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Circular RNA DLGAP4 Inhibits Ischemic Stroke-Induced Microglia M1 Polarization and Proinflammatory Cytokine Production, Possibly through the NF-κB Pathway

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Circular RNA DLGAP4 (circ_DLGAP4) participates in the progression of ischemic stroke (IS), but whether it could regulate microglia activation to affect IS injury is unclear. This study aimed to explore the effect of circ_DLGAP4 on IS-induced microglia polarization and inflammatory cytokines, and the underlying mechanism. BV-2 cells (microglia) were transfected with circ_DLGAP4 overexpression (oeCirc), short hairpin RNA plasmid (shCirc), or corresponding negative control plasmids (oeNC and shNC). oeCirc or oeNC transfected cells were also treated with phorbol 12-myristate 13-acetate (PMA). Subsequently, BV-2 cells were treated with oxygen-glucose deprivation and reperfusion (OGD/R) to mimic IS. Circ_DLGAP4 was reduced in OGD/R-stimulated microglia versus normal microglia. Circ_DLGAP4 overexpression decreased cluster of differentiation (CD)68 and CD86, but increased CD206 and arginase-1 in OGD/R-stimulated microglia, suggesting that circ_DLGAP4 overexpression might inhibit M1 but facilitate M2 polarization of microglia. Besides, circ_DLGAP4 overexpression reduced tumor necrosis factor-α, interleukin (IL)-1β, and IL-6, but elevated IL-10 in OGD/R-stimulated microglia, indicating that circ_DLGAP4 overexpression reduced proinflammatory cytokines but facilitated anti-inflammatory cytokines. Circ_DLGAP4 overexpression decreased p-nuclear factor kappa-B (NF-κB) and p-NF-κB inhibitor (IκB)-α in OGD/R-stimulated microglia, suggesting its inhibition of the NF-κB pathway. Notably, circ_DLGAP4 downregulation reversed the above phenomenon. PMA facilitated M1 polarization and proinflammatory cytokines but inhibited M2 polarization and anti-inflammatory cytokines in OGD/R-stimulated microglia. Interestingly, PMA attenuated the effect of circ_DLGAP4 overexpression on the above-mentioned processes in OGD/R-stimulated microglia. In conclusion, circ_DLGAP4 may attenuate IS injury by inhibiting microglia M1 polarization and proinflammatory cytokine production, which may be attributed to the inactivation of the NF-κB pathway.

Keywords: circular RNA DLGAP4; inflammatory cytokine; ischemic stroke; microglia polarization; NF-κB pathway
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Introduction

Ischemic stroke (IS) accounts for approximately 85% of all strokes, which is the second contributor to death and disability worldwide (Saini et al. 2021). Although some progress has been made in the treatment of IS, the prognosis of IS patients is still dismal (Mendelson and Prabhakaran 2021; Paul and Candelario-Jalil 2021). Microglia are the major immune cells in the brain, which are closely engaged in the pathology and progression of IS (Jurcau and Simion 2021; Var et al. 2021; Zeng et al. 2022). After IS, microglia are activated, and its M1 polarization facilitates the secretion of proinflammatory cytokines, which accelerates neuronal damage and secondary brain injury (Ma et al. 2017; Qiu et al. 2021; Xue et al. 2021); meanwhile, microglia M1 polarization is generally associated with the high expression of several cell markers, such as cluster of differentiation (CD)68 and CD86 (Jiang et al. 2020). Oppositely, the M2 polarization of microglia could facilitate the recovery following IS, which is characterized

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by the production of anti-inflammatory cytokines and expression of several markers, such as CD206 and arginase-1 (ARG1) (Ma et al. 2017; Jiang et al. 2020). Inspired by this, seeking a potential strategy that inhibits microglia M1 polarization and facilitates its M2 polarization may be meaningful to improve the management of IS patients.

Circular RNA DLGAP4 (circ_DLGAP4) is derived from exons 8, 9, and 10 of the DLGAP4 gene, which is also engaged in IS pathology and progression (Feng et al. 2023; Liu et al. 2023; Zhang et al. 2023). For instance, one previous study reports that circ_DLGAP4 attenuates oxygen-glucose deprivation (OGD)-stimulated human cortical neurons-2 cell injury and inflammation by regulating the microRNA (miR)-503-3p/neuronal growth regulator 1 pathway (Qiu et al. 2022). Another study indicates that circ_DLGAP4 modulates the miR-320/Krüppel-like factor 5 pathway to facilitate angiogenesis in IS rats (Feng et al. 2023). Moreover, circ_DLGAP4 serves as a sponge of miR-6085, which further regulates the growth differentiation factor 11 to inhibit oxygen-glucose deprivation and reperfusion (OGD/R)-stimulated human brain microvascular endothelial cell apoptosis and oxidative stress (Liu et al. 2023). However, whether circ_DLGAP4 could regulate microglia polarization to influence IS progression is currently unclear.

Accordingly, this study aimed to explore the engagement of circ_DLGAP4 in IS-induced microglia polarization and inflammatory cytokine production, as well as the underlying mechanism.

Methods

Cell culture and OGD/R

Normal BV-2 cells (Procell, Wuhan, China) were cultivated with 10% fetal bovine serum (FBS) (Procell) containing Dulbecco's Modified Eagle Medium (DMEM) (Procell) at 37°C, 21% O_2 and 5% CO_2. To mimic IS in vitro, OGD/R was conducted (Yao et al. 2022). In brief, BV-2 cells were incubated in DMEM without glucose/FBS at 95% N_2 and 5% CO_2 for 2 hours (h), followed by being maintained in 10% FBS containing DMEM at 21% O_2 and 5% CO_2 for 24 h.

Cell transfection

Circ_DLGAP4 overexpression (Geneseed, Guangzhou, China), short hairpin RNA plasmid (oeCirc and shCirc) (Geneseed), and relative negative control plasmids (oeNC and shNC) (Geneseed) were transfected into BV-2 cells. To complete transfection, Hieff Trans® Universal Transfection Reagent (Yeasen, Shanghai, China) was applied. The cells were collected at 48 h after transfection for OGD/R. BV-2 cells incubated with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h after being transfected with oeNC or oeCirc (24 h after transfection) (Zhao et al. 2016). Afterward, OGD/R was performed.

Immunofluorescence (IF)

After OGD/R, BV-2 cells were fixed by 4% paraformaldehyde (Beyotime, Shanghai, China). Then, permeabilization by 0.3% Triton X-100 and sealing by 5% bovine serum albumin (BSA) (Servicebio, Wuhan, China) were completed. Afterwards, cells incubated with CD68 (1:500; Abclonal, Wuhan, China), CD86 (1:100; Abclonal), CD206 (1:1,000; Proteintech, Wuhan, China) and ARG1 (1:100; Abclonal) overnight at 4°C. Cy3 conjugated secondary antibody (1:500, Servicebio) was successively incubated with cells for 1 h. The results were analyzed under an inverted fluorescence microscope (Motic, Xiamen, China).

Enzyme linked immunosorbent assay (ELISA)

The supernatant was collected after OGD/R. ELISA were completed according to tumor necrosis factor (TNF)-α (Solarbio, Beijing, China), interleukin (IL)-1β (Elabscience, Wuhan, China), IL-6 (Elabscience) and IL-10 (Beyotime) ELISA kits' protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated with an RNA Isolation Kit (cwbio, Shanghai, China). The linear RNA was removed by RNase R (Beyotime). Reverse transcription and qPCR were accomplished by RT Master Mix (Biomarker, Shanghai, China) and qPCR PreMix (Tiangen, Beijing, China), respectively. The following primers were applied: circ_DLGAP4 forward, 5′ AAGTGAACAAGGGACGCTGAC 3′, circ_DAGAP4 reverse, 5′ ACTGCTCTGGACTGTG ACTGA 3′; GAPDH forward, 5′ GTGCTGAGTATGTCGT GGAGTCT 3′, GAPDH reverse, 5′ GCATTGCTGA CAATCTTGAGTGAGT 3′.

Western blot

Protein was extracted by Total Protein Lysis Buffer (Sangon, Shanghai, China) and separated by SuperPAGE™ gel (Epizyme Biotech, Shanghai, China), followed by electrically transferred to nitrocellulose membrane. Next, the protein was probed with nuclear factor kappa B (NF-κB) antibody (1:5,000; Proteintech, Wuhan, China), p-NF-κB antibody (1:2,000; CST, Danvers, Massachusetts, USA), NF-κB inhibitor (IκB)-α (1:3,000; Proteintech, Wuhan, China) and p-IκB-α (1:2,000; CST, Danvers, Massachusetts, USA). At last, the horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Epizyme Biotech, Shanghai, China) was incubated with the membrane.

Statistical analysis

All data were analyzed by GraphPad Prism (GraphPad Software, Boston, Massachusetts, USA) and presented as mean ± standard deviation. Unpaired t-test and Tukey's test were used to perform analysis. $P < 0.05$ was considered statistically significant.

Results

Effect of circ_DLGAP4 on BV-2 cell polarization and cytokine production under OGD/R-stimulation

According to IF (Fig. 1A), CD68 ($P < 0.01$) (Fig. 1B), CD86 ($P < 0.01$) (Fig. 1C), CD206 ($P < 0.05$) (Fig. 1D), and ARG1 ($P < 0.05$) (Fig. 1E) were increased in the OGD/R group compared to the normal group. TNF-α ($P < 0.05$) (Fig. 1F), IL-6 ($P < 0.01$) (Fig. 1G), IL-1β ($P < 0.01$) (Fig. 1H), and IL-10 ($P < 0.05$) (Fig. 1I) were also elevated in the OGD/R group versus (vs.) the normal group. These findings indicated the IS cellular model was established successfully. Interestingly, circ_DLGAP4 was decreased in the OGD/R group vs. the normal group ($P < 0.01$) (Fig. 1J).

Of note, this study used short hairpin RNA plasmid to treat BV-2 cells. The significance of short hairpin RNA lied in its higher stability and longer duration of action compared to small interfering RNA during transfection. Therefore, the use of shRNA might make our findings more reliable. It was found that circ_DLGAP4 was increased in the oeCirc group compared to the oeNC group ($P < 0.001$), while it was decreased in the shCirc group vs. the shNC group ($P < 0.01$), indicating the transfection was successful (Fig. 2A). CD68 (Fig. 2B) and CD86 (Fig. 2C) were reduced in the oeCirc group compared to the oeNC group (both $P < 0.05$). CD206 (Fig. 2D) and ARG1 (Fig. 2E) were higher in the oeCirc group than the oeNC group (both $P < 0.05$). These findings indicated that circ_DLGAP4 overexpression might inhibit microglia M1 polarization and promote microglia M2 polarization. Representative images of CD68, CD86, CD206, and ARG1 detection by IF were exhibited in Fig. 2F. TNF-α (Fig. 2G), IL-1β (Fig. 2H), and IL-6 (Fig. 2I) were decreased in the oeCirc vs. the oeNC group (all $P < 0.05$). On the contrary, IL-10 was increased in the oeCirc vs. the oeNC group ($P < 0.05$) (Fig. 2J). These findings indicated that circ_DLGAP4 overexpression inhibited proinflammatory cytokines, but promoted anti-inflammatory cytokines. Of note, circ_DLGAP4 downregulation exhibited the opposite effects as its overexpression.

Effect of circ_DLGAP4 on the NF-κB pathway in BV-2 cells under OGD/R stimulation

NF-κB, p-NF-κB, IκB-α, and p-IκB-α were detected by western blot (Fig. 3A). It was found that p-NF-κB/NF-κB ($P < 0.01$) and p-IκB-α/IκB-α ($P < 0.05$) were decreased in the oeCirc group vs. the oeNC group. However, p-NF-κB/NF-κB ($P < 0.01$) and p-IκB-α/IκB-α ($P < 0.05$) were increased in the shCirc group compared to the shNC group (Fig. 3B). These findings suggested that circ_DLGAP4 overexpression inactivated the NF-κB pathway in BV-2 cells under OGD/R stimulation; its downregulation showed an inverse trend.

Effect of PMA on circ_DLGAP4-regulated NF-κB pathway in BV-2 cells under OGD/R stimulation

Circ_DLGAP4 was not different between the PMA group and the oeNC group ($P > 0.05$), as well as between

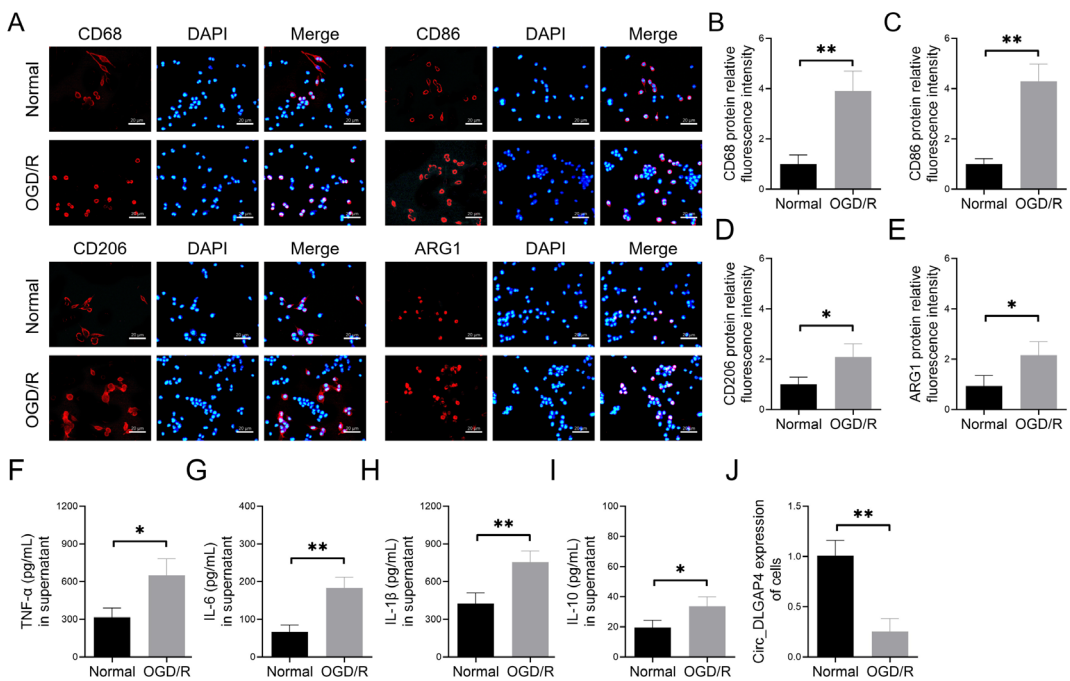

Fig. 1. Circ_DLGAP4 was reduced in BV-2 cells under OGD/R stimulation.
Representative images of IF-detected CD68, CD86, CD206, and ARG1 (A) and comparison of protein relative fluorescence intensity of CD68 (B), CD86 (C), CD206 (D), ARG1 (E) between the OGD/R and normal groups. Comparison of TNF-α (F), IL-6 (G), IL-1β (H), and IL-10 (I) level in supernatant between the OGD/R and normal groups. Comparison of circ_DLGAP4 expression of cells between the OGD/R and normal groups (J). *$P < 0.05$; **$P < 0.01$.

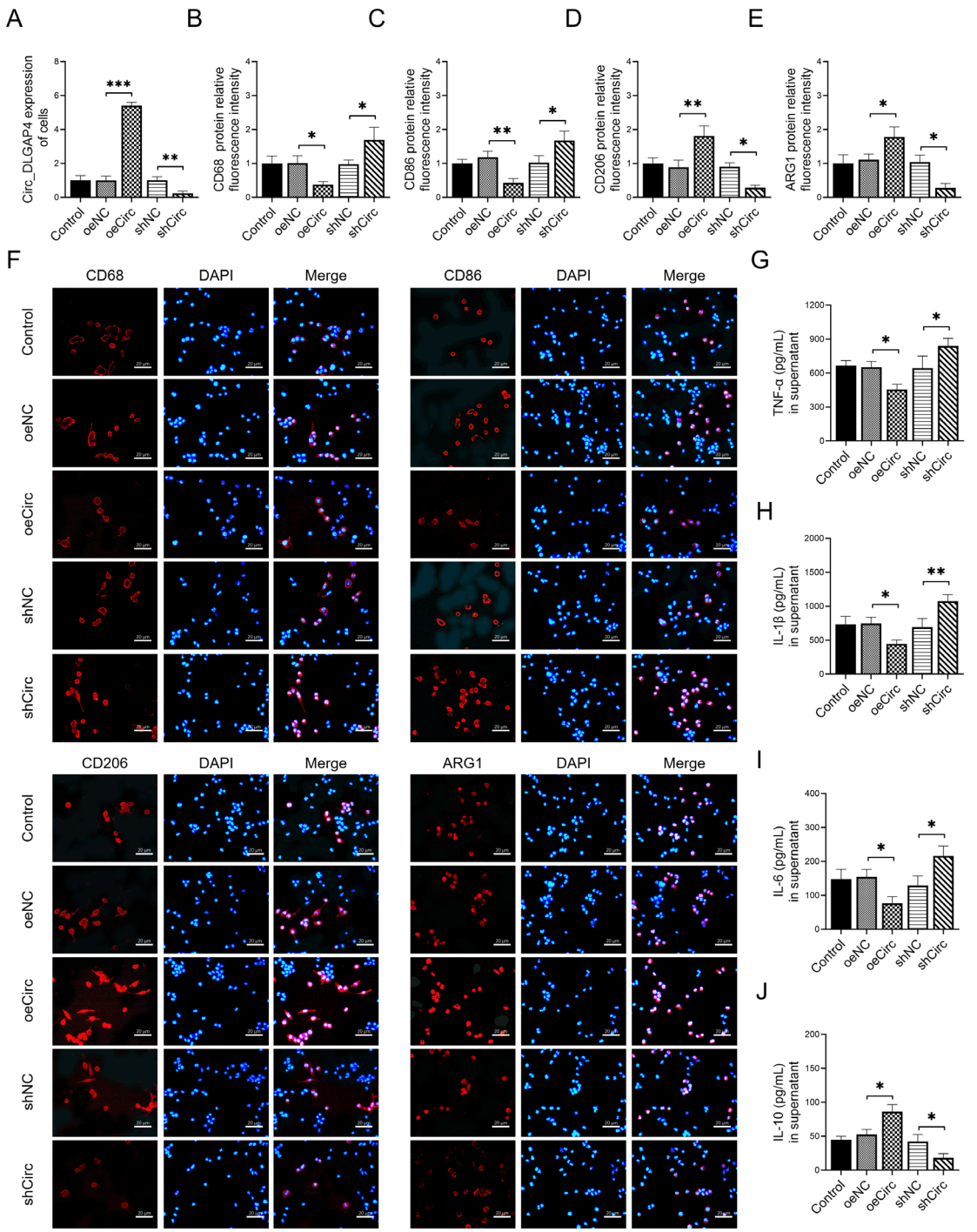

Fig. 2. Circ_DLGAP4 inhibited BV-2 cell M1 polarization and proinflammatory cytokine production under OGD/R stimulation. Comparison of circ_DLGAP4 expression of cells among the control, oeNC, oeCirc, shNC, and shCirc groups (A). Comparison of protein relative fluorescence intensity of CD68 (B), CD86 (C), CD206 (D), and ARG1 (E), as well as representative images of CD68, CD86, CD206, and ARG1 by IF (F) among the control, oeNC, oeCirc, shNC, and shCirc groups. Comparison of TNF-α (G), IL-1β (H), IL-6 (I), and IL-10 (J) level in supernatant among the control, oeNC, oeCirc, shNC, and shCirc groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the oeCirc + PMA group and the oeCirc group ($P > 0.05$), suggesting that PMA did not affect circ_DLGAP4 expressions (Fig. 4A). According to western blot (Fig. 4B), p-NF-κB/NF-κB ($P < 0.01$) and p-IκB-α/IκB-α ($P < 0.01$) were increased in the PMA group vs. the oeNC group, suggesting that PMA activated the NF-κB pathway. Notably, p-NF-κB/NF-κB ($P < 0.001$) and p-IκB-α/IκB-α ($P < 0.05$) were also elevated in the oeCirc + PMA group vs. the oeCirc

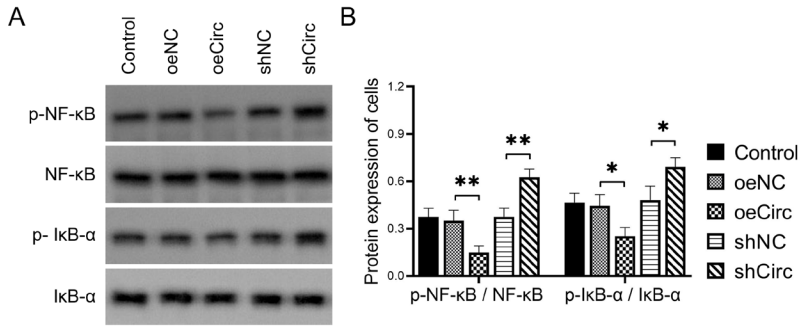

Fig. 3. Circ_DLGAP4 inactivated the NF-*κ*B pathway in BV-2 cells under OGD/R stimulation. Representative images of NF-*κ*B, p-NF-*κ*B, I*κ*B-*α*, and p-I*κ*B-*α* by western blot (A) and comparison of protein expression of p-NF-*κ*B/NF-*κ*B and p-I*κ*B-*α*/I*κ*B-*α* (B) among the control, oeNC, oeCirc, shNC, and shCirc groups. **P* < 0.05; ***P* < 0.01.

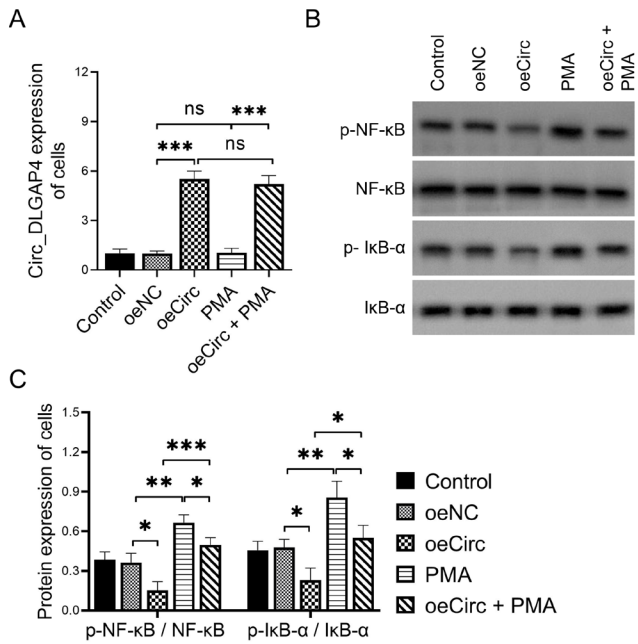

Fig. 4. PMA attenuated the effect of circ_DLGAP4 on inhibiting the NF-*κ*B pathway in BV-2 cells under OGD/R stimulation. Comparison of circ_DLGAP4 expression of cells among the control, oeNC, oeCirc, PMA, and oeCirc + PMA groups (A). Detection of NF-*κ*B, p-NF-*κ*B, I*κ*B-*α*, and p-I*κ*B-*α* by western blot (B), and comparison of protein expression of p-NF-*κ*B/NF-*κ*B and p-I*κ*B-*α*/I*κ*B-*α* (C) among the control, oeNC, oeCirc, PMA, and oeCirc + PMA groups. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant.

group, suggesting that PMA attenuated the effect of circ_DLGAP4 overexpression on the NF-*κ*B pathway (Fig. 4C).

Effect of PMA on circ_DLGAP4-regulated BV-2 cell polarization and cytokine production under OGD/R stimulation

CD68 (Fig. 5A) and CD86 (Fig. 5B) were increased in the PMA group vs. the oeNC group (both $P < 0.01$), and elevated in the oeCirc + PMA group vs. the oeCirc group (both $P < 0.01$). CD206 (Fig. 5C) and ARG1 (Fig. 5D) were reduced in the PMA group vs. the oeNC group (both $P < 0.05$), and reduced in the oeCirc + PMA group vs. the oeCirc group (both $P < 0.05$). These findings indicated that PMA facilitated microglia M1 polarization and inhibited microglia M2 polarization; in addition, PMA reversed the effect of circ_DLGAP4 overexpression on microglia M1 and M2 polarization. Representative images of CD68, CD86, CD206, and ARG1 detection by IF were shown in Fig. 5E. TNF-*α* (Fig. 5F), IL-1*β* (Fig. 5G), and IL-6 (Fig. 5H) were increased in the PMA group vs. the oeNC group (all $P < 0.01$), and elevated in the oeCirc + PMA group vs. the oeCirc group (all $P < 0.05$). However, IL-10 was reduced in the PMA group vs. the oeNC group ($P < 0.05$), and decreased in the oeCirc + PMA group vs. the oeCirc group ($P < 0.01$) (Fig. 5I). These findings suggested that PMA facilitated proinflammatory cytokines but inhibited anti-inflammatory cytokines; meanwhile, PMA reversed the effect of circ_DLGAP4 overexpression on proinflammatory and anti-inflammatory cytokines.

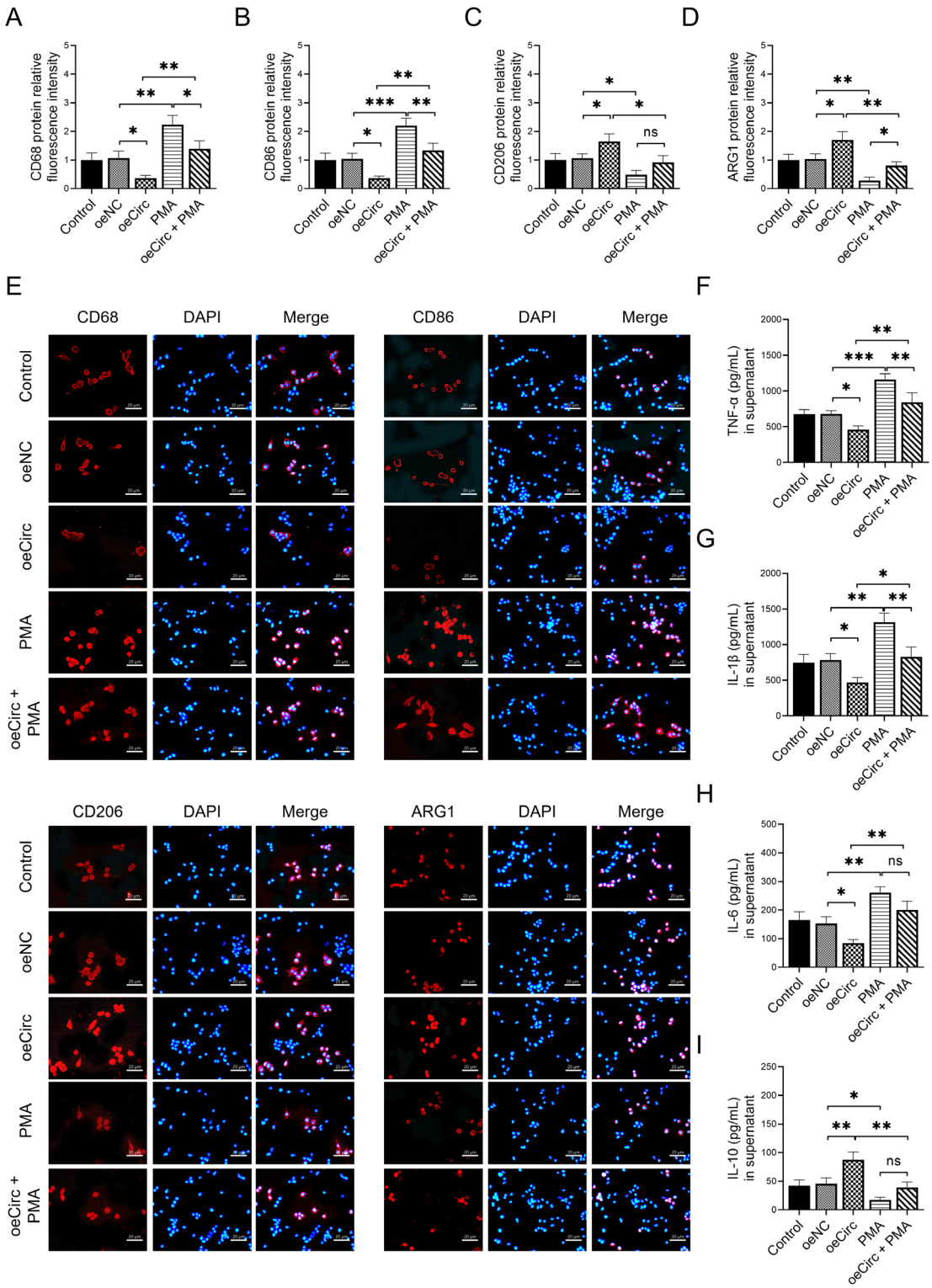

Fig. 5. PMA attenuated the effect of circ_DLGAP4 on inhibiting BV-2 cell M1 polarization and proinflammatory cytokine production under OGD/R stimulation.
Comparison of protein relative fluorescence intensity of CD68 (A), CD86 (B), CD206 (C), and ARG1 (D), and representative images of the above markers detected by IF (E) among the control, oeNC, oeCirc, PMA, and oeCirc + PMA groups. Comparison of TNF-α (F), IL-1β (G), IL-6 (H), and IL-10 (I) level in supernatant among the control, oeNC, oeCirc, PMA, and oeCirc + PMA groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

Discussion

Circ_DLGAP4 is involved in the pathology and progression of IS, and its dysregulation has been reported by some previous studies (Bai et al. 2018; Zhu et al. 2019; Qiu et al. 2022; Liu et al. 2023). For example, a clinical study reports that blood circ_DLGAP4 is lower in IS patients than controls (Zhu et al. 2019). Besides, circ_DLGAP4 is decreased in the plasma of IS patients compared to non-stroke controls, as well as reduced in the mouse stroke model compared with the sham (Bai et al. 2018). Notably, a few *in vitro* studies also explore the dysregulation of circ_DLGAP4 in IS cellular models (Qiu et al. 2022; Liu et al. 2023). According to a study, circ_DLGAP4 is decreased in human cerebral cortical neuronal cells after OGD stimulation (Qiu et al. 2022). Another study finds out that OGD/R stimulation reduces circ_DLGAP4 in human brain microvascular endothelial cells (Liu et al. 2023). Considering the involvement of microglia in IS (Zeng et al. 2022), it is meaningful to investigate the dysregulation of circ_DLGAP4 in microglia, but no relevant evidence is found. In the current study, it was found that circ_DLGAP4 was decreased in OGD/R-stimulated microglia compared to normal microglia, which was consistent with previous *in vitro* studies (Qiu et al. 2022; Liu et al. 2023). A possible reason might be that the occurrence of IS created an environment of hypoxia and nutritional deficiency, which might lead to changes in gene expression in cells (Mitroshina et al. 2021). Therefore, IS resulted in a decrease in circ_DLGAP4 in microglia. Subsequently, abnormal expression of circ_DLGAP4 may further exacerbate IS injury by promoting inflammation and oxidative stress (Qiu et al. 2022; Liu et al. 2023). Therefore, the decrease in circ_DLGAP4 might be a sign of IS injury. However, this speculation should be validated by subsequent studies.

According to previous studies, microglial activation is also closely engaged in the pathology and progression of IS (Luo et al. 2022; Qiu et al. 2023). For instance, one study reports that microglial activation leads to aggravated inflammation and neuronal apoptosis in cerebral ischemia-reperfusion rats (Qiu et al. 2023). Another study elucidates that microglial activation exacerbates locomotor deficits and neuronal pyroptosis in middle cerebral artery occlusion/reperfusion injury mice (Luo et al. 2022). In terms of circ_DLGAP4, it also participates in the progression of IS (Bai et al. 2018; Qiu et al. 2022; Liu et al. 2023), but evidence regarding the regulation of circ_DLGAP4 on microglia activation in IS injury is currently unclear. This study discovered that circ_DLGAP4 inhibited M1 polarization and proinflammatory cytokine production, but promoted M2 polarization and anti-inflammatory cytokine production in OGD/R-stimulated microglia. In contrast, its downregulation exhibited the opposite effects. The potential reasons would be that: (1) circ_DLGAP4 could bind to AU-rich element RNA-binding factor 1 to inhibit microglia M1 polarization and promote M2 polarization (Liu et al. 2022). (2) circ_DLGAP4 could regulate the miR-503-3p/neuronal growth regulator 1 pathway to suppress proinflammatory cytokine production but accelerate anti-inflammatory cytokine production (Qiu et al. 2022). Therefore, circ_DLGAP4 could regulate polarization and inflammatory cytokine production in OGD/R-stimulated microglia. Notably, CD206 and ARG1 were increased by circ_DLGAP4 in OGD/R-stimulated microglia, which was considered as microglia M2 polarization (Plastira et al. 2016; Zhang et al. 2019). However, it might be better to use double staining with type 2 cytokines (such as IL-4 and IL-10) to confirm the microglia M2 polarization, which could be a study direction for our subsequent studies.

NF-κB pathway is closely engaged in the inflammation process, and its role in IS has been reported by some previous studies (Kong et al. 2022; Pu et al. 2022). For instance, the NF-κB pathway facilitates nerve injury and neuroinflammation in both *in vivo* and *in vitro* IS models (Xian et al. 2021). At the same time, a previous study states that the NF-κB pathway modulates the production of inflammatory cytokines, such as TNF-α, IL-1α, IL-1β, and IL-6, as well as microglia activation after cerebral ischemia-reperfusion injury (Pu et al. 2022). Additionally, the NF-κB pathway facilitates microglia polarization toward the M1 phenotype and inhibits microglia M2 polarization in middle cerebral artery occlusion mice and OGD/R-induced microglia cells (Kong et al. 2022). In the current study, it was found that circ_DLGAP4 inactivated the NF-κB pathway in OGD/R-stimulated microglia, and its downregulation showed the opposite effects. Besides, the addition of PMA reversed the effect of circ_DLGAP4 on NF-κB pathway, polarization, and inflammatory cytokine production in OGD/R-stimulated microglia. These findings suggested that circ_DLGAP4 might inactivate the NF-κB pathway to inhibit OGD/R-induced microglia M1 polarization and proinflammatory cytokine production, but promote microglia M2 polarization and anti-inflammatory cytokine production. However, according to previous studies, PMA is an NF-κB pathway activator, as well as a microglia M1 polarization stimulator (Jin et al. 2021; Kushwah et al. 2022; Lei et al. 2023). Therefore, the reversal of circ_DLGAP4' impact on microglia polarization and inflammatory cytokine production by PMA might not be exclusively attributed to the activation of the NF-κB pathway. Hence, our findings should be validated by subsequent studies. Additionally, it should be clarified that we only applied IF to detect CD68, CD86, CD206, and ARG1, and further studies should consider using the data of mRNA or western blot to further confirm the findings of this study. Moreover, microglial cloned BV-2 cells were used in this study. Although BV-2 cells are commonly used microglial cell lines, the morphologies, adhesion properties, and proliferation rates of BV-2 cells are different from primary microglia (Wang et al. 2021). Meanwhile, some microglia-specific genes, such as integrin subunit beta 5 and MER proto-oncogene, tyrosine kinase, are rarely expressed in BV-2 cells (Butovsky et al. 2014;

Wang et al. 2021). Therefore, BV-2 cells might not fully represent primary microglia characteristics. Further studies should consider applying other microglial cell lines, such as N9 cells and human immortalized microglia (HMO6) to validate the findings of this study.

Inspiringly, our study found that circ_DLGAP4 was reduced in microglia after IS injury, which suggested that circ_DLGAP4 might have the potential to serve as a biomarker for IS patients (Zhu et al. 2019). At the same time, we further discovered that circ_DLGAP4 might inactivate the NF-κB pathway to inhibit microglia M1 polarization and proinflammatory cytokines, which might be helpful in attenuating IS injury. Clinically, although revascularization therapies made advances in treating IS patients, the prognosis of IS patients was still unsatisfactory (approximately 3.29 million deaths were caused by IS in 2019, worldwide) (GBD 2019 Stroke Collaborators 2021). Considering secondary brain injury was a crucial cause for poor prognosis in IS patients, and circ_DLGAP4 might help to ameliorate this process, our findings may enhance the management of IS patients.

To sum up, circ_DLGAP4 is decreased after IS; its overexpression inhibits IS injury by suppressing microglia M1 polarization and proinflammatory cytokine production, which may be possibly due to the inactivation of the NF-κB pathway. Our findings may provide a theoretical reference that circ_DLGAP4 may be beneficial in attenuating IS progression. However, the detailed mechanism should be validated by further studies.

Conflict of Interest

These authors declare no conflict of interest.

Referrences

Bai, Y., Zhang, Y., Han, B., Yang, L., Chen, X., Huang, R., Wu, F., Chao, J., Liu, P., Hu, G., Zhang, J.H. & Yao, H. (2018) Circular RNA DLGAP4 Ameliorates Ischemic Stroke Outcomes by Targeting miR-143 to Regulate Endothelial-Mesenchymal Transition Associated with Blood-Brain Barrier Integrity. *J. Neurosci.*, **38**, 32-50.

Butovsky, O., Jedrychowski, M.P., Moore, C.S., Cialic, R., Lanser, A.J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P.M., Doykan, C.E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J.D., Ransohoff, R.M., et al. (2014) Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nat. Neurosci.*, **17**, 131-143.

Feng, J., He, W., Xia, J., Huang, Q., Yang, J., Gu, W.P., Zhang, N. & Liu, Y.H. (2023) Human umbilical cord mesenchymal stem cells-derived exosomal circDLGAP4 promotes angiogenesis after cerebral ischemia-reperfusion injury by regulating miR-320/KLF5 axis. *FASEB J.*, **37**, e22733.

GBD 2019 Stroke Collaborators (2021) Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet Neurol.*, **20**, 795-820.

Jiang, C.T., Wu, W.F., Deng, Y.H. & Ge, J.W. (2020) Modulators of microglia activation and polarization in ischemic stroke (Review). *Mol. Med. Rep.*, **21**, 2006-2018.

Jin, P., Deng, S., Sherchan, P., Cui, Y., Huang, L., Li, G., Lian, L., Xie, S., Lenahan, C., Travis, Z.D., Zhang, J.H., Gong, Y. & Tang, J. (2021) Neurokinin Receptor 1 (NK1R) Antagonist Aprepitant Enhances Hematoma Clearance by Regulating Microglial Polarization via PKC/p38MAPK/NFkappaB Pathway After Experimental Intracerebral Hemorrhage in Mice. *Neurotherapeutics*, **18**, 1922-1938.

Jurcau, A. & Simion, A. (2021) Neuroinflammation in Cerebral Ischemia and Ischemia/Reperfusion Injuries: From Pathophysiology to Therapeutic Strategies. *Int. J. Mol. Sci.*, **23**, 14.

Kong, L., Li, W., Chang, E., Wang, W., Shen, N., Xu, X., Wang, X., Zhang, Y., Sun, W., Hu, W., Xu, P. & Liu, X. (2022) mtDNA-STING Axis Mediates Microglial Polarization via IRF3/NF-kappaB Signaling After Ischemic Stroke. *Front. Immunol.*, **13**, 860977.

Kushwah, N., Woeppel, K., Dhawan, V., Shi, D. & Cui, X.T. (2022) Effects of neuronal cell adhesion molecule L1 and nanoparticle surface modification on microglia. *Acta Biomater.*, **149**, 273-286.

Lei, P., Li, Z., Hua, Q., Song, P., Gao, L., Zhou, L. & Cai, Q. (2023) Ursolic Acid Alleviates Neuroinflammation after Intracerebral Hemorrhage by Mediating Microglial Pyroptosis via the NF-kappaB/NLRP3/GSDMD Pathway. *Int. J. Mol. Sci.*, **24**, 14771.

Liu, C., Gu, J. & Yu, Y. (2023) Celastrol assuages oxygen-glucose deprivation and reoxygenation-induced damage in human brain microvascular endothelial cells through the circDLGAP4/miR-6085/GDF11 pathway. *Metab. Brain Dis.*, **38**, 255-267.

Liu, H., Zheng, W. & Song, Z. (2022) circDlgap4 Alleviates Cerebral Ischaemic Injury by Binding to AUF1 to Suppress Oxidative Stress and Neuroinflammation. *Mol. Neurobiol.*, **59**, 3218-3232.

Luo, L., Liu, M., Fan, Y., Zhang, J., Liu, L., Li, Y., Zhang, Q., Xie, H., Jiang, C., Wu, J., Xiao, X. & Wu, Y. (2022) Intermittent theta-burst stimulation improves motor function by inhibiting neuronal pyroptosis and regulating microglial polarization via TLR4/NFkappaB/NLRP3 signaling pathway in cerebral ischemic mice. *J. Neuroinflammation*, **19**, 141.

Ma, Y., Wang, J., Wang, Y. & Yang, G.Y. (2017) The biphasic function of microglia in ischemic stroke. *Prog. Neurobiol.*, **157**, 247-272.

Mendelson, S.J. & Prabhakaran, S. (2021) Diagnosis and Management of Transient Ischemic Attack and Acute Ischemic Stroke: A Review. *JAMA*, **325**, 1088-1098.

Mitroshina, E.V., Savyuk, M.O., Ponimaskin, E. & Vedunova, M.V. (2021) Hypoxia-Inducible Factor (HIF) in Ischemic Stroke and Neurodegenerative Disease. *Front. Cell Dev. Biol.*, **9**, 703084.

Paul, S. & Candelario-Jalil, E. (2021) Emerging neuroprotective strategies for the treatment of ischemic stroke: An overview of clinical and preclinical studies. *Exp. Neurol.*, **335**, 113518.

Plastira, I., Bernhart, E., Goeritzer, M., Reicher, H., Kumble, V.B., Kogelnik, N., Wintersperger, A., Hammer, A., Schlager, S., Jandl, K., Heinemann, A., Kratky, D., Malle, E. & Sattler, W. (2016) 1-Oleyl-lysophosphatidic acid (LPA) promotes polarization of BV-2 and primary murine microglia towards an M1-like phenotype. *J. Neuroinflammation*, **13**, 205.

Pu, Z., Xia, S., Shao, P., Bao, X., Wu, D. & Xu, Y. (2022) Regulation of Microglia-Activation-Mediated Neuroinflammation to Ameliorate Ischemia-Reperfusion Injury via the STAT5-NF-kappaB Pathway in Ischemic Stroke. *Brain Sci.*, **12**, 1153.

Qiu, J., Guo, J., Liu, L., Liu, X., Sun, X. & Chen, H. (2023) Vav1 promotes inflammation and neuronal apoptosis in cerebral ischemia/reperfusion injury by upregulating microglial and NLRP3 inflammasome activation. *Neural Regen. Res.*, **18**, 2436-2442.

Qiu, L., He, J., Chen, H., Xu, X. & Tao, Y. (2022) CircDLGAP4 overexpression relieves oxygen-glucose deprivation-induced neuronal injury by elevating NEGR1 through sponging miR-503-3p. *J. Mol. Histol.*, **53**, 321-332.

Qiu, M., Xu, E. & Zhan, L. (2021) Epigenetic Regulations of

Microglia/Macrophage Polarization in Ischemic Stroke. *Front. Mol. Neurosci.*, **14**, 697416.

Saini, V., Guada, L. & Yavagal, D.R. (2021) Global Epidemiology of Stroke and Access to Acute Ischemic Stroke Interventions. *Neurology*, **97**, S6-S16.

Var, S.R., Shetty, A.V., Grande, A.W., Low, W.C. & Cheeran, M.C. (2021) Microglia and Macrophages in Neuroprotection, Neurogenesis, and Emerging Therapies for Stroke. *Cells*, **10**, 3555.

Wang, Y., Peng, Y. & Yan, H. (2021) Commentary: Neuroinflammatory In Vitro Cell Culture Models and the Potential Applications for Neurological Disorders. *Front. Pharmacol.*, **12**, 792614.

Xian, M., Cai, J., Zheng, K., Liu, Q., Liu, Y., Lin, H., Liang, S. & Wang, S. (2021) Aloe-emodin prevents nerve injury and neuroinflammation caused by ischemic stroke via the PI3K/AKT/mTOR and NF-kappaB pathway. *Food Funct.*, **12**, 8056-8067.

Xue, Y., Nie, D., Wang, L.J., Qiu, H.C., Ma, L., Dong, M.X., Tu, W.J. & Zhao, J. (2021) Microglial Polarization: Novel Therapeutic Strategy against Ischemic Stroke. *Aging Dis.*, **12**, 466-479.

Yao, M., Luo, Y., Li, H., Liao, S. & Yu, J. (2022) LncRNA Tug1 Contributes Post-stroke NLRP3 Inflammasome-Dependent Pyroptosis via miR-145a-5p/Tlr4 Axis. *Mol. Neurobiol.*, **59**, 6701-6712.

Zeng, J., Bao, T., Yang, K., Zhu, X., Wang, S., Xiang, W., Ge, A., Zeng, L. & Ge, J. (2022) The mechanism of microglia-mediated immune inflammation in ischemic stroke and the role of natural botanical components in regulating microglia: A review. *Front. Immunol.*, **13**, 1047550.

Zhang, J., Zheng, Y., Luo, Y., Du, Y., Zhang, X. & Fu, J. (2019) Curcumin inhibits LPS-induced neuroinflammation by promoting microglial M2 polarization via TREM2/ TLR4/ NF-kappaB pathways in BV2 cells. *Mol. Immunol.*, **116**, 29-37.

Zhang, X., Wan, M., Min, X., Chu, G., Luo, Y., Han, Z., Li, W., Xu, R., Luo, J., Li, W., Yang, Y., Ma, Y., Jiao, L. & Wang, T. (2023) Circular RNA as biomarkers for acute ischemic stroke: A systematic review and meta-analysis. *CNS Neurosci. Ther.*, **29**, 2086-2100.

Zhao, B., Wang, J., Liu, L., Li, X., Liu, S., Xia, Q. & Shi, J. (2016) Annexin A1 translocates to nucleus and promotes the expression of pro-inflammatory cytokines in a PKC-dependent manner after OGD/R. *Sci. Rep.*, **6**, 27028.

Zhu, X., Ding, J., Wang, B., Wang, J. & Xu, M. (2019) Circular RNA DLGAP4 is down-regulated and negatively correlates with severity, inflammatory cytokine expression and pro-inflammatory gene miR-143 expression in acute ischemic stroke patients. *Int. J. Clin. Exp. Pathol.*, **12**, 941-948.