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Regulation of Microglial Polarization through the NF-kB Pathway by activating Rho/ROCK Pathway induced by LPS Priming in the 5xFAD Mouse Model of AD

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Abstract. The symptoms of Alzheimer's disease (AD) are the deposition of beta-amyloid plaques and chronic neuroinflammation caused by microglia. Its pathological process is closely related to microglia. Microglia have innated immune memory (IIM) and have different roles that vary with animal models of neurodegenerative diseases such as AD. However, how IIM-mediated microglia function is well validated in the 5*FAD model. We want to know if lipopolysaccharide (LPS)-induced IIM in the preclinical period of AD alters microglia polarization. We did this by injecting LPS into 5*FAD mice at 6 weeks (before plaque formation). After 140 days, we assessed microglia polarization and activation of the Rho/ROCK pathway and NF-κB pathway in 5*FAD-initiated and non-initiated mice. Furthermore, the activation of them in microglia was also evaluated. ROCK2 was overexpressed in primary microglia by lentivirus transfection. Then, ROCK2 overexpression model was constructed by CRISPR/Cas9 based on 5*FAD mice. Cross with Tmem119-CreERT2 mice to obtain microglia overexpressing ROCK2 mice. The activation of microglia, and these two pathways were evaluated after LPS-induced IIM. Our expected result is that LPS-induced IIM changes microglial polarization from M1 to M2 by the inhibition of them. The paper only provides theoretical experiment design and possible results about alterations in microglia polarization and their mechanisms after LPS priming stimulates innate immune memory, which needs further research in its mechanisms. Suggestions for more long-term effects and potential therapeutic applications in humans also has been listed for reference.

Keywords: Alzheimer’s disease, LPS, Priming, innate immune memory, microglial, polarization, Rho/ROCk pathway, NF-κB pathway.

1. Introduction

Alzheimer's disease (AD) is a disease that can lead to the degeneration of nerve cells. It occupies a high proportion in all dementia cases (60~80%) [1]. The pathological features of AD are neurofibrillary tangles with intracellular hyper-phosphorylated tau protein and extracellular deposition of misfolded amyloid-βeta plaques [2]. A-β plaque-activated glial cells that surround the plaque and release inflammatory mediators. Thus, the neuroinflammatory response is also one of the case features of AD

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[3]. Current research suggests an essential role in how disease develops and evolves. [4]. Impaired microglia phagocytosis leads to A-β deposition, which can be classified as one of the causative factors of AD. Aβ deposition leads to microglia activation, neuroinflammation, and subsequent neurodegeneration [5, 6]. Pro-inflammatory cytokines are produced in large quantities during neuroinflammation. The major cytokines contain interleukins (IL-1β, IL-6, IL-18), chemokine ligands (CCL1, CCL5), small molecule messengers (prostaglandins, NO), etc.

The clear function of microglia cells in the initial and middle phases of the AD pathology remains unknown. However, microglia are significant in the pathogenesis and disease evolution of AD. It is an immune cell in the central nervous system (CNS). Microglial activity synchronously paces with the growth, maturation, and aging of the CNS throughout its lifetime via the collaboration of different regulatory networks. It is also indispensable for developmental synaptic pruning, neuronal apoptosis, management of synaptic plasticity, and immune monitoring. [7], [8]. Impaired immune surveillance appears to be strongly associated with neurodegenerative diseases[8]. Injury can cause other microglia to become activated into different states, M1 and M2. Through the activation, microglia will result in two phenotypes: the pro-inflammatory phenotype (M1) involved in inducing nerve injury, then the anti-inflammatory phenotype (M2) role in inflammatory alleviation [9]. Microglia have a long lifespan; for example, the average lifespan of microglia in the adult mouse cortex is more than 15 months [10] [11], and greater than 4.2 years in most human brains[12]. Microglial cells' longevity makes it possible to "remember" their inflammatory history thus causing either a trained or tolerant to the stimulus that can be treated for the second time based on microglia innate immune memory (IIM), which possibly guides the development of neural degeneration disorders in the end. IIM has been shown to be related to multiple neurodegenerative diseases. [13] [14] [15]. Microglia has emerged as the primary point of attention as recent AD research approves the significance of the innate immune response and neuroinflammation in driving neurodegenerative diseases [16].

It has recently been found that the progressive accumulation of Lipopolysaccharide (LPS) in the AD brain is related to neuropathology and affects gene expression [15], [17].LPS can bind to toll-like receptor 4 (TLR4) which is a receptor sitting on the top layer of many immune cells including the microglia [18]. Subsequently, TLR4 can directly activate NF-κB pathway pathways, to maintain a mild and systemic inflammatory response [19]. LPS priming activates the NLRP3 inflammasome in macrophages and guides to the production of pro-inflammatory cytokine and exacerbated inflammation [20, 21]. Infectious stimulation is a clear inducer of IIM, so it promotes remodeling of innate cellular responses in AD.[22]. It has been shown that the results of microglia IIM count specifically on both the duration and intensity of the stimulus. That being said, IIM can either inhibit or promote the immune response to secondary inflammatory stimuli [23, 24].

Reduced activation of microglia and suppressed neuroinflammation with LPS-induced 5*FAD was reported in a recent study. Other obviously inhibited proteins in Primed-5xFAD microglia were found to be enriched with the Ras pathway by proteomic assays. The researchers found that Rho expression was reduced [25]. ROCK, known as Rho-associated protein kinase is a serine/threonine kinase that may regulate the microglial phenotype. [26]. Rho is the core enzyme of the Rho GTPases group, which is a member of the Ras superfamily; and Rho can be activated ahead ROCK. [27] [28]. ROCK 2 enzyme, as one of the isoforms of ROCK, has been rendered as a crucial regulator of microglial polarization. Under conditions of reduced ROCK2 activity or when it inhibits the NF-κB pathway, microglia tend to shift from the M1 to M2[28]. NF-κB is an important transcription factor which has been extensively studied. It typically binds to the NF-κB inhibitory protein-α (IκB-α) as a p50-NF-κB dimer. and exists in the cytoplasm in a dormant state. Upon stimulation, IκB-α is phosphorylated and degraded, and NF-κB separates from p50 and rapidly undergoes transactivation, connecting to the κB complex on the promoter of regulatory genes and initiating the transcription process of pro-inflammatory genes. Rho/ROCK phosphorylates the downstream target IκB-α, which then dissociates from the complex with NF-κB and no longer inhibits NF-κB from entering the nucleus [29]. Thereby affecting the polarization process of microglia. Our goal is to investigate whether or not the modulation of the ROCK Pathway based on the activation of NF-κB Pathway brings microglia changes within the 5*FAD mice induced by LPS.

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Early pathological stages have received increasing attention due to the unsuccessfully clinical trials targeting advanced stages of the disease. Therefore, we hope to explore new directions in the treatment of the disease from the preclinical stage. According to previous reports, microglia exhibit pro-inflammatory activation as early as 6 weeks before a β accumulation in a 5*FAD mouse model [30]. Administration of LPS intraperitoneally at 6 weeks reduced microglia activation, attenuated neuroinflammation, and guided to a decrease of the disease phenotype in mice [25]. Therefore, we would like to investigate the mechanism of microglia changes.

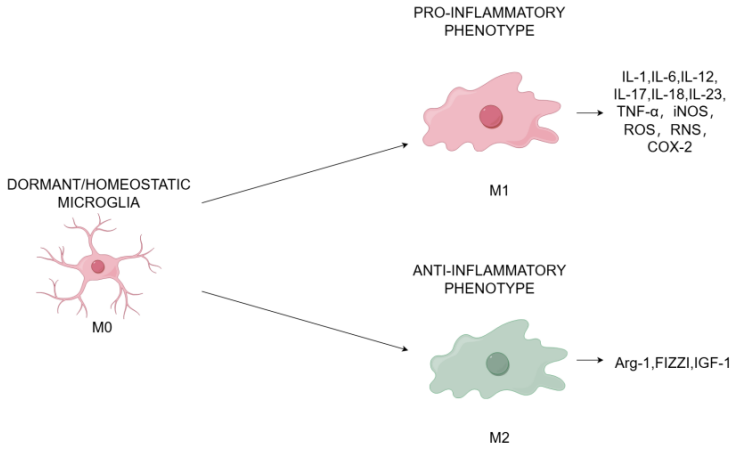

Figure 1. Microglia's two primary activation states. (By Figdraw)

2. Methods and Expected Results

We wanted to verify whether LPS priming ahead of plaque deposition in a 5*FAD AD mouse model could inhibit the Rho/ROCK pathway and thus regulate microglia M1/M2 polarization via the NF-κB pathway. Therefore, we performed cellular experiments to test our hypothesis after detecting changes in microglial cell polarization in an animal model.

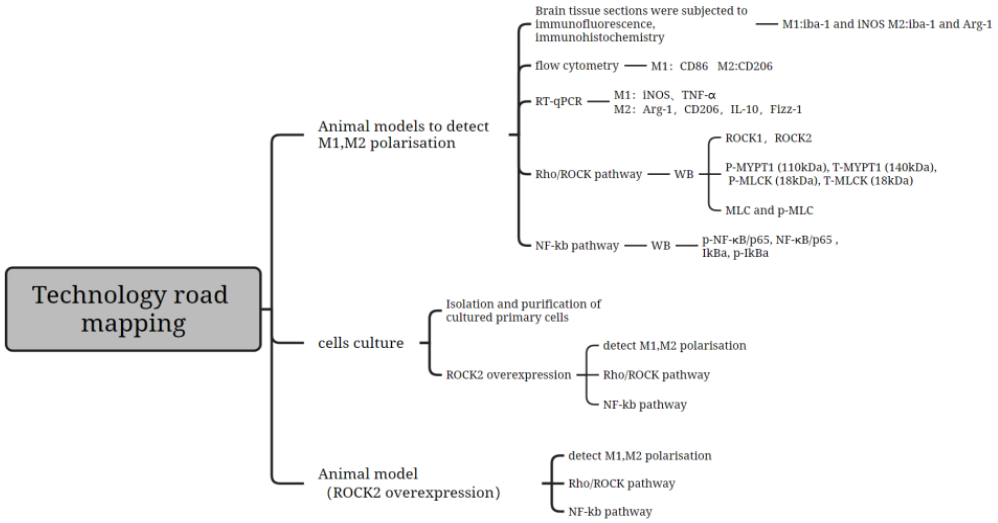

Figure 2. Technology road mapping.

2.1. Detection of changes in polarization of mouse microglia

2.1.1. Experimental Grouping

We divided 60 5*FAD mice equally into two separate groups. The first group was injected with LPS, and another was injected with an equal amount of saline. An equal amount of saline was also injected

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into 30 C57/B6 mice. Immunofluorescence staining, immunohistochemical staining, and RT-qPCR were performed on mouse brain tissue at 6 months[25].

2.1.2. Detection of microglia polarization

Ionized calcium-binding adapter molecule 1 (Iba1) is an exclusive protein that bounds to calcium for both microglia and macrophage and it is involved in cell membrane ruffle formation and phagocytosis in microglia after activation. Iba1 has been used as a microglia marker due to its specific expression in the CNS[31]. The microglial pro-inflammatory M1 state is described by notable levels of some proteins that induce inflammation such as inducible nitric oxide synthase (iNOS) and COX2, classical surface markers like CD68, CD86, and morphological transition from resting branching cells to amoeboid phagocytosis [32, 33]. CD206 is the primary marker for the identification of M2 macrophages. Arginase 1 (Arg1) is also an ideal phenotypic indicator for the identification of M2 macrophages. CD206 is the primary marker for the identification of M2 microglia[34]. Arg1 is highly expressed in M2 microglia and is therefore also an ideal phenotypic indicator for the identification of M2 macrophages[35]. Meanwhile, interleukin (IL-10) secretion by M2 microglia is upregulated. Inflammatory zone 1(Fizz-1), on the other hand, is an M2 gene [36].

Immunofluorescence staining was performed on brain tissue sections, and each section was stained with iba-1, iNOS, arginase 1(Arg-1), and DAPI at the same time. After staining, observation was performed using a confocal microscope. The amount of marker iba-1, iNOS double positive and iba-1, Arg-1 double positive cells were counted respectively. And calculate the two-cell ratios[37].

Immunohistochemical staining and immunofluorescence staining were performed using the same markers to count the number of both types of double-positive cells separately after staining and calculate the ratio of the two types of cells [37].

Afterward, CD86 positive cells and CD206 positive cells were screened and counted using flow cytometry, and the ratio of the two types of cells was calculated.

Quantification of M1, M2 microglia-associated RNAs such as iNOS, tumor necrosis factor α (TNF-α) Arg-1, CD206, IL-10, Fizz-1 using RT-qPCR. Thereby detecting polarization changes in microglia.

Our predicted experimental outcome was a decrease in the ratio of the amount of iba-1, iNOS double-positive microglia to the amount of iba-1, Arg-1 double-positive microglia in retrosplenial cortex (RSC) compared with 5*FAD mice without LPS priming. RT-qPCR shows a reduction in M1 microglia-associated markers and an acceleration in M2 microglia-associated markers. However, because of the reduction in the total number of microglia activated [25], there is also possible to be a reduction in both cell-associated markers, but a smaller proportion of the reduction in M2 microglia-associated markers.

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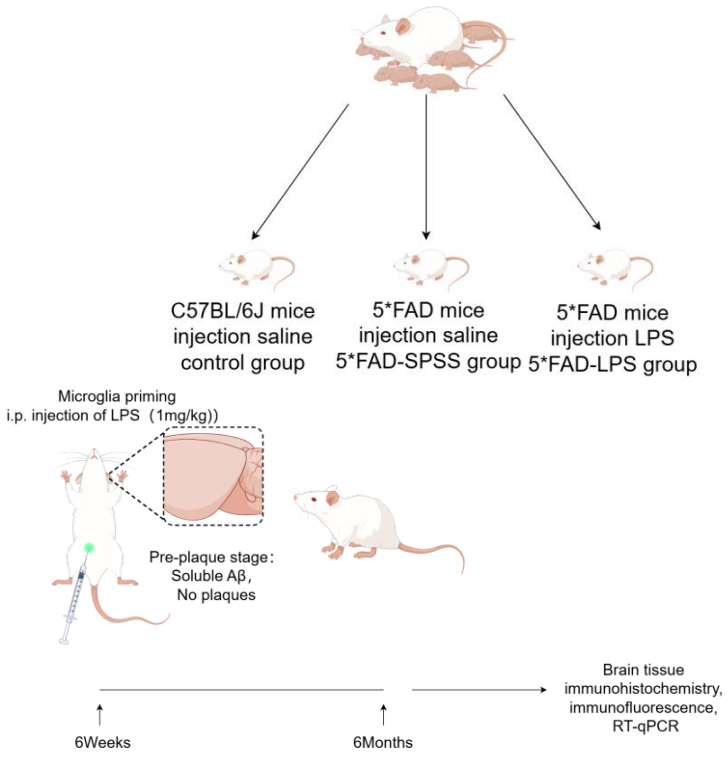

Figure 3. Experimental design of object recognition tasks. (By Figdraw)

2.2. Detection of Rho/ROCK pathway inhibition

ROCK is distributed throughout the body and there are two types of ROCK, which are ROCK 1 that mainly being exist in non-neural tissues, and ROCK2 dominates in brain and muscle tissues [37]. ROCK activation phosphorylates downstream molecules, mainly myosin light chain (MLC), myosin phosphodiesterase target subunit (MYPT) 1, and myosin light chain phosphatase (MLCP) [29]. Phosphorylated MYPT1 promotes MLC phosphorylation, whereas phosphorylated MLCP loses its role in promoting MLC dephosphorylation. MYPT1 and MLCP regulate the amount of phosphorylated MLC by increasing the source and inhibiting the destination [38]. The Rho/ROCK pathway regulates microglial cell function mainly by regulating phosphorylated MLC. Therefore, phosphorylated MLC expression is often used to determine the degree of activating the Rho/ROCK signaling pathway [39].

The contents of M1 and M2 microglia-related proteins in cerebrum tissue were detected by Western blot (WB) following using flow cytometry to sort out the activated microglia. The content of ROCK1(158kDa) and ROCK2 (160kDa) is first detected, using GAPDH (36kDa) as the internal parameter. Then, P-MYPT1 (110kDa), T-MYPT1 (140kDa), P-MLCK (18kDa), and T-MLCK (18kDa) were respectively detected, and GAPDH (36kDa) was also used as the internal parameter [37]. Total MLC and p-MLC expression levels were detected, and β-acting was used as the internal parameter [40].

We expected no difference in ROCK1 expression between the two controls, but the 5*FAD-LPS had more ROCK2 than the control group and less rock2 than the 5*FAD-SPSS. Compared with the 5*FAD-SPSS, p-MYPT1/t-MYPT1, p-MLCK/t-MLCK, and p-MLC/MLC in the 5*FAD-LPS decreased.

2.3. Detection of NF-κB pathway inhibition

Similarly, Western Blotting was performed after screening microglia to detect the expression of relevant proteins. NF-κB, a regulatory factor, is very important in the polarization of neurotoxic microglia [41]. NF-κB downstream of ROCK is a regulator of microglia polarization. NF-κB P65 gains access to the nucleus upon phosphorylation. Microglia polarization is promoted when NF-κB nuclear translocation occurs, and cell polarization is inhibited when NF-κB P65 is inhibited [42]. In the cytoplasm, the NF-

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κB transcription factor complex remains inactive and bound to IκB. When IκB is targeted for ubiquitination, the NF-κB transcription factor can be phosphorylated to enter the nuclear zone [43]and regulate microglia polarization. We, therefore, assayed p-NF-κB/p65, NF-κB/p65, IkBa, p-IkBa, and used β-acting as an internal reference [44]. After immunofluorescence staining of NF-κB P65 then confocal microscopy to observe whether it metastasizes into the nucleus [45].

Our expected results were a decrease in NF-κB/p65 translocation in nuclear and a decrease in the ratio of p-NF-κB/p65 and p-IkBa by contrasting to the 5*FAD-SPSS later than LPS priming. The NF-κB pathway was inhibited. The 5*FAD-SPSS had an increase in NF-κB/p65 nuclear translocation and an increase in the ratio of -NF-κB/p65 and p-IkBa versus the control.

2.4. Changes in microglial polarization are produced by suppression of the NF-κB pathway via the Rho/ROCK pathway

2.4.1. Cellular experiment

2.4.1.1. Cells culture

Mouse microglia were isolated and cultured by enzymatic digestion using 1-3 day old 5*FAD mice. Mouse microglia were subsequently purified from mixed glial cell cultures [40]. Thus, primary microglia were obtained.

2.4.1.2. Setup of ROCK2-overexpression (ROCK-OE) stable cells

Construction of ROCK2 overexpression minis using lentiviral transfection. ROCK2 was duplicated into the OE vector PcSLenti-CMV-MCS-3xFLAG-PGK-puro-WPRE3 to produce CMV-Rock2 [37].

2.4.1.3. Experimental Grouping

Stimulation with LPS (25 ng/mL) was first induced into microglia for 3 hours, after which they were washed and incubated for one day (24h). After the incubation, they were treated with stimulation by Aβ42 (3 μM) for three hours [46, 47].

Microglia were distributed into 4 different groups, and the first group was transfected with lentivirus to construct ROCK2-OE cells; after this LPS stimulation and Aβ stimulation were performed in the same way as described above. The second group was transfected with ROCK2. Then an equal amount of H2O to stimulate the cells after that we add Aβ. They were named asROCK2-LPS group, and ROCK2-H2O group, respectively. The third and fourth groups were not transfected with ROCK2 and were stimulated with LPS and H2O, named LPS group, and H2O group, respectively. We perform cell collections 3 hours after Aβ stimulation, and then detection of to ensure the Rho/ROCK and NF-κB pathways activation. Meanwhile, changes in microglia polarization were observed. They were analyzed as described previously.

There is an increasing proportion of M2 microglia in the LPS compared to the proportion in the H2O. This suggests that the stimulation method can elicit innate immune memory. In comparison between the H2O and the ROCK2-H2O, it was observed that the H2O had less activation of the Rho/ROCK pathway and more M2 microglia. This indicates that ROCK2 overexpression was successful. The LPS demonstrated less activation of it, which was inhibited, and less activation of the NF-κB pathway compared to the ROCK2-LPS. The percentage of M2 microglia increased in ROCK2-LPS versus ROCK2-H2O. Meanwhile, the activation of the Rho/ROCK pathway was reduced, the activation of the NF-κB pathway was lowered, and the indexes of the ROCK2-LPS were in the middle of the LPS and the ROCK2-H2O.

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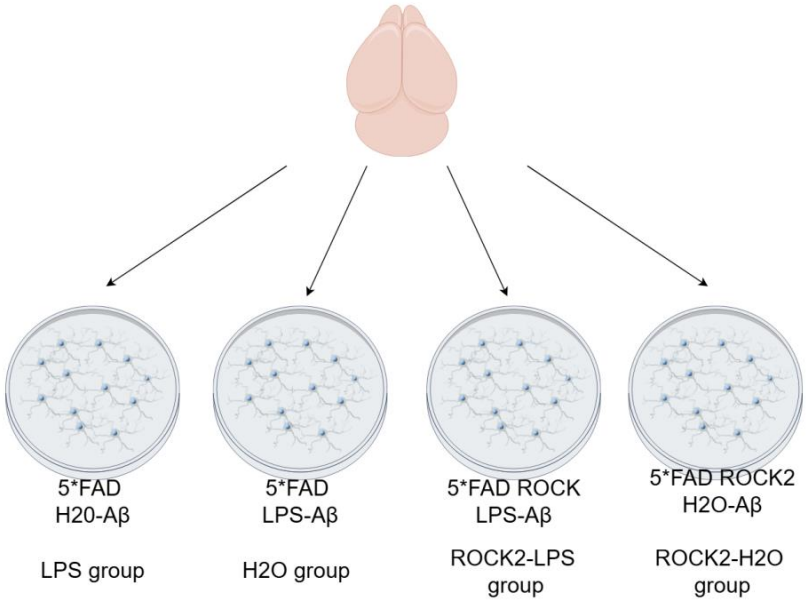

Figure 4. Grouping diagram.(By Figdraw)

2.4.2. Mice Model

2.4.2.1. Construction of ROCK2 overexpressing mice

A conditional overexpression mouse model of the Rosa26 targeted ROCK2 gene was constructed. CRISPR/Cas9 technology was used to target ROCK2 insertion at the Rosa26 locus by homologous recombination. The promoter -LSL-ROCK2-2A-EGFP-WPRE-polyA was inserted into the Rosa26 locus of 5*FAD mice. This was followed by crossbreeding with Tmem119-CreERT2 mice. A mouse model of microglia-specific overexpression of ROCK2 was obtained after Tamoxifen induction in 4-week-old mice.

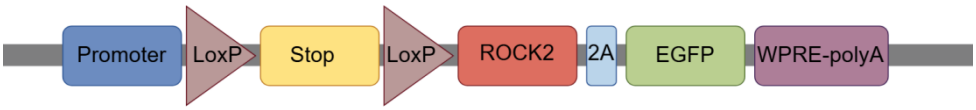

Figure 5. Design Schematic.(By Figdraw)

2.4.2.2. Experimental Grouping

At 6 weeks, the mice were subdivided into two groups, one group was injected intraperitoneally with LPS, and the other group was injected intraperitoneally with an equal amount of saline, named 5*FAD-ROCK2-LPS group and 5*FAD-ROCK2-SPSS group, respectively. At the same time, equal amounts of saline and LPS were injected intraperitoneally into 5*FAD mice, respectively, which were named as5*FAD -SPSS group and 5*FAD-LPS group. Brain tissues of mice were taken at 6 months to observe the M1, M2 ratio. The two pathways mentioned earlier activation was detected. The methods were the same as the previous experiments.

The prediction results were similar to those of the cell experiment. Our expected result was inhibiting the Rho/ROCK pathway when ROCK2 was overexpressed, caused by innate immune memory, attenuated and enhanced NF-κB Pathway Activation Compared With Normal ROCK2 Expression in 5*FAD Mice.

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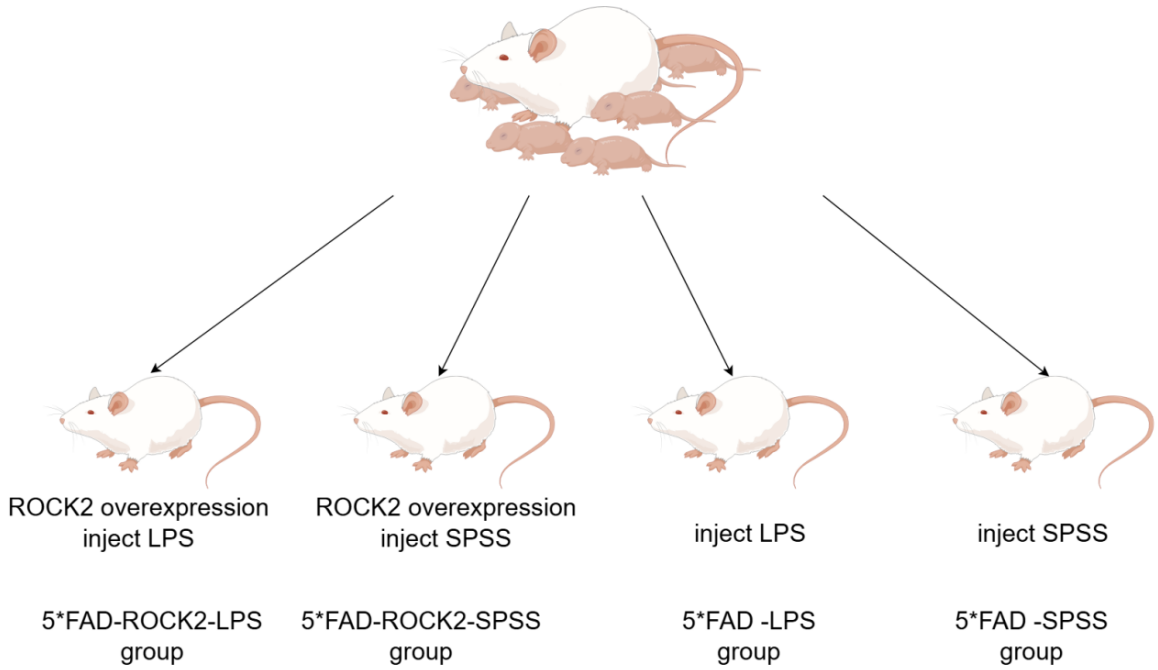

Figure 6. Grouping diagram.(By Figdraw)

2.5. Methods

2.5.1. Animal

We used 5*FAD mice and C57/BL6-SJL mice for our experiments. The mice used in all experiments were males. These mice express human amyloid precursor protein (APP) with 3 mutation sites and human presenilin 1 (PSEN1) with two mutation sites, all under the Thy-1 promoter associated with neurons, modeling familial Alzheimer's disease (FAD). Mice were divided into groups of five, with a 12-hour cyclic period switching back and forth from light to dark, and had access with no obstruction to their food and water. At the age of 6 weeks old, LPS were in injected into mice's peritoneal (1 mg/kg), or an equal amount of vehicle (saline) was injected to the same position of peritoneal as a control. We guaranteed there was no fatality or sickness in mice behavior due to the LPS concentration being observed [25]. We distinguish the mice into two treatment groups: with or without LPS(control group, n = 20; 5*FAD SPSS, n = 20; 5*FAD LPS, n = 20;5*FAD ROCK2 SPSS, n = 20; 5*FAD ROCK2 LPS, n = 20).

2.5.2. RT-qPCR

After extraction, RNA concentration was determined. Then we synthesise first-strand cDNA from RNA. PCR primers for CD-206, Fizz-1, IL-10, Arg-1, iNOS, and TNF-α were designed with reference to OriGene Technologies and literature. Mutations like Pde6b rd1, which are known for causing early retinal degeneration were screened and excluded from breeders to prevent confounding behavioral results [48].

2.5.3. Brain section

5% isoflurane with oxygen was used to anesthetize the mice. Cold PBS was then perfused via the heart. Fixed it in paraformaldehyde and sucrose sequentially. Then cerebrum sections were made at a 30 μm thickness, gathered in groups of 6 or 12, conserved in a solution that has the effect of cryoprotective and storage at -20℃ and consisted of sucrose, ethylene glycol, phosphate buffer in a 3:3:4 ratio [25].

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2.5.4. Immunofluorescence (IF) staining

Cells from brain sections and cover slides were added to PBS with 10% donkey serum and 0.3% Triton X-100 at 25°C for 1 hour and 20 minutes, respectively, and then sent to incubate at 4°C overnight with primary antibodies. After development, the section was treated with Cy3-supported secondary antibody for 2 hours [37].

2.5.5. Immunohistochemical staining

Tissue sections were deparaffinized. To block endogenous peroxidase enzyme activity, endogenous peroxidase blocker was added for 15 minutes. The sections were pre-incubated for 10 minutes with 10% normal donkey serum before the incubation of the primary antibody, anti-hepatic arginase antibody and anti-inducible nitric oxide synthase antibody at 4°C overnight. The results were then titrated for 10 minutes with 100μL goat anti-rabbit IgG polymer labeled with the enhancer. The findings have been demonstrated by light microscopy [37].

2.5.6. flow cytometry

Collect the brain tissue and cut it into 2 to 4-mm pieces with scissors or a scraper. Follow the instructions for the enzyme. Add the appropriate amount of enzyme dissolved in PBS and incubate at the appropriate temperature. The cells should be gently pipetted and filtered through the cell filter to remove any clumps or debris. Collect the cell suspension in the conical tube. Centrifugation of cells was performed. After the removal of the supernatant, the cell sediments were re-suspended in PBS. Follow the step-by-step method to centrifuge the cells. Repeat the above two steps.

First, FcRblock was incubated (1μg/test), 4°C, 5-10min; Then the CD86 surface was stained at 4°C for 30min. After washing the cells with PBS for 2 to 3 times, the Fixation Buffer (500ul) was added and fixed at room temperature for 30 min away from light. Then centrifuge 150g for 5min and discard the fixative solution, add 2 ml of 10×Intracellular Staining Permeabilization Wash Buffer diluted with ddH2O 10 times to reinsert cells. Centrifuge 150g for 5min to discard the supernatant, and repeat the steps 2~3 times. Finally, 100μL1×Intracellular Staining Permeabilization Wash Buffer was re-suspended, CD206 antibody was added (0.5μg/tests,), and incubated at 25°C for 30min with no light. After incubation, 2ml1×Intracellular Staining Permeabilization Wash Buffer was washed 2 to 3 times, and 500μL cell staining buffer was added.

2.5.7. Brain homogenization

Brain tissues including the prefrontal cortex and hippocampi were altogether dissected and immediately placed on ice. The brain tissues were then treated with homogenization with ice-cold RIPA buffer that was covered with protease inhibitors and phosphatase inhibitors [49]. Homogenization was performed using either Lysis Matrix D 2 ml tubes in a FastPrep™-24 Classic homogenizer in a 6 m/s speed for 2 cycles of 10s each [25] or by ultrasonic crushing at 5% power for 20 minutes on ice [50, 51]. The homogenized substance was then incubated on ice for 0.5 hours to ensure the completion of protein extractions. The incubated samples were then centrifuged, and the supernatant containing soluble proteins was carefully assembled. The remaining pellet containing insoluble proteins was resuspended in 70% formic acid and sonicated for 1 minute at 60% amplitude [25, 49]. Both fractions were subsequently used for protein quantification, and the soluble fraction was analyzed by using a BCA protein assay [25, 49-51]. While the insoluble fraction was analyzed by using a Coomassie Plus assay[25]. At last, the samples were prepared for SDS-PAGE by adding Laemmli buffer and warming for 5 minutes at 95°C. Next, we load it for Western blotting [49].

2.5.8. WB

Total protein was extracted using the RIPA buffer kit. The membranes were then marked by the following antibodies: primary antibody, anti-myosin light chain 2 (MLCK2), anti-phosphomyosin light chain 2 (p-MLCK2), anti-mypt1, anti-rock1, anti-rock2 and GAPDH. GAPDH will be used as an internal parameter.

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We will also label it with anti-Myosin Light Chain (MLC), anti-phosphomyosin light chain (p-MLC), anti-phospho-NF-κ b p65 (p-NF-κ b p65), anti-NF-κ b p65 (NF-κ b p65), anti-i-κ b α (i-κ b α), anti-phospho i-κ b α (p-IkBa), and β-actin antibodies. The cells were separately incubated overnight at 4°C and then incubated with the corresponding secondary IgG (H + L) antibodies at various dilutions for 2 hours at 25°C. Visualize and capture protein bands. ImageJ software was put into use to quantify the optical density. It was normalized to the intensity of GAPDH [40]

2.5.9. Localizing NF-κB using immunofluorescence

Glass-capped synoviocytes were incubated in HBSS at 37°C for 60 minutes. Fixation by using 4% paraformaldehyde was treated, subsequently permeabilized with 0.1% TX100, and blocked with serum albumin. Then the cells were sent to incubate at 4 °C with a primary antibody all-night. After three washes with PBS, incubate with cy3-labeled secondary antibody for 120 minutes at 25°C. The cells were then analyzed by fluorescence microscopy after labeling with 2 lM DAPI for 5 minutes [45].

2.5.10. Primary cell culture

After removing the mouse brain tissue, the cerebrum was transferred to a petri dish with a D-Hanks balanced salt solution. After the pia and dural membranes were removed, two-milliliter trypsin/EDTA (0.05%:0.02%) was added to a new petri dish and the brain was chopped into a fine paste. The brain/trypsin suspension was gently transferred to a 15ml centrifuge tube and the same volume of trypsin /EDTA was added (0.05%/0.02%). Tubes are then incubated for 8-10 minutes at human body temperature in a humidified environment containing 5% CO2. Equal amounts of 10% FBS and DMEM will be added to the suspension. Gently agitate the single-cell suspension. After leaving at room temperature for one minute, filter twice using a 70μm nylon cell filter. Then centrifuge 190 x g in a cooled (4°C) centrifuge for 8 minutes. The cell particles were suspended in 15ml DMEM containing 15% FBS. The suspension cells were added to a poly-D-lysine coated flask. The medium was prepared by adding 10%FBS to DMEM. For three days, discard the old DMEM and add 15 ml of the new medium. Then equal parts of the new medium are mixed with the supernatant of the medium in the bottle. Replace the medium daily with the mixture. When about 80-90% of the cells are fused, they can be used for purification. 20 hours before decomposition, replace the medium with fresh DMEM with 15% FBS. The following day, the flask was placed on a heated orbital shaker at a speed of 200-240 rpm and a temperature of 37°C for 2 hours. Translocate the suspension to a 50ml centrifuge tube and centrifuge 190xg for 8min. Cells were resuspended in DMEM with 15% FBS. The cells were then inoculated on 24-well tissue culture plates. The culture plates were pre-coated with 5ml polyd-lysine (10μg/ml). Cultured in a 5% CO2 atmosphere that has been humidified at 37°C. The substrate is replaced every 2 days with the new medium. The medium is then replaced every 2 days with a 1:1 mixture of DMEM with 10% FBS and the supernatant from the flask medium mentioned above. It is then possible to repeat the purification process one week later [52].

2.5.11. To establish stable cells with ROCK2 overexpression (ROCK-OE)

The cells were cultured in six-well plates. Lentivirus was then used for plasmid transfection when the fusion reached 40%. We filter cells with purinomycin hydrochloride before transfection. ROCK2 was replicated into the OE vector pcslentii - cmv - mcs - 3xflag - pgk -puro- wpre3 to obtain cmv - ROCK2. The principal target sequences of amplified ROCK2 shRNA and primers are shown in Table 1[37].

3. Discussion

Studies now demonstrate that microglia have a key role in AD [7]. Innate immune memory of microglia influences disease progression in AD. It has been shown that after activation of innate immunity by LPS priming in mice the disease phenotype is attenuated and microglia activation is reduced. Other significant inhibitory proteins in microglia were observed to be enriched around the Ras pathway [25]. ROCK2 can affect microglia polarization through the NF-κB pathway [29]. However, the mechanisms underlying the changes in microglia induced by LPS priming before amyloid plaque formation have not

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been fully elucidated. In our experiments, we first used immunofluorescence staining, immunohistochemical staining, and flow cytometry to validate LPS priming-induced changes in microglia polarization. Subsequently, primary microglia overexpressing ROCK2 were constructed. The polarization changes of microglia were examined by the above methods. The accumulation of activation of the Rho/ROCK and NF-κB pathway was also examined using WB. After that, we CRISPR/Cas9 technology constructed a new ROCK2 overexpression mouse model and obtained a mouse model of microglia overexpressing ROCK2 by crossing with Tmem119-CreERT2 mice. We used the same method to detect microglia polarization and pathway activation. This could demonstrate the important role of the Rho/ROCK pathway and observe the effect of this pathway on the NF-κB pathway.

Previous studies have demonstrated the participation of the Rho/ROCK and NF-κB signaling pathways in microglial polarization[29]. The Rho/ROCK pathway can regulate microglia polarization through the NF-κB pathway. Inhibition of these two pathways can change the process of microglial polarization from M1 to M2 [25]. In a study, it was observed that microglia were altered after LPS priming in 5*FAD mice. Rho expression was found to be reduced after proteomic analysis. Therefore, we would like to investigate the mechanism of the changes in microglia. However, our experiments still have limitations. Firstly, we used all male mice. So we're not sure if the same phenomenon would occur in female mice. Furthermore, the Rho/ROCK pathway acts in a variety of ways, and we did not test whether it affects microglia polarization in other ways. Deeper research is needed in the future to explore the specific mechanisms involved in this process.

However, our results tend to combine common mechanisms and provide a comprehensive synthesis among neuroinflammatory diseases, including Alzheimer's Disease (AD) [53]. There are studies suggesting that LPS prompts an inflammation-enhancing activity in microglia by activating NF-κB and contributes the neuroinflammation [54]. At the same time, the Rho/ROCK signaling pathway participates as a modulator in this process and aggravates inflammation by transiting the microglial polarization state into an M1 subtype which produces pro-inflammatory cytokines [53]. Researchers found that by inhibiting this pathway with Fasudil, there is a trend that indicates the reduction of amyloid-β (Aβ) production and suppression of the inflammatory response in the mouse models[55]. These inhibitors block ROCK activity thus reducing downstream NF-κB activation. As a result, the inhibitors are responsible for shifting microglia to an anti-inflammatory M2 state [54]. This modulation of neuroinflammation implies that targeting the Rho/ROCK and NF-κB pathways could slow AD progression by preventing excessive neuroinflammatory responses and promoting neuroprotection. It is worth mentioning that since its human-like amyloid pathology, the 5xFAD mouse model is pivotal for us to understand the therapeutic effects of these inhibitors [56].

We suggest that future research looking into this therapeutic strategy should focus on optimizing the timing and application of Rho/ROCK inhibitors like Fasudil [55]. These inhibitors have been proven to be efficient in alleviating neurodegenesis by not only reducing Aβ levels but also limiting cognitive decline in preclinical studies in various models [56]. The potential to influence neuroinflammation in the beginning of the disease progression is encouraging for preventing further neuronal damage [54]. Additionally, ROCK inhibition is often correlated with enhanced autophagy and reduced oxidative stress. Both of them are critical factors in AD pathology [55]. With researchers further exploring the ROCK inhibitors, they need to pay attention on improving bioavailability and safety profiles to maximize their clinical application [53]. As the prevailing preclinical studies, we believe Rho/ROCK pathway inhibitors are powerful candidates for future therapeutic regimens for AD and relevant neurodegenerative diseases [55].

Microglia activation plays an important role in AD. LPS priming-induced innate immune memory reduces microglial activation and attenuates the disease phenotype. If the results of our experiments are as expected, this implies that the mechanism by which microglia are altered in LPS within innate immune memory is the Rho/ROCK pathway regulating microglia polarization through the NF-κB pathway. The implication of this potential mechanism is still yet to be determined through further experiments in 5*FAD mice and human patients.

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Table 1. The core target sequence of shRNA and the design of primer amplification ROCK2 are discussed.

shRNA Construct Sequences

Gene	Sequence
ROCK2	1) 5'-GGTTTATGCTATGAAGCTT-3'
	2) 5'-GCAGCAATTTCGATGACAT-3'
	3) 5'-GCAACTGGCTCGTTCAATT-3'
	4) 5'-GCACCTTGCAAAGTATATT-3'
Control	5'-TTCTCCGAACGTGTCACGT-3'

Primers for amplification

Gene	Primer
ROCK2	1) 5'- CGCAAATGGGCGGTAGGCGTG -3'
	2) 5'- CAGCGGGGCTGCTAAAGCGCATGC -3'

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