroinflammation in neurodegenerative diseases such as PD (Fig. 1).

2. Materials and methods

2.1. Cell culture

The BV2 microglia, Hela, and SH-SY5Y were provided by the

Table 1
Primers used in RT-PCR analysis.

Gene	Primer sequence (5′-3′)
IL-6	Forward- AGTGGCTAAGGACCAAGACC
	Reverse- ACCACAGTGAGGAATGTCCA
iNOS	Forward- ATGCGAAAGGTCATGGCTTC
	Reverse- CCCAAATGTGCTTGTCACCA
MCP-1	Forward- GCTGGAGAGCTACAAGAGGA
	Reverse- ACCTTAGGGCAGATGCAGTT
IL-1β	Forward- AGCTCTCCACCTCAATGGAC
	Reverse- TTGCTTGGGATCCACACTCT
TNF-a	Forward-ACAAGATGCTGGGACAGTGA
	Reverse- ACATTCGAGGCTCCAGTGAA
GAPDH	Forward- AATGTGTCCGTCGTGGATCT
	Reverse- AGACAACCTGGTCCTCAGTG
Ubc	Forward-GGTCGATGCCAGTGAAACTAGCAAGAAGG
	Reverse-CCCCCAGCACACCCTTGAACAAGCACAAG

Table 2
Primary antibodies and fluorescein-labeled antibodies used in frozen sections.

Antibodies	Dilution	Catalog number
PRAK	1:250	Immunoway, YT3848
NeuN	1:300	Abcam, ab177487
Iba-1	1:250	Wako,019-19741
GFAP	1:250	Abcam, ab68428
IL-1β	1:200	SANTA CRUZ, sc-52012
ARG-1	1:200	SANTA CRUZ, sc-271430
Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L)	1:500	Abcam, ab150077
Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L)	1:500	Abcam, ab150117
Alexa Fluor 594-conjugated goat anti-rabbit IgG (H + L)	1:500	Thermo Fisher, A-11037

Table 3
Antibodies used in Western blotting.

Antibodies	Dilution	Catalog number
iNOS	1:1000	Abcam, ab49999
PRAK	1:1000	Immunoway, YT3848
PRAK (phosphorylated Thr182)	1:1000	Immunoway, YP0820
PRAK (phosphorylated Thr182)	1:1000	Invitrogen, PA5-99147
HSP27	1:1000	Immunoway, YT2252
ARG-1	1:1000	Proteintech, 66129-1-Ig
Anti-AIP1/Alix	1:1000	Millipore, ABC40
CD9	1:500	Immunoway, YT0782
CD63	1:1000	Immunoway, YT5525
GAPDH	1:1000	TRANS, HC301
HRP-conjugated goat anti-rabbit IgG(H + L)	1:2000	Biodragon, BF03008
HRP-conjugated goat anti-mouse IgG(H + L)	1:2000	Biodragon, BF03001

Department of Immunology, School of Basic Medical Sciences, Lanzhou University. 293T cells were provided by the Department of Pathology, Peking University Health Science Center. BV2 microglia and SH-SY5Y neurons were cultured in F-12/DMEM (Gibco, 12800-017) containing 10 % FBS (Gibco, 10099-141), 1 % PS (Gibco, 10378-016). Hela and 293T cells were cultured in RPMI 1640 (Gibco, 11875093) containing 10 % FBS (Gibco, 10099-141), and 1 % PS (Gibco, 10378-016). Cells were cultured overnight and media were replaced by F-12/DMEM or RPMI 1640 (free of FBS and PS) on the second day for further treatment.

Primary microglia were isolated from neonatal ICR mice and cultured as published [19].

2.2. α-Synuclein oligomer and $A\beta_{1\text{-}42}$ oligomer preparation

α-Synuclein protein (SinoBiological, 12093-HNAE) was diluted and identified according to the manufacturer's instruction, and α-synuclein oligomer was prepared as published [19]. $A\beta_{1\text{-}42}$(1 mg/ml) (Abcam, ab120301) were prepared in F12/DMEM media at a concentration of 2uM and incubated in 4℃ for 24 h before being used in BV2 culture to promote the formation of oligomer [21].

2.3. Cell viability measurement

PRAK inhibitor GLPG0259 was provided by Prof. Yu Zhang from the School of Basic Medical Sciences, Peking University [22]. BV2 microglia were treated with F12/DMEM containing a ranging concentration of GLPG0259 including 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM and 64 μM, and incubated for 6 h, 12 h, 24 h, and 48 h. Cell viability was assessed by CCK-8 assay kit (Beyotime, C0038).

2.4. The inhibitory effect of GLPG0259 on various cell types

BV2 microglia were treated with LPS + IFN-γ (LPS, 100 ng/ml, IFN-γ, 20 ng/ml), TNF-α (25 ng/ml), α-synuclein oligomer (400 pg/ml), $A\beta_{1\text{-}42}$ (2uM) for 12 h. For induction of PRAK in various cell types, Hela cells were treated with TNF-α (100 ng/ml) for 20 min [14], SH-SY5Y neurons were treated with oligomeric $A\beta_{1\text{-}42}$ (2uM) for 6 h [15], 293 T cells were treated with H_2O_2 (400 μM) for 2 h.

For GLPG0259 treatment, we chose a concentration of 8uM in BV2 microglia and 4uM to 32uM in SH-SY5Y, 293T and Hela cells. Pre-treatment with GLPG0259 was conducted 2 h before stimulation of BV2 microglia or inductions of PRAK in other three cell types.

2.5. RT-PCR

Total RNA was isolated from BV2 microglia using TRIZOL reagent (Invitrogen, 15596-026). The first cDNA was synthesized from about 1 μg total RNA using FastQuant RT Kit with gDNase (MCE, HY-K0511). 1 μl reverse-transcribed cDNA was used in real-time PCR with PowerUp SYBR® Green Master Mix (Applied Biosystem, A25741). The average expressions of GAPDH and Ubc were used as a control for normalizing the number of mRNAs. The primers used are presented in Table 1. All results were analyzed using $2^{-\triangle\triangle Ct}$ and were presented as mean ± s.e.m.

2.6. Immunofluorescence staining

Cells were plated on microscopy-grade Petri dishes (Jet biofilm, BDD002035) at a density of 2×10^5/ml and were treated as described in *2.1*. For frozen sections, coronal or sagittal sections of 30 μm were prepared and stained as previously published [19]. Nuclei were stained with DAPI or hoechst33258.

Images were acquired under a confocal microscope (ZEISS, LSM780). The antibodies and their paired fluorescein-labeled antibodies used are presented in Table 2.

2.7. EVs preparation, nanoparticle tracking analysis, and RNA sequencing analysis

EVs derived from microglia were isolated as previously described [20]. NTA was performed using a NanoSight instrument and NAT Version 3.2 Dev Build 3.2.16 software to quantify EVs and their dispersion and size distribution [20]. miRNA sequencing and analysis were performed by Shanghai Bioprofile Technology Company Ltd, China.

2.8. EVs labeling with Dil

EVs were labeled with Dil for tracing in subsequent experiments. Briefly, a total volume of 300 μl EVs was labeled with Dil at a concentration of 5 μM for 20 min at 4℃. To remove free Dil, Dil-EVs were washed with PBS two times and collected at 35,000 rpm, 4℃, for 2 h

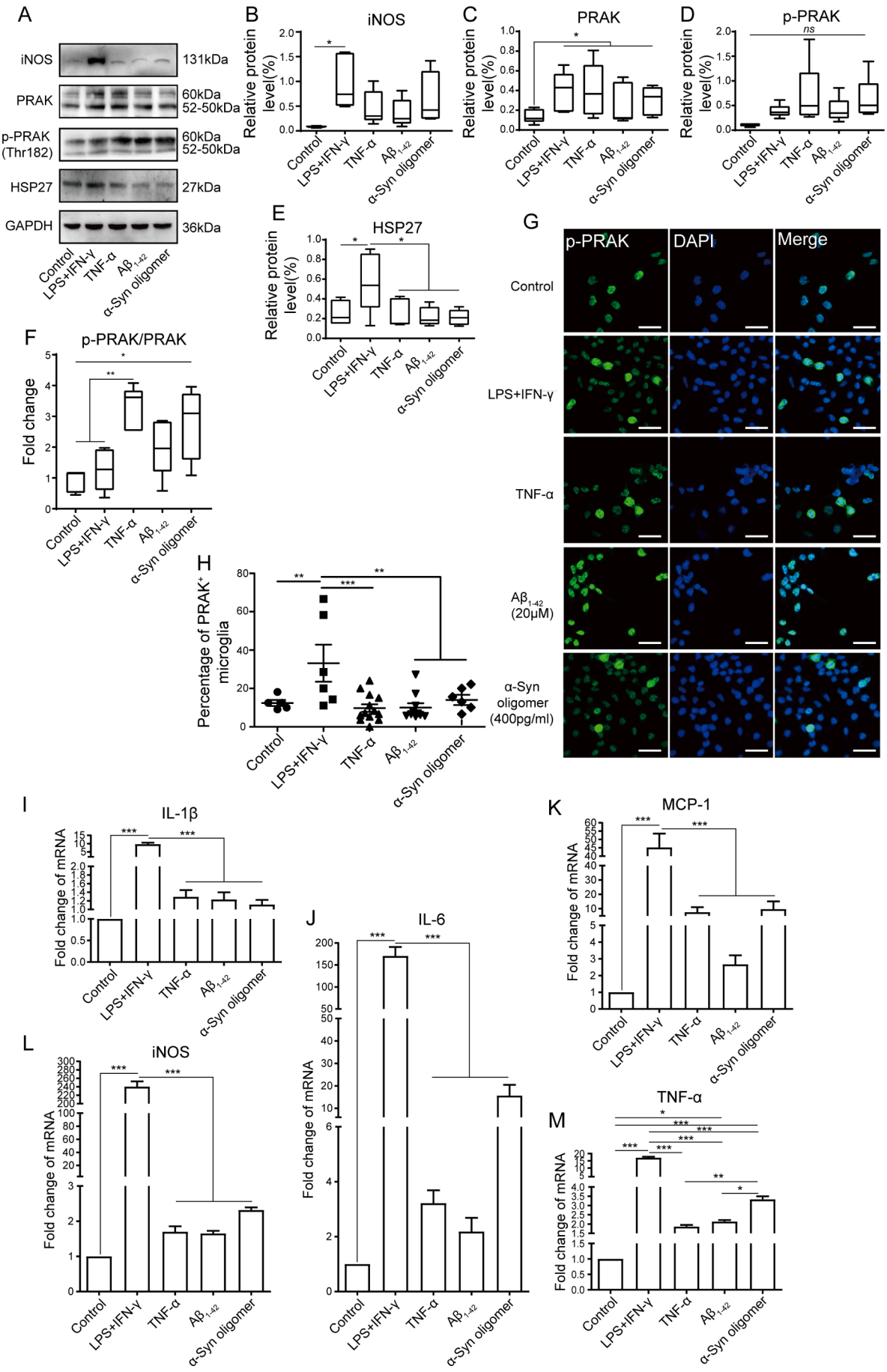

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Fig. 2. Comparison of exogenous factors in activating microglia. A. Expressions of iNOS, PRAK, p-PRAK, and downstream HSP27 in microglia challenged with stimuli. B-E. Densitometry of iNOS, PRAK, p-PRAK and HSP27 in blots (n = 3). F. Ratio of p-PRAK to total PRAK. G. Fluorescent labeling of p-PRAK in BV2 microglia challenged with stimulating factors (scale bar = 50 μm). H. Percentage of microglia with p-PRAK expression. I-M. The levels of mRNA of (I) IL-1β, (J) IL-6, (K) MCP-1, (L) iNOS and (M) TNF-α in BV2 microglia challenged with exogenous factors. Data are presented as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

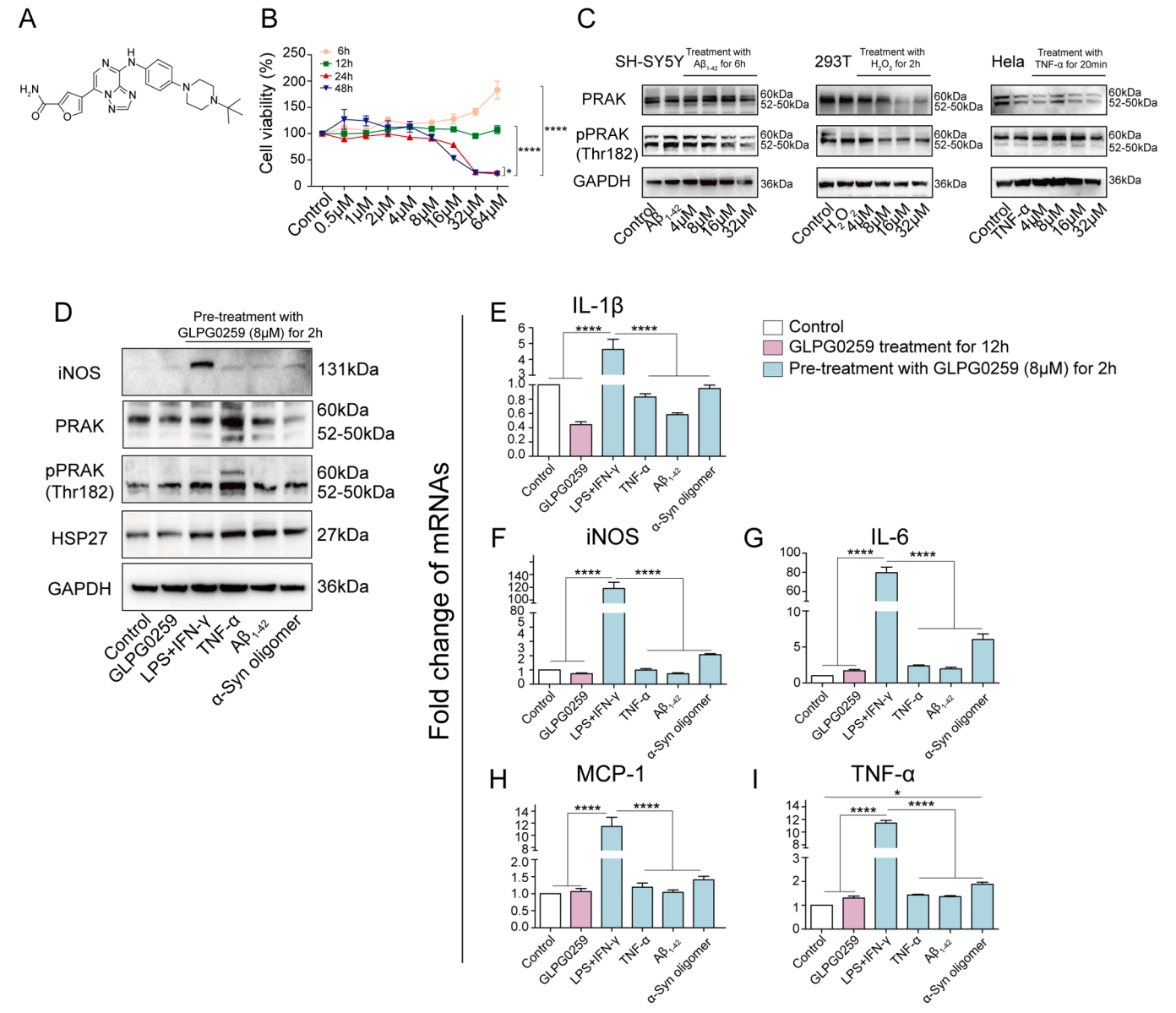

Fig. 3. Inhibition of PRKA ameliorated pro-inflammation in BV2 microglia. A. Structure of PRAK inhibitor GLPG0259. B. Cytotoxicity of GLPG0259 (0.5 μM-64 μM) on BV2 microglia at 6 h, 12 h, 24 h and 48 h. C. Inhibitory effect of GLPG0259 on SH-SY5Y, 293 T, and Hela. D. Expressions of iNOS, PRAK, p-PRAK and downstream HSP27 in microglia with pre-treatment of GLPG0259 under exogenous stimulations were detected by western blotting (n = 3). E-I. The level of mRNAs of (E) IL-1β, (F) iNOS, (G) IL-6, (H) MCP-1, (I) TNF-α in BV2 microglia with pre-treatment of GLPG0259 under exogenous stimulations. Data are shown as mean ± s.e.m. * $p < 0.05$, **** $p < 0.0001$.

after each wash. Dissolving Dil in PBS or the supernatant free of Dil was taken as parallel controls, other procedures were identical to that of Dil labeled EVs outlined above.

2.9. BV2 microglia incubation with Dil-EVs and the internalization of EVs

Dil-EVs were added into BV2 microglia and co-cultured for 12 h at 30 μg/ml (50 μl). Fluorescent signals internalized into microglia were observed and acquired under a confocal microscope (ZEISS, LSM780).

2.10. Electron microscopy

EVs were applied to a copper grid and negatively stained with 2 % phosphotungstic acid for 5 min at RT. The morphology of EVs was examined by transmission electron microscope (TEM) (FEI Tecnai G2 Spirit Bio-Twin).

2.11. Western blotting

Proteins were extracted from cells using cell lysis buffer (RIPA, HARVEYBIO, C1503; Halt Protease & Phosphatase Inhibitor Cocktail

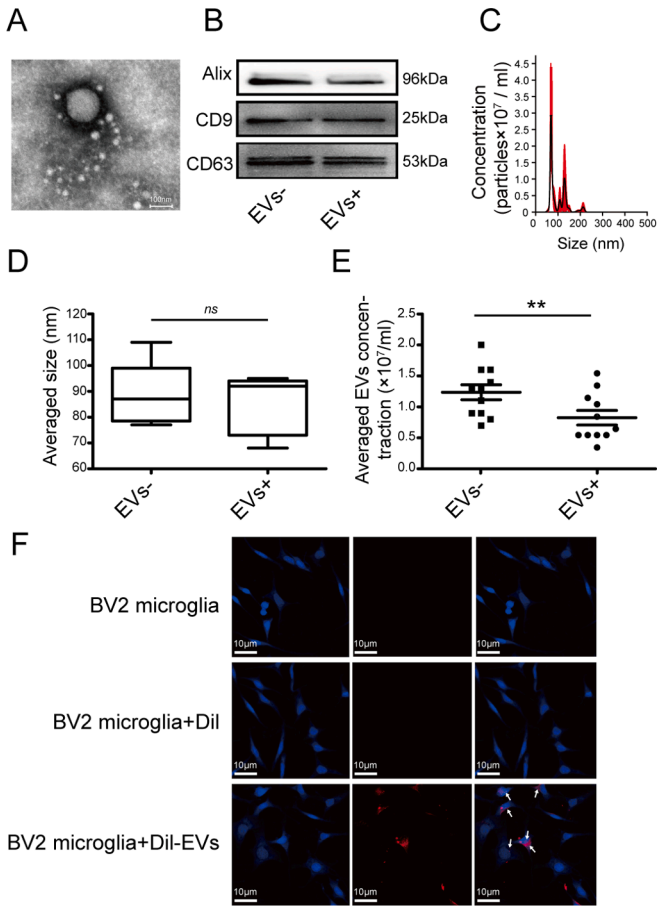

Fig. 4. Identification of microglia-derived EVs and the internalization of EVs by microglia. A. Morphology of EVs was examined by transmission microscopy (scale bar = 100 nm). B. The existence of EVs was verified with Alix, CD9, and CD63 by western blotting (n = 3). C. Nanoparticle tracking analysis of EV concentrations and distribution. D. Comparison of average size between EVs- and EVs +. E. Comparison of EV concentrations between EVs- and EVs +. F. Dil fluorescent signals were observed inside BV2 microglia in the cytoplasm or overlapping with nuclear (scale bar = 10 μm). Data are shown as mean ± s.e.m. ** $p < 0.01$.

(100X), Thermo, 78444). A total content of 35 μg EVs was loaded onto SDS-PAGE. Western blotting was carried out routinely [20]. The antibodies used are presented in Table 3.

2.12. Flow cytometry

BV2 microglia were treated with LPS + IFN-γ, IL-4 + IL-13 or co-cultured with EVs- and EVs+ respectively for 12 h. Cells were incubated with PE Anti-Mouse CD206 Antibody (Elabscience, E-AB-F 1135UD) and FITC Anti-Mouse CD16/32 antibody (Elabscience, E-AB-F 0997C). FACS was performed by NovoCyte Quanteon Flow Cytometer (Agilent) and data was analyzed by Kaluza.

2.13. ELISA and nitrate measurement

The levels of IL-1β, TNF-α, and IL-10 and nitrate in the supernatant of microglia were examined routinely [19]. ELISA kits used included mouse IL-10 ELISA Kit (Beyotime, PI522), mouse TNF alpha ELISA Kit (Proteintech, KE10002), and mouse-IL-1-beta-ELISA-Kit, (Beyotime, PI301). Nitrate concentrations were measured using the Nitric Oxide Assay Kit (Beyotime, S0021).

2.14. Animals and experimental design

SNCA-KO mice (B6;129X1-Snca^{tm1Rosl}/J) lacking endogenous α-synuclein expression were purchased from Nanjing BioMedical Research Institute at Nanjing University [23]. Both SNCA-KO mice and the control wild-type C57/BL6 mice were maintained in a specific pathogen-free facility with a 12-hour light–dark cycle and free access to food and water. All animal experiments and care were strictly performed in accordance with the protocols approved by the ethics committee of Lanzhou University (jcyxy20220306).

Dil-labeled EVs, and parallel controls (PBS, Dil, or supernatant) were intravenously injected into wild-type C57/BL6 mice 3 h before IVIS spectrum imaging (PerkinElmer, Waltham, MA, USA).

LPS (Sigma, L2630; ≧500,000 Endotoxin Units/mg) was reconstituted in water at a concentration of 1 mg/ml. LPS was administered via intraperitoneal (i.p.) injection when mice (SNCA-KO) were 8 weeks old at t = 0 h, t = 6 h, t = 24 h with a dose of 3 mg/kg diluted by 200 μl PBS. Approximately 10 μg (100 μl) of EVs+ was given via intravenous injection (IV) following the last LPS injection and mice were sacrificed at t = 36 h. The brains from both mice were removed and prepared for frozen sections [19].

2.15. Prediction of miRNAs targeting PRAK

Four bioinformatic algorithms (miRWalk, miRPathDB, miRmap, and TargetScan) and Venn diagrams were selected and analyzed to predict miRNAs targeting PRAK.

2.16. Statistical analysis

ANOVA analysis or *t*-test (Mann-Whitney test) was performed for comparisons among groups using GraphPad Prism 8. Data were shown as their mean ± s.e.m. *$p < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. Activation of microglia by exogenous factors and the involvement of PRAK

We first examined the activation of microglia by exogenous factors, the LPS + IFN-γ, which classically induces pro-inflammatory microglia, and the TNF-α, a robust mediator in M1-microglia-induced BBB disruption [24]. Besides, pathological proteins $Aβ_{1-42}$ oligomer and α-synuclein oligomer were used. All factors increased iNOS expression, indicating a pro-inflammatory status in microglia (Fig. 2A, B). The activation of microglia was related to the PRAK pathway, showing elevated expression of PRAK and phosphorylated PRAK in BV2 microglia. PRAK expression demonstrated a significant difference between the control group and the other four groups, phosphorylated PRAK also increased but no statistical significance was achieved (Fig. 2A, C, D). The ratio of phosphorylated PRAK to total PRAK in the control group vs TNF-α treatment, control group vs α-synuclein oligomer treatment and LPS + IFN-γ treatment vs TNF-α treatment showed significantly different (**$p < 0.01$ or * $p < 0.05$, respectively) (Fig. 2F). As a downstream of PRAK, HSP27 increased in LPS + IFN-γ and TNF-α treatments, but not oligomeric $Aβ_{1-42}$ or α-synuclein treatment (Fig. 2A, E), suggesting that oligomeric $Aβ_{1-42}$ and α-synuclein might influence other downstream molecules regulated by PRAK such as CREB, HSP25, Tip60, Hsp40/DnaJB1 [11,25,26]. Fluorescent labeling of PRAK in microglia showed increased expression of PRAK under α-synuclein oligomer treatment (Fig. 2G, H), this was largely in accordance with PRAK levels in Fig. 2A.

When examining cytokines, the mRNAs of pro-inflammatory cytokines including IL-1β, IL-6, iNOS, TNF-α, and chemokine MCP-1 were dramatically elevated in LPS + IFN-γ treatment. α-Synuclein oligomer treatment promoted mRNA expressions of IL-6, iNOS, TNF-α, and MCP-1

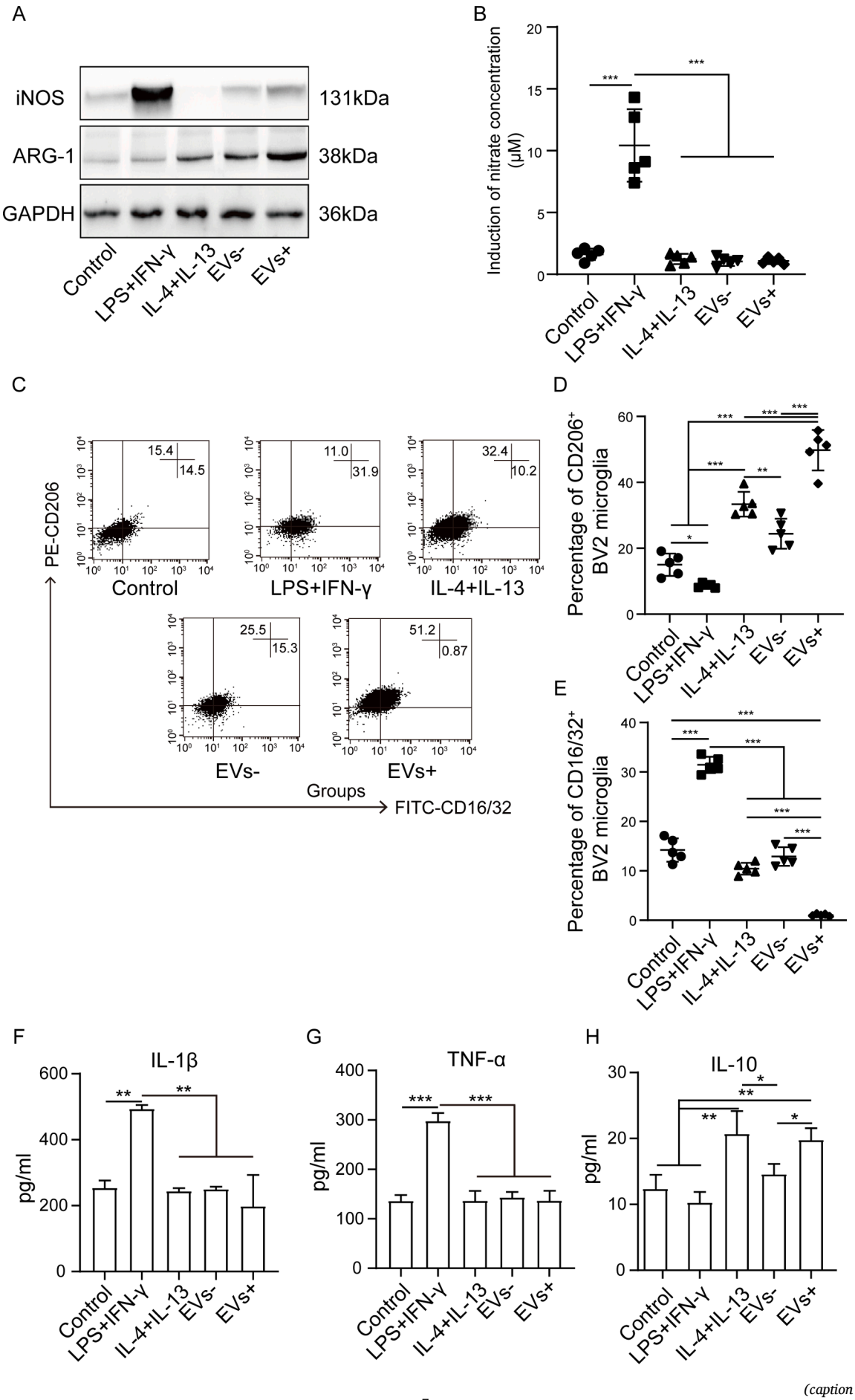

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Fig. 5. EVs+ promoted microglia towards anti-inflammatory phenotype in vitro. A. EVs from BV2 microglia promoted ARG-1 expression and inhibited iNOS expression in primary microglia following 12 h treatment. B. Nitrate concentration in the supernatant in primary microglia (n = 5 with 3 replications). C. BV2 microglia, stained with CD206 and CD16/32 were analyzed by flow cytometry. D-E. Scatter plots indicated the expression of CD206 (D) and CD16/32 (E) on BV2 microglia. F-H. Levels of cytokines IL-1β (F), TNF-α (G), and IL-10 (H) in the supernatant of BV2 microglia by ELISA (n = 3 with 3 replications). Data are shown as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

compared with other groups except for LPS + IFN-γ treatment (Fig. 2I-M). These suggested oligomeric α-synuclein might induce pro-inflammatory microglia through activation of PRAK.

3.2. Inhibition of PRKA ameliorates pro-inflammation in BV2 microglia

As exogenous α-synuclein oligomer induced pro-inflammatory microglia, and this process was closely related to activation of PRAK, it is intriguing to explore the inhibition of PRAK in microglial inflammation. We first treated BV2 microglia with a specific PRAK inhibitor GLPG0259 (Fig. 3A) and found a maximum of 16 μM within 12 h-treatment maintained the viability of BV2 at 100 % (Fig. 3B). We next employed the inhibitory effect of GLPG0259 in three cell lines, and found GLPG0259 prohibited PRAK expression and its phosphorylation in a dose-dependent manner in SH-SY5Y, 293 T cells and Hela [14], while Hela needed a dose above 32 μM for the aim of PRAK inhibition (Fig. 3C).

We next sought to determine whether GLPG0259 could alter microglial overactivation. In all groups except for the LPS + IFN-γ treatment, GLPG0259 attenuated iNOS expression in microglia (Fig. 3D and supplementary Fig. 1A). There was obvious inhibition of PRAK by GLPG0259 in $A\beta_{1\text{-}42}$ oligomer and α-synuclein oligomer treatments, nearly to the level of PRAK in GLPG0259 treatment alone and control group, and this inhibitory effect was more robust in α-synuclein oligomer treatment (Fig. 3D and supplementary Fig. 1B). The same trends of p-PRAK and HSP27 were also observed (Fig. 3D and supplementary Fig. 1C, D). Pre-treatment with GLPG0259 inhibited mRNAs of IL-1β, iNOS, IL-6, MCP-1, and TNF-α in TNF-α and Aβ 1–42 oligomer treatments (Fig. 3E-I). Though high mRNA levels were still maintained in LPS + IFN-γ and α-synuclein oligomer treatments, GLPG0259 pre-treatment dramatically reduced the mRNAs of these inflammatory mediators in both groups (Supplementary Fig. 2A-E). Together, these suggested inhibition of PRAK by GLPG0259 resulted in decreased mRNAs of inflammatory mediators in microglia.

3.3. Monomeric α-synuclein modulates anti-inflammatory microglia via delivery of EVs in vitro

We previously demonstrated that monomeric α-synuclein regulated microglia towards anti-inflammatory phenotype [19] and EVs+ appeared to be protective involving neuronal mitochondrial fission [20]. In this study, we wanted to elucidate the exact component of EVs+ that might regulate anti-inflammatory microglia.

We first characterized EVs as membrane-bound morphology by TEM (Fig. 4A). The presence of EVs was verified by markers of Alix, CD9, and CD63 (Fig. 4B). The NTA analysis demonstrated the diameter distribution around 100 nm (Fig. 4C). Surprisingly, the release of EVs by microglia treated with monomeric α-synuclein dramatically decreased as evaluated by the expression of Alix, CD9, and CD63 (Fig. 4B), further calculation indicated the average concentration, not the size of EVs demonstrating a significant difference between EVs- and EVs+ (Fig. 4D, E). Confocal microscopy analysis showed clearly the presence of Dil-labeled EVs in the cytoplasm of microglia (Fig. 4F).

We next wanted to clarify the function of EVs+ in modulating microglial activation. We found both EVs- and EVs+ increased ARG-1 expression and inhibited iNOS expression in primary microglia (Fig. 5A). Nitrate concentrations measured in culture media were consistent with the iNOS levels (Fig. 5B). We also chose another two markers CD16/32 and CD206 as a microglial pro-inflammatory marker or anti-inflammatory marker, and examined their expressions on microglia by flow cytometry. The percentage of $CD206^+$ microglia was significantly elevated (***$p < 0.001$ compared with other groups) (Fig. 5C, D). In contrast, the percentage of $CD16/32^+$ microglia was dramatically reduced (***$p < 0.001$ compared with other groups) under EVs+ treatment (Fig. 5C, E). When detecting cytokines, EVs+ treatment dramatically reduced the pro-inflammatory cytokines IL-1β and TNF-α (Fig. 5F, G). and increased the anti-inflammatory cytokine IL-10 (Fig. 5H). These results suggested EVs+ tended to inhibit pro-inflammatory microglia and modulate microglia towards an anti-inflammatory phenotype.

3.4. EVs cross the BBB and regulate microglia towards an anti-inflammatory phenotype in vivo

We next sought to determine whether EVs could cross BBB and induce anti-inflammatory microglia in vivo. By intravenous injection of the Dil-labeled EVs into wild-type mice, we found the Dil-labeled EVs penetrated through the BBB three hours post-injection, with the strongest fluorescent signal in the Dil-EVs group (Fig. 6A, B). To determine the cell-specific locations of EVs, we used NeuN, Iba-1, and GFAP as markers of neurons, microglia and astrocytes, respectively. Immunofluorescence images showed Dil-EVs were present in all cells with an average of 1–3 Dil signals in one cell in the cortex, hippocampus or midbrain (Fig. 6C), there was no significant difference in the distribution of EVs among these cells.

Without being interfered with by endogenous α-synuclein, EV injections were carried out in mice lacking endogenous α-synuclein. We examined microglial expressions of ARG-1 and IL-1β following LPS injection, which induced microglial activation via peripheral administration [27]. LPS injection induced high levels of microglia positive for Iba-1and IL-1β (53.19 %) (Fig. 7A, B) and deficient levels of $Iba\text{-}1^+/ARG\text{-}1^+$ microglia (8.39 %) (Fig. 7C, D). However, when LPS and EVs+ were injected, the percentage of $Iba\text{-}1^+/ARG\text{-}1^+$ microglia dramatically increased (61.68 %) (Fig. 7C, D), the percentage of $Iba\text{-}1^+/IL\text{-}1\beta^+$ microglia decreased (34.96 %) (Fig. 7A, B), indicating EVs+ were able to regulate microglia towards an anti-inflammatory phenotype in vivo.

3.5. The EVs+ associated miRNAs mostly target PRAK

As stable and multiple biological functions, miRNAs in EVs could be transferred into recipient cells. We hypothesized that EVs-associated miRNAs play a role in inducing anti-inflammatory microglia. We performed a miRNA-sequencing analysis of EVs. Based on the profiling results, of 901 miRNAs, 10 with increase and 10 with decrease as determined by the value of logFC (more than two-fold change), and their features related to neuroprotective or anti-inflammatory functions were also analyzed (Fig. 8A and Supplementary Table 1). To further elucidate the PRAK as an intersection in microglial activation challenged by monomeric and oligomeric α-synuclein, we screened four bioinformatics tools (miRWalk, miRPathDB, miRmap and TargetScan) to predict miRNAs targeting PRAK and the overlapping of the targeted miRNAs with the changed 20 miRNAs (Fig. 8B). Of the 20 miRNAs, 14 miRNAs targeting PRAK were confirmed by bioinformatic predictions. Of 14 miRNAs, miR-466b-3p, miR-466c-3p and miR-669c-5p possessed neuroprotective or anti-inflammatory functions (Fig. 8C and Supplementary Table 1) [28–31]. WB results further showed it was EVs+, not EVs-, α-synuclein monomer, or α-synuclein oligomer that inhibited PRAK and its phosphorylation (Fig. 8D). In conclusion, miRNAs in EVs+

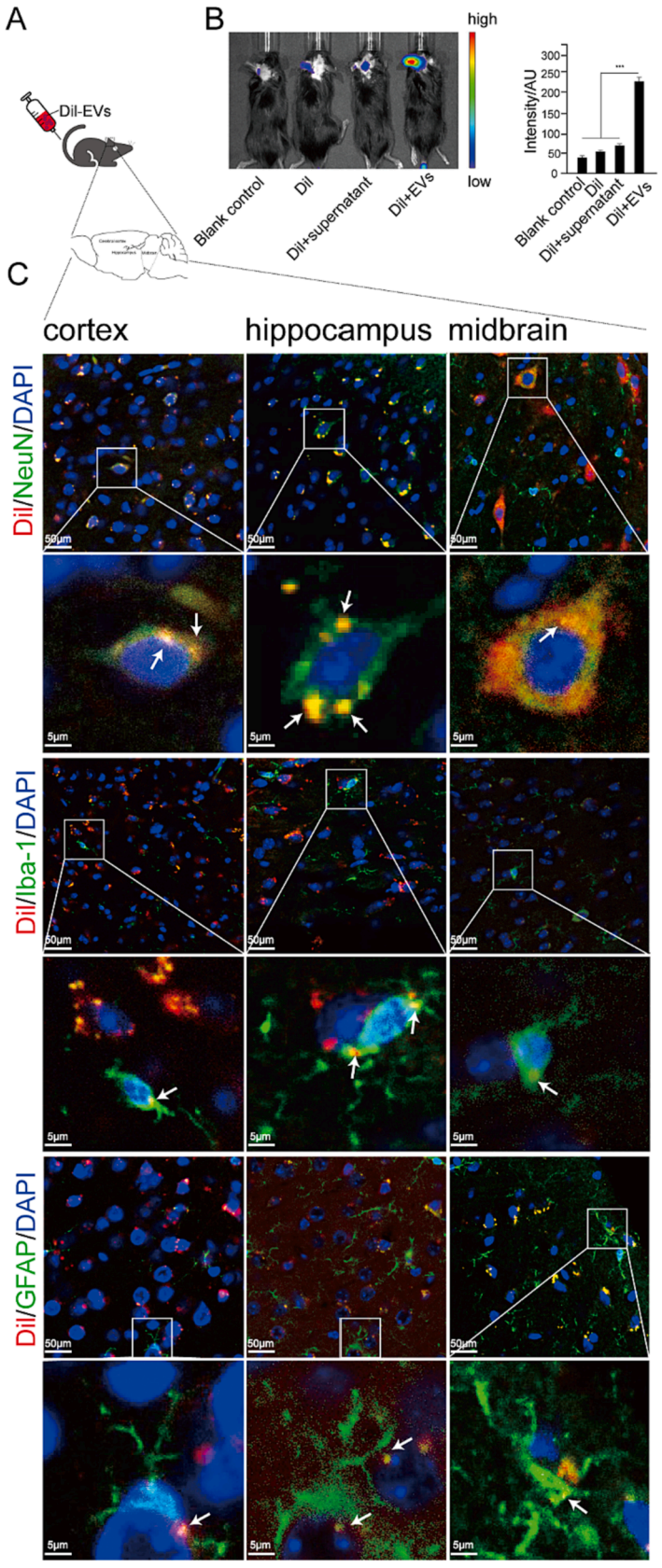

Fig. 6. EVs+ **cross the BBB and were internalized by neurons and glia in vivo.** A. Indication of intravenous injection of Dil-labeled EVs into wild-type or SNCA-KO mice. B. IVIS spectrum imaging of the wild-type mouse brains 2-hour post injections and the intensity of fluorescent signals. C. Representative images of Dil-labeled EVs+ colocalized with neurons, microglia and astrocytes in the cerebral cortex, hippocampus and midbrain of wild-type mice. The arrows indicate Dil-labeled EVs+ (red) in cells. (Upper scale bar = 50 μm, lower scale bar = 5 μm, n = 5 mice). One-way ANOVA with Tukey's multiple comparison test. Bar graphs show mean ± s.e.m. ***$p < 0.0001$.

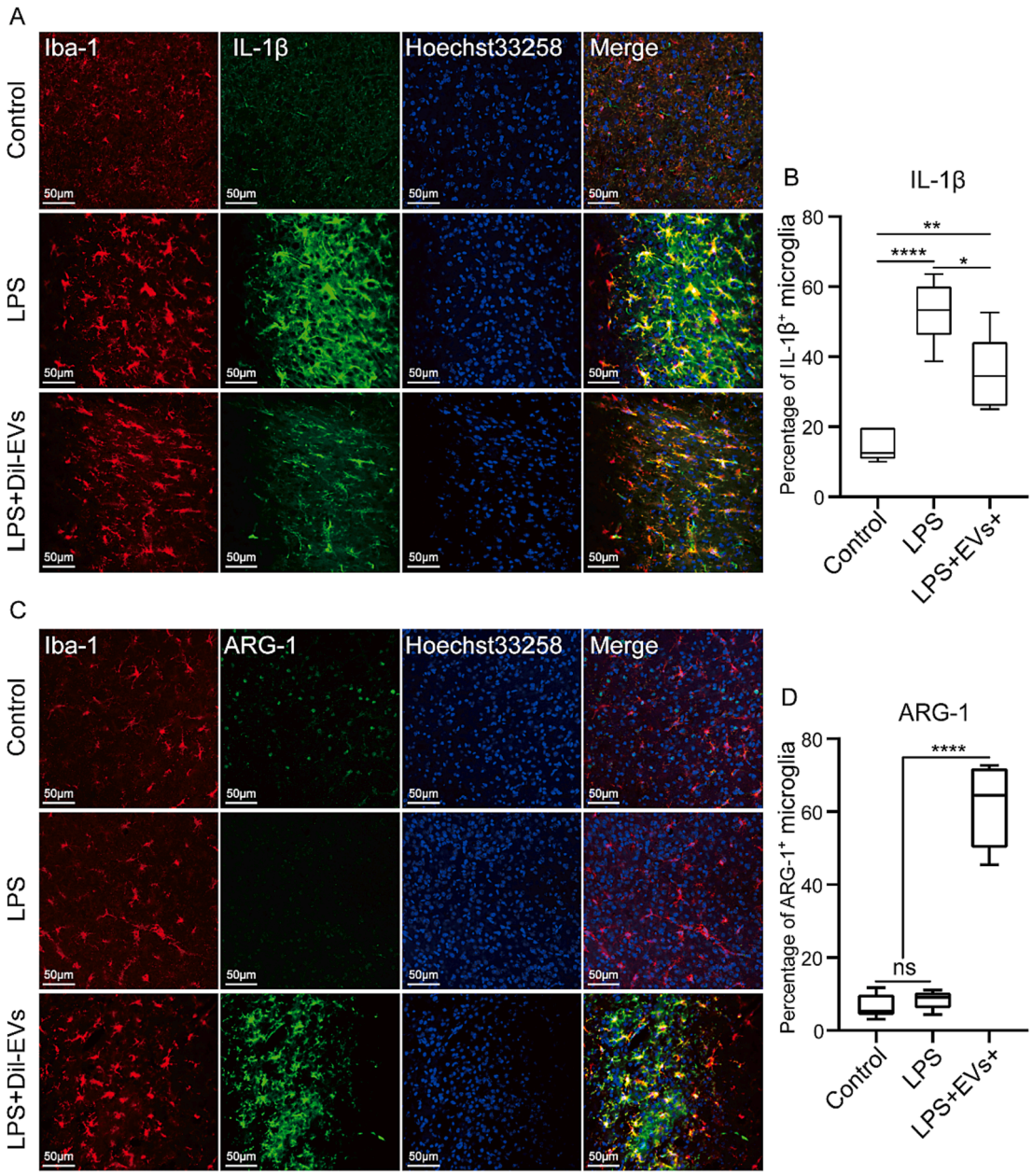

Fig. 7. EVs+ **promoted microglia towards anti-inflammatory phenotype in vivo.** A. Immunofluorescent staining for IL-1β (green) in Iba-1$^+$ (red) microglia, nuclei were stained with hoechest33258 (blue) (scale bar = 50 μm) (n = 3 SNCA-KO mice). B. Immunofluorescent staining for ARG-1 (green) in Iba-1$^+$ (red) microglia, nuclei were stained with hoechest33258 (blue) (scale bar = 50 μm) (n = 3 SNCA-KO mice). C. Percentage of IL-1β$^+$ cells in Iba-1$^+$ microglia. D. Percentage of ARG-1$^+$ cells in Iba-1$^+$ microglia. One-way ANOVA with Tukey's multiple comparison test. Bar graphs show mean ± s.e.m. *p < 0.05, **p < 0.01, ****p < 0.0001.

induced anti-inflammatory microglia via inhibition of PRAK. However, the validation of EVs+ associated miR-466b-3p, miR-466c-3p and miR-669c-5p targeting PRAK and related molecular mechanisms warranted further investigation.

4. Discussion

The most important discovery of this study is uncovering the PRAK as an intersection in microglial activation confronting aggregated or monomeric α-synuclein, the underlying mechanism was related to the delivery of EVs+ miRNA-466b-3p, −466c-3p and −669c-5p targeting PRAK into microglia. Neuroinflammation has attracted considerable attention to its role in degenerative diseases, and the role of p38 MAPK in microglial activation has previously been suggested [32,33]. PRAK is one of the substrates regulated by p38α [34]. Targeting p38α related kinases rather than individual factors may provide promise in ameliorating or balancing neuroinflammation [12].

Studies have demonstrated MK2 in microglial inflammation and neurodegeneration, but the role of PRAK(MK5) in microglial activation has not been reported, besides being phosphorylated by p38α, PRAK can also be phosphorylated by ERK [35]. As an important agent in Parkinson's disease and related LB pathology, aggregated α-synuclein has been proven to insult pro-inflammatory polarization of microglia [36]. In our study, the overactivation of microglia was induced by α-synuclein oligomer treatment (Fig. 2I-M), and this was related to the PRAK pathway (Fig. 2A, C, D, and F). The PRAK inhibitor could mitigate microglial inflammation by specifically inhibiting the activation of PRAK and its downstream HSP27 (Fig. 3D and Supplementary Fig. 2).

Activation of microglia exerts increased secretions of pro-inflammatory factors such as IL-1β, TNF-α, NO, PGE_2, MCP-1 and IL-6,

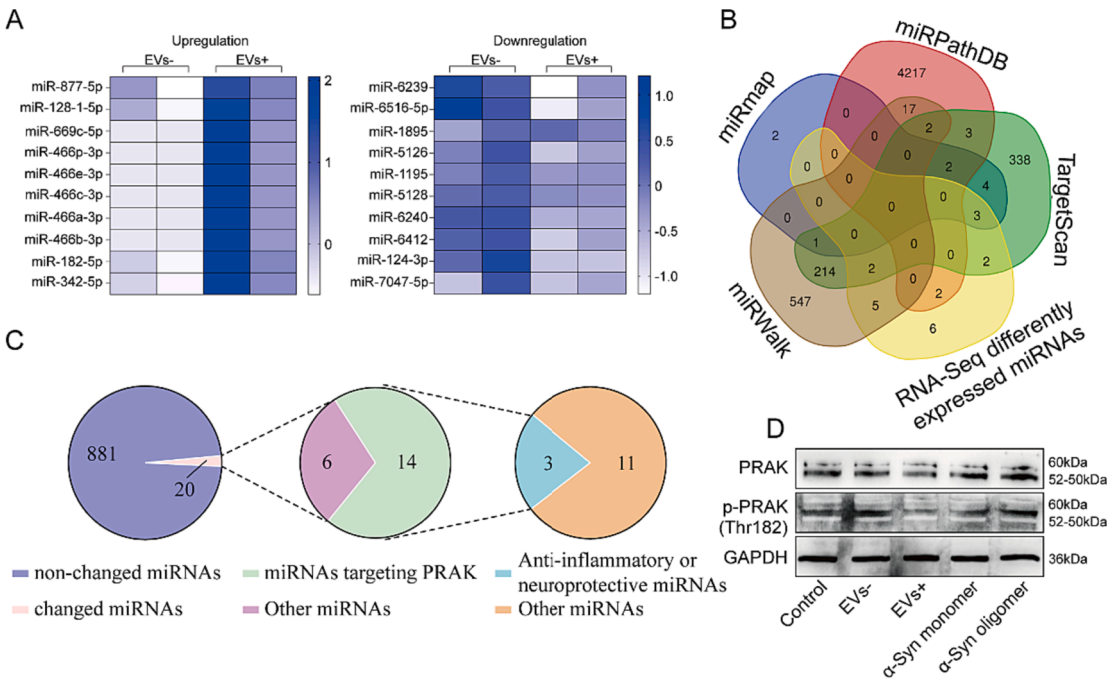

Fig. 8. EVs+ **associated miRNAs targeted PRAK predicted by bioinformatic analysis** A. A heatmap displayed up-regulated and down-regulated miRNAs in EVs+ and EVs-. B. An intersection of the Venn diagram showed the overlapping of miRNAs targeting PRAK among miRWalk, miRPathDB, miRmap, TargetScan and miRNA sequence analysis. C. Diagram showing EVs+ associated miRNAs targeted PRAK and participated in anti-inflammation or neuroprotection. D. Protein levels of PRAK and p-PRAK in microglia with treatments of EVs-, EVs+, α-synuclein monomer or α-synuclein oligomer.

which proved to be involved in many neuroinflammation and neurodegenerative diseases [37]. Here the utility of PRAK inhibitor GLPG0259 showed obvious downregulations of IL-1β, IL-6, MCP-1, iNOS and TNF-α (Fig. 3E-I and Supplementary Fig. 2). The molecular networks in microglia with stimuli of these exogenous factors have been fully elucidated. Simultaneous triggering of TLR4 by LPS and IFN-γ signaling pathways induce pro-inflammatory microglia [38,39], LPS stimulates phosphorylation of p38 MAPK, JNK, c-Jun, ERK and NF-κB to promote pro-inflammatory phenotype in BV2 microglia [40], but LPS exposure alone was not enough to induce MAPK pathway activation in SN of mice [41]. TNF-α originates from microglia or other sources could reactivate microglia in positive feedback through binding to TNFR1 (p55) and/or TNFR2 (p75) to mediate various biological processes including inflammation [42]. $A\beta_{1\text{-}42}$ induced microglial activation via TLR4 and related to MAPK activation [43,44]. α-Synuclein aggregates activate the NLPR3 inflammasome of microglia through recognition by TLR2 and TLR4 [36], which insults microglial activation and is related to PRAK activation (Fig. 2A-F). In contrast to the aforementioned exogenous agents, monomer α-synuclein, as a native state of this protein, can be internalized into microglia via ganglioside GM1 and in lipid raft-dependent, but clathrin-, caveolae-, dynamin-independent manner [45]. We have previously confirmed monomeric α-synuclein interacts with ERK and promotes the anti-inflammatory phenotype of microglia through ERK/NF-κB/PPARγ pathways [19]. This may explain why monomeric α-synuclein could inhibit PRAK phosphorylation as PRAK serves as a substrate of ERK (Fig. 8D). PRAK inhibitor GLPG0259 showed a robustly inhibitory effect on PRAK and p-PRAK in microglia treated with α-synuclein oligomer, indicating the important role of PRAK in microglial activation challenged with oligomeric α-synuclein (Fig. 3D). However, we just showed observations in a microglial cell line BV2, other cell types used were also common cell lines, further investigation in vivo in animal models is still necessary.

The EVs derived from microglia have been reported to exert multifaceted effects in the CNS including the development of AD and PD [46–49]. Different stimuli such as LPS, aggregative α-synuclein and IL-4 have proved to enhance EVs secretion from microglia [50–52]. However, we found monomeric α-synuclein reduced EVs release from microglia (Fig. 4E), indicating this physiological form of α-synuclein participated in the cell process but was not immediately degraded by the ubiquitin–proteasome system and autophagy-lysosome pathway as aggregative form does [36]. Further study confirmed the EVs could be internalized into BV2 microglia in vitro (Fig. 4F). And penetrate the BBB to colocalize with neurons, astrocytes and microglia in different parts of the brain in vivo (Fig. 6). EVs+ promoted BV2 microglia towards an anti-inflammatory phenotype (Fig. 5), this was confirmed in an in vivo animal model lacking endogenous α-synuclein expression (Fig. 7). As we previously demonstrated monomeric α-synuclein promoted M2 microglia. Yang GY has proved M2 microglia-derived EVs promote white matter repair and functional recovery via miRNA [53]. Release of EVs from microglia under treatment with aggregative α-synuclein facilitates α-synuclein transmission in PD [54]. These suggest the function of EVs derived from microglia depends on the stimuli and the status of microglia.

EVs-associated miRNAs could be transferred from cell to cell and regulate target genes to modulate the functions of recipient cells [55]. EVs+ -associated miR-466b-3p, miR-466c-3p and miR-669c-5p might exert neuroprotective or anti-inflammatory functions by targeting PRAK (Fig. 8A, B, C and Supplementary Table 1) [28–31]. The exact mechanism and the crosstalk of EVs-associated miRNAs and their targets still need further investigation. Nonetheless, our results elucidate the importance of PRAK in microglial activation, understanding the miRNA-PRAK axis and the utility of EVs+ may pave the way for ameliorating neuroinflammation.

5. Conclusion

In summary, we compared the exogenous stimuli in activating microglia and the role of PRAK in microglial activation. The application of the specific PRAK inhibitor GLPG0259 and EVs+ may provide great value in controlling microglia-related inflammation if further investigation is validated.

CRediT authorship contribution statement

Na Li: Conceptualization, Methodology, Investigation, Writing – original draft. **Yang Huang:** Methodology, Formal analysis. **Yufeng Wu:** Methodology, Formal analysis. **Qilong Wang:** Methodology, Formal analysis. **Pengyu Ji:** Conceptualization, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2023.137562.

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