Microglia phagocytosis regulated by RAC2 in Alzheimer's disease brains

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Abstract. Alzheimer's disease (AD) is a degenerative neurological disease characterized by memory impairment, agnosia, and loss of motor function. Although treatment is available to alleviate symptoms, there is no cure due to limited understanding of its pathogenesis. Amyloid protein amyloid-β (Aβ) is believed to be the core mechanism of disease onset. And several studies reported that Aβ plaque could be effectively cleared by microglia, thereby preventing AD progression. Yet as a double-edged sword, microglia over-activation would cause unexpected neuroinflammation in patients with AD. To understand the dysfunction of microglia in AD, single-cell RNA-sequencing (scRNA-seq) was used to precisely determine the gene expression of microglia populations. Several genes involved phagocytosis showed decreased expression in microglia of AD brains. The upregulated RAC2, a small Rho-GTPase (GTP enzyme), was selected to explore its function in microglia activation. Results from in vitro experiments further revealed the overexpression of RAC2 in microglia of AD patient may increase the microglia phagocytosis to Aβ. Therefore, RAC2 overexpression can potentially reduce the level of Aβ protein in AD patients and delay the progression of the disease. We hope that the results of the experiment will provide more helpful information for the treatment of AD.

Keywords: Alzheimer's disease, microglia, phagocytosis, RAC2 overexpression.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by memory and cognitive decline. The onset of disease is induced by chronic apoptosis of neurons [1]. The core mechanism involves the accumulation of amyloid-β (Aβ) peptides and tau protein, blocking intercellular transportation in the brain [2]. There is increasing evidence indicating that inflammation represents a key pathological component of AD. Thus far, there has been no cure for this disease due to incomplete understanding of its pathogenesis.

Microglia are the resident macrophages of the central nervous system (CNS) and perform essential roles in diverse aspects such as early in the neurodegenerative process. The microglia are an indispensable part of immunity due to their function of removing dead cells and cell debris to maintain brain homeostasis [3]. The microglia are characterized by highly dynamic morphology, allowing them to flexibly reach any corners in brain tissue. The microglia are activated upon exposure to stimulus such as pathogen-associated molecular patterns or endogenous damage-associated molecular patterns. After activation, they can differentiate into two subcategories, M1 or M2 [4]. M1 microglia lead to inflammation and neurotoxicity through the production of certain proinflammatory cytokines, while M2 microglia participate in anti-inflammatory and recovery processes [5]. Healthy microglia also help prune superfluous synapses, via both promoting synapse formation and by targeting specific synapses for elimination and phagocytosis. Interestingly, alterations in microglia terminal differentiation are implicated in lots of neural disorders such as AD.

Under healthy condition, the microglia are responsible for engulfing and removing waste, as well as releasing degrading enzymes or performing local Aβ degradation to remove excess Aβ peptides[6]. This process is frequently complemented by other distinct mechanism. During AD pathology, microglia are less able to remove Aβ fragments, allowing them to accumulate and harm neurons. Thus, increased numbers of research proposed that over-activated microglia are a well-characterized histological observation indicator from AD brains [7]. Studying the link between microglial phagocytosis and Aβ clearance could provide useful insight for AD treatment.

The clear mechanisms in microglia for Aβ are mediated by many pathways. This study aims to explore how these risk genes or factors in microglia participate in the pathogenesis of AD. We used bioinformatics analysis to find risk genes involved in regulating the functional changes of AD microglia in brain tissues from clinical AD and control patients. we selected RAC2 as a marker gene because of its important role in phagocytosis. In order to explore the role of RAC2 in the pathogenesis of AD, we constructed a microglia model of AD in vitro, simulated the up-regulated RAC2 in the brain of AD patients, and compared the changes in microglia activation and phagocytosis after the up-regulation of RAC2. The results of this project advanced our understanding of the functional changes of microglia in the pathogenesis of AD, shedding a light on novel targets for AD treatment.

2. Method

2.1. scRNAseq data analysis

GSE160936 [8] was used for single cell RNA-seq data analysis to understand the specific genes and associated pathways of AD. Samples were collected from the entorhinal and somatosensory cortex tissue from 6 non-diseased control cases (Braak stage 0–II) and 6 AD cases (Braakstage III–VI). Cellenics (http://scp.biomage.net/repository) was used to show the details of different expressed genes in microglia which were collected for downstream analysis. A gene is declared differentially expressed when the difference is significant (FDR<0.05). Gene ontology (GO) is an effort to unify the terminology of gene function. To understand the specific pathway of the upregulated and downregulated genes, gene lists [symbol, Log2(Fold Change)] were fed into SRplot (http://www.bioinformatics.com.cn/srplot) to perform GO analysis.

2.2. Cell culture

BV2 cells were purchased from iCell Bioscience Inc (Shanghai, China). Cells were cultured in dishes (d = 6 cm) with DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mg/ml amphotericin in a 37°C, 5% CO_2/95% air incubator. The cell medium was changed every two days.

Timeline of the experiment:

The cryopreserved cells were resurrected on day one. Over the next two days (day2-3) the cell status was observed and ensured to be stable, with no excess cells dying or unable to stick to the wall. On the fourth day, the medium fluid was changed once, because a few days later, a large amount of infection precipitated in the culture medium, which was not conducive to the continued growth of the cells. On the fifth to seventh day, when the cell density is around 80%, passage process was required. In two days after the passage, we continued to observe the cell status, and the fluid was changed every 2-4 days according to the specific situation.

2.3. RAC2 expression plasmids construction

Small segment of mouse *Rac2* gene (General Biol., Chuzhou, China) is added into a specific cell by circular DNA molecule (pLVX-mCherry-C1). The added segments encoded various trait for Amp and Puro resistance. After the insertion of the genes, 293T cells were used to package the virus. BV cells will be treated strictly under controlled condition in laboratory, where temperature should be kept constant and nutrient rich liquid are placed in the vessels. In transfection, the 6-well plate was transfected with 5 uL virus per well (titer 2×10^8 TU/ml). The process was as follows: after 48h, Puromycin (2 μg/mL) screening was added to filter the plasmids expressing cells for 4 days. The suitable environment allows cells with plasmids to replicate with different DNAs and grow into larger number. The genes experience overexpression so that they have the ability to be transcribed and translated under human-controlled conditions in large scale. Lastly, RAC2 overexpression's function is explored.

2.4. Aβ treatment and enzyme-linked immunosorbent assay (ELISA)

Aβ oligomer were grown from synthetic $A\beta_{1-42}$ peptides [8]. Firstly, to dissolve 5 mg of synthetic peptide into cooled 1.1 mL HFIP and incubate at room temperature for 60 minutes. After the peptide-FTFIP solution was placed on ice for 10 minutes, the HFIP was volatilized in the cabinet at room temperature. Then the trace HFIP residue was removed by vacuum evaporation for 60 min. Peptide was added to fresh anhydrous 100% DMSO to form peptide film, diluted with culture solution at 4℃ for 24 h, and the final order was 50 μM. BV cells were plated in 96-well plates at $4x10^4$ cells/ml and were incubated with Aβ oligomer for 24 h. ELISA help us test if RAC2 overexpression made microglia engulf more Aβ proteins.

2.5. RNA extraction and real-time PCR

RNA was extracted from BV2 cells using standard extraction methods with Trizol. Extracted RNA was reverse transcribed into cDNA through reverse transcriptase. Real-time PCR was performed to quantify the level of mRNA. Components needed for PCR are Template cDNA, primers (10 μM), 2*AceQ universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). In real-time PCR, we make sure the primers are complementary to template strand (Table 1). Repetition of PCR processes support us by amplify and test the genes of interest using the program described below: pre-denaturation at 95℃ for 5 min, 40 cyclic reactions with 95℃ for 30 s then 60℃ for 30 s. The result can be detected by using fluorescent dye so that we can observe them from the PCR machine. The changed ratio was analyzed with the $2^{-\Delta\Delta ct}$ method.

Table 1. Primers for the study

primers	sequence (5'-3')
Tgfb1-F	TGATACGCCTGAGTGGCTGTCT
Tgfb1-R	CACAAGAGCAGTGAGCGCTGAA
Il10 -F	CGGGAAGACAATAACTGCACCC
Il10 -R	CGGTTAGCAGTATGTTGTCCAGC
CD206-F	GTTCACCTGGAGTGATGGTTCTC
CD206-R	AGGACATGCCAGGGTCACCTTT
TNF-a-F	GGTGCCTATGTCTCAGCCTCTT
TNF-a-R	GCCATAGAACTGATGAGAGGGAG
Il1b -F	TGGACCTTCCAGGATGAGGACA
Il1b -R	GTTCATCTCGGAGCCTGTAGTG
Cd86-F	ACGTATTGGAAGGAGATTACAGCT
Cd86-R	TCTGTCAGCGTTACTATCCCGC
iNos -F	GAGACAGGGAAGTCTGAAGCAC
iNos -R	CCAGCAGTAGTTGCTCCTCTTC
Arg1-F	CATTGGCTTGCGAGACGTAGAC
Arg1-R	GCTGAAGGTCTCTTCCATCACC

3. Results

3.1. Differential expressed genes in microglia of AD brains

The topic of our experiment was first to study how the transcript levels in the brains of people with AD differ from those of normal people. We collected published scRNA-seq samples from the cortex in both AD patients and control [9]. Samples were collected from the entorhinal and somatosensory cortex tissue from 6non-diseased control cases (Braak stage 0–II) and 6 AD cases (Braakstage III–VI) without cell portion wise difference. We classified brain the scRNA seq data by cell type based on marker genes' expression levels. And cell clustering was used to further classify differentially expressed genes (Fig. 1A). Uniform manifold approximation and projection (UMAP) 2D visualization showed the clustering of 45,828 single nuclei from the 12 brain samples. The cell

clusters were arranged in different parts in the UMAP graph, the microglial cells were marked with purple and spread a large amount. We would only focus on gene expression in microglia during latter experiment.

To better understand the relationship between microglia function and AD, we explored the differential expressed genes in microglia clusters. A gene is declared differentially expressed when the difference is significant (FDR<0.05). The volcano map showed the genetic differences between normal and AD patients, and the results showed that 65 genes were down-regulated and 85 were up-regulated. Most of the genes show no significance (Fig. 1B). With the acquisition of these genes, it is believed that these genes were involved in the regulation of altered microglia function in the brain of AD patients.

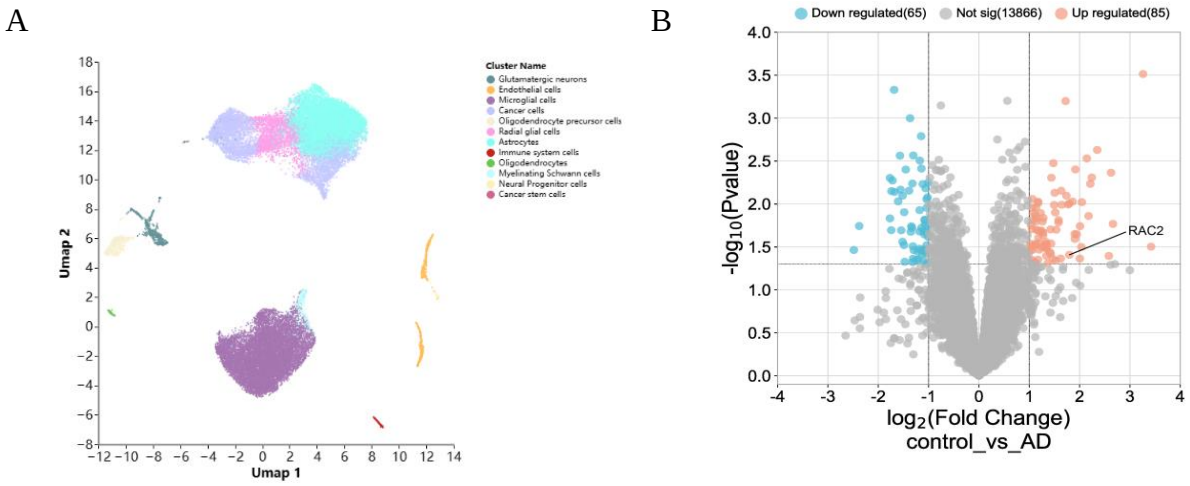

Fig. 1 Single-cell RNA sequencing of the cortex in AD patients and control brains revealed changes in gene expression.

(A) Uniform Manifold Approximation and Projection (UMAP) plot showing major cell types pooled from the scRNAseq data in AD and control groups. Each dot represents a single cell. (B) Volcano plots showing differential expressed genes in microglia of AD brains compared to control.

3.2. The differentially expressed genes of microglia in AD brain are aggregated in phagocytic related cell functions and cell components

To compare which functions of these 150 differentially expressed genes regulate microglia in the AD brain, we performed Gene ontology (GO) analysis of these differentially expressed genes. The experimental results show enrichment scores GO analysis, where the size of the dots represents the number and the color represents the *p*-value of enrichment score (Fig. 2).

The Cellular Component graph shows that most of the differentially expressed genes clustered in the cellular components associated with phagocytic vesicles (Fig. 2A). It can be seen that the number of genes enriched in cortical cytoskeleton was the largest. As shown in this bubble diagram, most significantly enriched biological processes were with *p* value around 1e-04. Protein localization to plasma membrane is with high number count and low error. In contrast regulation of T cell differentiation is with less count of number and higher p value. In Biological Process graph (Fig. 2B), most of the differential expressed genes are associated with cell membrane activity, such as early endosome membrane, integral component of endoplasmic reticulum membrane, and endocytic vesicle. When it comes Molecular Function (Fig. 2C), most of the differential expressed genes focus on the regulatory processes of transporter function and kinase functional activity, such as bicarbonate transport, inorganic anion transport and regulation of T cell differentiation.

During endocytosis and phagocytosis, the plasma membrane of the cell forms a pocket around the material to be internalized. The pocket closes and then separates from the plasma membrane to form vesicles or endosome. These results suggest that the genes expressed by different microglia in AD may affect the immune function of microglia, especially the phagocytosis function of microglia. The

cellular pathway involved may be related to the regulation of transporter function and kinase activity. Therefore, we then selected genes related to the phagocytosis function of microglia for follow-up studies.

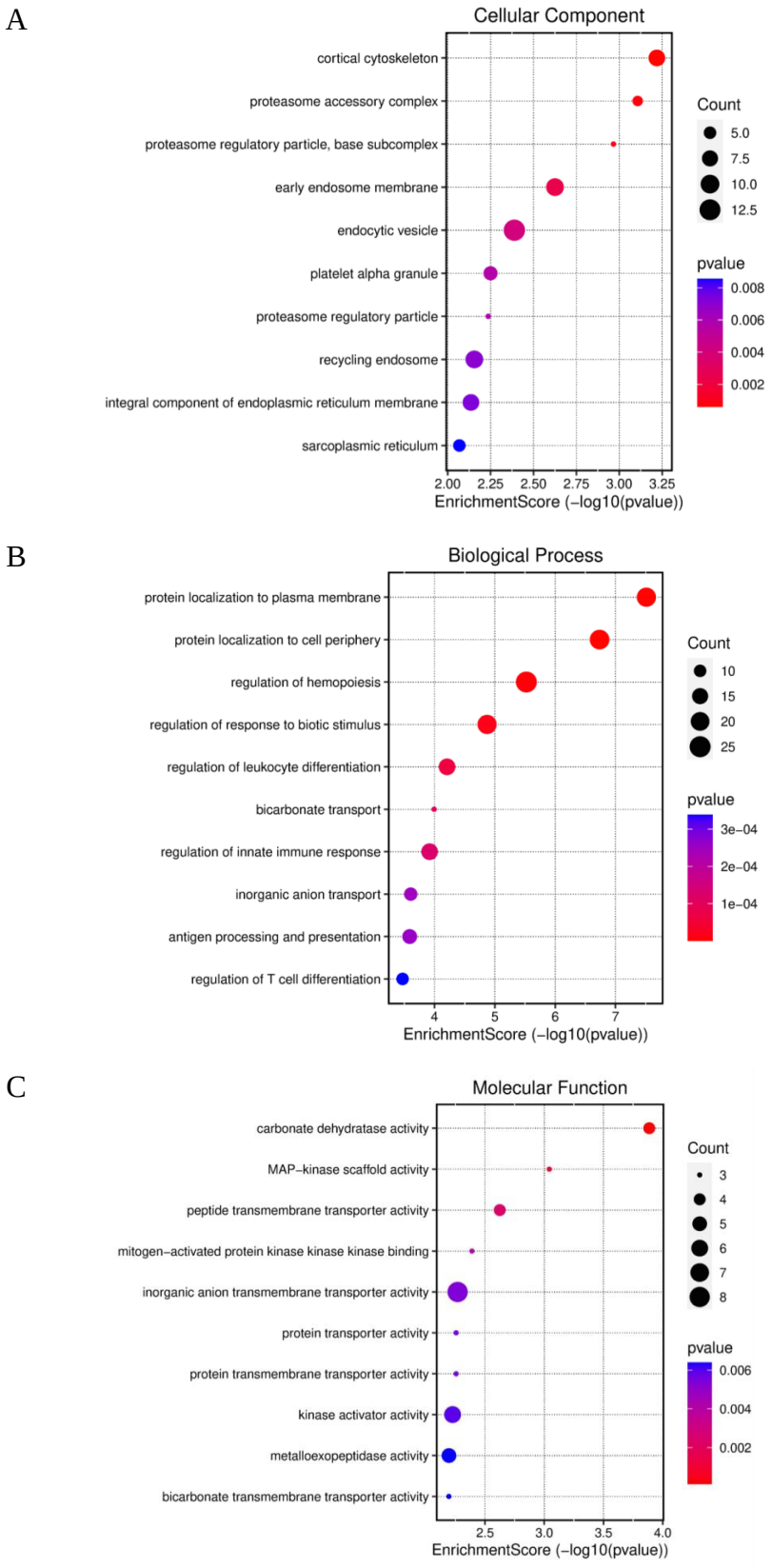

Fig. 2 The result of GO analysis for the different expressed genes by microglia in AD.

(A) Cellular component enrichment, endocytic vesicle count for the most part in cellular component. (B) Biological process enrichment, protein localization to plasma membrane, cell

periphery, regulation of hemopoiesis and regulation of response to biotic stimulus count form similar number and all have similar p value. (C) Molecular function enrichment, transmembrane transporter activity account the most.

3.3. Plasmids overexpressing RAC2 was constructed successfully

In these differentially expressed genes, we figured out those related to the microglia phagocytosis regulation and consuming related genes, including MCOLN1, DLG4, GRIA2, CD9, RAC2, TAPBP. Among that, RAC2 is involved in immune response and microglia activation to deal with protein buildup. In Fig.1B, RAC2 was illustrated with log2 (Fold Chang) = 1.7005, -log10 (p value) = 1.4660. Thus, we selected RAC2 to observe its function on phagocytosis.

Since RAC2 is upregulated in AD brain microglia, we first constructed RAC2-overexpressed microglia cells through recombination. Next, we cultured microglia and infected the microglia with plasmids in the form of lentiviruses, making them overexpress RAC2 (Fig4) to describe the morphological changes.

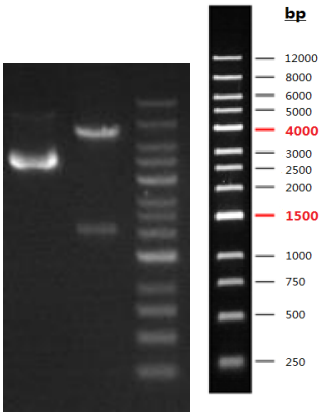

Fig. 3 DNA gel electrophoresis indicated that RAC2 overexpression plasmid was successfully constructed

Lane1: Plasmid DNA, Lane2: Plasmid digested by KpnI, Lane M: DNA Marker

3.4. RAC2 overexpression promotes the transformation of BV2 cells to M2 activation

Lentiviral infection cells carrying RAC2 overexpression plasmids were performed. After RAC2 overexpressing, BV2 cells showed a broad activated response with more protrusions (Fig. 4).

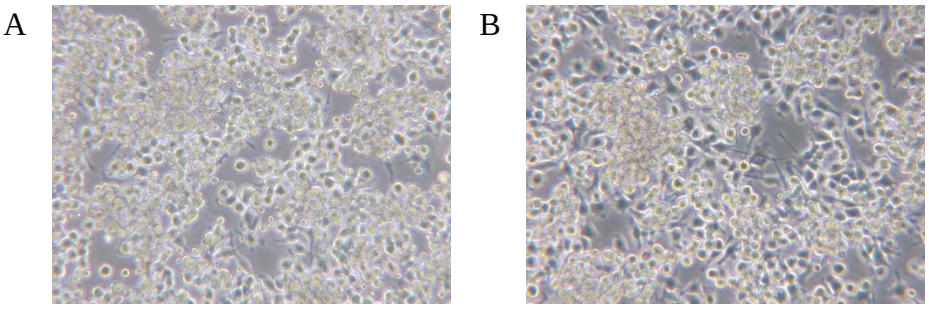

Fig. 4 Morphological changes of BV2 cells after transfection with RAC2 plasmid

(A) control (B) RAC2 overexpression

Microglia may be differentiated into two phenotypes. M1 type activated microglia could release inflammatory factors and cause neuroinflammatory responses. When microglia shift into the alternative activated M2 phenotypes, it will play a role in neuroprotection. Since the previous results (Fig. 1) suggested that RAC2 is upregulated in AD patients, we wanted to further compare the effects of RAC2 upregulation on the types of microglia activated. Here, RAC2 overexpression induced M2 activation of microglia and downregulated M1 activation (Fig. 5). Because the inflammatory factors of BV2 cells overexpressing RAC2 were all decreased, such as TNF-α, IL-1β, CD86 and iNOS.

Meanwhile, the typical cytokine in M2 type were increased after RAC2 overexpression, including TGF-β1, IL10 and CD206.

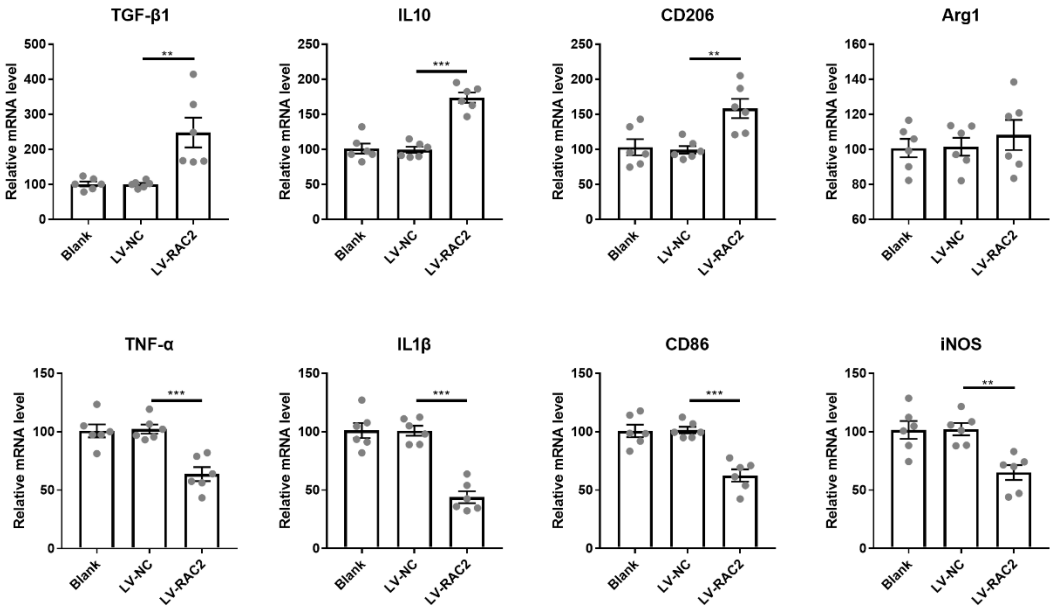

Fig. 5 Result of real time PCR

The eight images above reflect the data of real-time PCR. TGFβ1, IL10 and CD206 in RAC2 group is upregulated, because its data is significantly higher than the other two groups. The last four images present similar data since RAC2 group is significantly lower than the other groups as the bar did not overlap. However, the result of Arg1 shows no significant. ANOVA test, $^{**}p<0.01$, $^{***}p<0.001$.

3.5. RAC2 upregulation increased the phagocytosis of microglia

In order to compare the effect of RAC2 overexpression on the phagocytosis function of microglia, a microglia model of AD was constructed by incubation with Aβ oligomer to simulate the pathological environment of Aβ plaque aggregation in the brain of AD patients. Next, ELISA was used to detect the allowance of Aβ oligomer in supernatant medium and compare the phagocytosis ability of microglia. Apparently, the RAC2-infused group had a much higher phagocytic effect than the other group, so less protein was left in the brain (Fig. 6). This means that RAC2 can break down and reduce Aβ in the brain, and better solve the situation of AD disease.

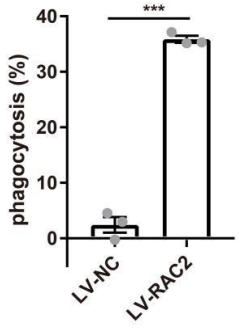

Fig. 6 The level of Aβ in the supernatant of BV2 cell culture medium was detected by ELISA.

The ability of phagocytosis is equal to the percentage of engulfed Aβ of the total protein. Unpaired t test, $^{***}p<0.001$.

3.6. Effects of RAC2 overexpression on F-actin dynamics during Aβ phagocytosis

All forms of phagocytosis require F-actin recruitment and re-arrangement promoting engulfment. Note phagocytic particle capture in phagocytosis involves F-actin-rich membrane ruffles that require

RAC2 signaling. After Aβ oligomer incubation, the overexpressed RAC2 was localized in the membrane (Fig. 7A). Altering abundance of specific membrane RAC2 may facilitate its release from Rho-GDI, promoting in turn its membrane insertion and activation [10].

In addition, Arp2/3 complex proteins localize to F-actin beneath surface-bound particles during phagocytosis. Here, the level of Arp2/3 complex proteins were increased when RAC2 was overexpressed (Fig. 7B). Thus, it suggested that overexpressed RAC2 after Aβ oligomer incubation orchestrate the remodeling of actin during phagocytosis. Activation of RAC2 promoted assembly of branched F-actin network which is required for the formation of engulfment endosome.

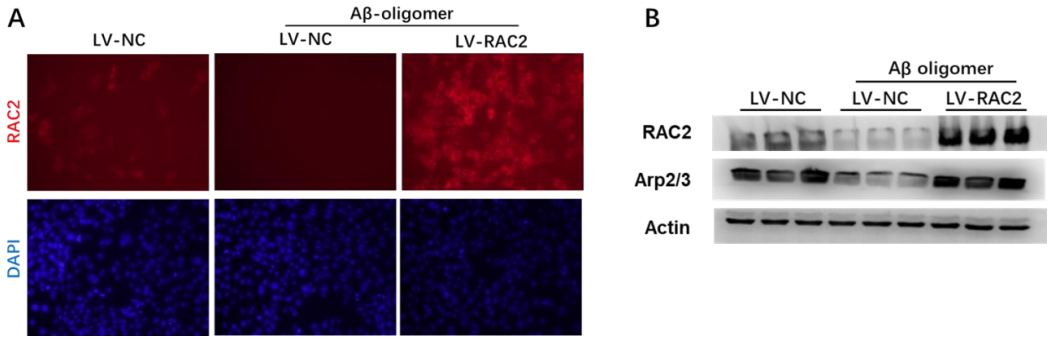

Fig. 7 RAC2 overexpression promoted F-actin dynamics during Aβ phagocytosis

(A) Cellular localization of RAC2 in BV2 cells under Aβ oligomer incubation. (B) Levels of Arp2/3 were upregulated after RAC2 overexpression.

4. Discussion

Using single cell RNA sequencing (scRNAseq) data analysis, we found that a small Rho-GTPase, RAC2, was upregulated in microglia of AD patients' brains. For microglia and immune genes, RAC2 is related to low novelty responding dorsal raphe. In an experiment, researchers [11] use mice model to test if the different level of RAC2 producing different amount of protein have effects on individuals. It was reported that RAC2 was up-regulated in rat models under chronic stress [11]. In neuropathic pain, Gene Ontology and pathway enrichment analyses indicated that RAC2 was closely related to immune and inflammatory responses [12]. The relationship between RAC2 and microglia has also been studied. RAC2 could enhance activation of microglia and astrocytes, inflammatory response, and studies have shown that RAC2 is upregulated in the brain in chronic constriction injury-induced neuropathic pain [13]. In tau P301S transgenic mice, γ-adducin, a cytoskeleton associated protein that is cleaved to produce γ-adducin fragment. Expression of the fragment in the hippocampus of tau P301S transgenic mice resulted in significant AD-like pathology and cognitive deficits gamma adducting fragments. And γ-adducin fragment downregulates the expression of RAC2, leading to defects in neurite outgrowth [14].

Previous research has proved that upregulated expression of RAC2 is closely related with microglia inflammatory (explore this aspect to discuss future direction of research) responses and AD. RAC2 overexpression also aggravated the inflammatory response, induced activation of microglia and astrocytes, and enhanced apoptosis, whereas knockdown of Rac2 had the opposite effects. RAC2 suppressed SIRT1 expression via activating the c-Jun N-terminal kinase (JNK) signaling pathway [12]. And in our scRNAseq data analysis and in vitro experiments, RAC2 was over-expressed in AD brains and promote microglia phagocytosis. However, the underlying molecular mechanism need more studies in the future. The JNK signaling pathway is a vital component of the MAPK signaling pathway, which can be triggered by stress stimuli and the upregulation of proinflammatory mediators. More experiments are needed to demonstrate whether JNK/SIRT1 signaling axis is the mechanism underlying the RAC2-mediated pathogenesis of AD.

Though RAC2 has been proven in cell tests to decrease neuroinflammation and enhance microglia clearance of peptides, we are not sure whether the RAC2-expressing gene therapy used in the human

brain will have the same effect as in a controlled laboratory environment and need to investigate the accurate proportion of the RAC2 added in actual usage.

5. Conclusion

In summary, we identified RAC2 as a causal gene in AD progression and designed a series of experiments to confirm its effects. Firstly, the scRNA-seq analysis of AD brains data sets demonstrated different expression genes enrichment in microglia phagocytosis. Among that, we finally found that RAC2 can indeed clear Aβ proteins faster, so it can also promote the treatment of Aβ accumulation. This provides a novel way of thinking and possibility for the treatment of AD disease.

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