MiR-124 Promotes Microglial M2 Polarization Through TLR4/MyD88/NF-κB p65/NLRP3 Signaling In Palmitic Acid Treated-BV2 Cells

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Research Article

Keywords: High-fat diet, Inflammasome, Microglia, MiR-124, Neuroinflammation, NLRP3

Posted Date: June 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-597326/v1

Abstract

Aim

Neuroinflammation is an explanation why obesity or high-fat diet induce central nervous system disorders. MiR-124, as a highly expressed microRNA in brain, might alleviate neuroinflammation through regulating microglial M1/M2 polarization, but its mechanism is unclear. The aim of the study was to explore whether miR-124 exerted its effect mentioned above through TLR4/MyD88/NF-κB p65/NLRP3 signaling in palmitic acid treated-microglia.

Methods

Prepared BV2 cells were treated with palmitic acid to establish an in vitro model of high-fat diet. MiR-124 mimic and inhibitor were adopted to up-regulate and down-regulate the expression of miR-124. TAK-242 and NLRP3 siRNA were used to down-regulate the expression of TLR4 and NLRP3. Expression of miR-124, signaling proteins (TLR4, MyD88, NF-κB p65), inflammasome markers (NLRP3, IL-1β) and microglial polarization markers (CD206, Arg-1, CD86, iNOS) was measured by qPCR and western blotting. Pyroptosis rate was assessed using flow cytometry.

Results

First, palmitic acid up-regulated the TLR4/MyD88/NF-κB p65 signaling, increased the NLRP3 expression, elevated the pyroptosis rate and inhibited the M2 polarization in BV2 cells. Second, miR-124 mimic and inhibitor separately alleviated and aggravated the effect of palmitic acid on microglial polarization and NLRP3 expression. MiR-124 mimic also down-regulated the TLR4/MyD88/NF-κB p65 signaling. Third, TAK-242 did not affect the expression of miR-124, but can simulate the protective effect of miR-124 mimic on microglial polarization and NLRP3 expression. Fourth, NLRP3 siRNA can also promoted M2 polarization in BV2 cells.

Conclusion

MiR-124 promoted microglial M2 polarization through TLR4/MyD88/NF-κB p65/NLRP3 signaling in palmitic acid treated-BV2 cells

Introduction

An increasing number of evidence suggest that obesity or high-fat diet cause cognitive impairment, and neuroinflammation is involved in such pathogenic process [1–4]. However, regulatory mechanism of neuroinflammation in high-fat model has not been fully clarified. But, academics agree that microglia play an irreplaceable role in it [5, 6]. Microglia are one kind of nerve cells which are distributed in central nervous system, and the main function is to regulate immune inflammatory response and to protect brain tissue from diseases and infections [7, 8]. Normally, the cells are dormant, but can be activated by inflammatory response. Activated microglia polarize to one functional state: M1 or M2 phenotype. The

former promotes the inflammation, while the latter exerts an anti-inflammatory effect [9]. So, promoting M2 polarization is a theoretically feasible strategy against neuroinflammation under high-fat diet condition.

In addition to microglia, NLRP3 inflammasome is also related to regulation of neuroinflammation induced by obesity and high-fat diet [10, 11]. Inflammasomes are a class of multi-component protein complexes which play a crucial role in natural immune response, and NLRP3 inflammasome is one important member of them [12]. Briefly, inflammasome is able to identify endogenous and exogenous risk molecules, and leads to release of inflammatory cytokines (i.e. IL-1β and IL-18) and development of pyroptosis [12]. The process promotes strong neuroinflammation, which protects brain tissue against the risk molecules in initial stage and begins to play a harmful role when the inflammatory response gets out of control [12]. Interestingly, Cui et al. suggest that inhibition of NLRP3 signaling promotes microglial M2 polarization in Alzheimer's disease [13]. The findings might shed light on functional relationship between microglia and NLRP3 inflammasome, but further research is needed to draw a final conclusion, especially in high-fat diet model.

MicroRNA-124 (miR-124) is an important member of microRNA family, and is highly expressed in central nervous system [14]. Previous studies reveal that miR-124 might promote anti-inflamed microglial M2 polarization and inhibit NLRP3 inflammasome expression in other acute and chronic brain events [15, 16]. And, TLR4 is one kind of conserved natural pattern recognition receptor, and is involved in regulation of M2 polarization and NLRP3 inflammasome activation in microglia [17, 18]. More importantly, miR-124 has been proved to regulate the activity of TLR4, and the latter seems to be a bridge between the former and microglia [17, 19]. Therefore, miR-124/TLR4 signaling pathway may be a potential regulatory mechanism of neuroinflammation in high-fat diet condition, which should be fully verify.

Therefore, we conducted an in vitro experiment using a high-fat treated-BV2 cell line. The aim of the study was to reveal the potential effect and regulatory mechanism of miR-124/TL4/NLRP3 signaling pathway on microglial M2 polarization, and to provide the therapeutic strategy against neuroinflammation and cognitive impairment induced by obesity and high-fat diet.

Materials And Methods

2.1 Cell culture

A mouse BV2 microglial cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All reagents and materials in the process were obtained from Life Technologies (CA, USA). The cells were cultured in minimum Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified environment with 5% carbon dioxide (CO_2). The medium was renewed every 48 hours. In addition, the cells were regularly treated with 0.25% trypsin to transfer and place them in more culture bottles until enough cells were obtained. Finally, monolayers of the BV2 microglial cells were cultured on 24-well plates (1×10^5 cells/well) for further experiments.

2.2 Reagents and preliminary experiments

Palmitic acid (PA, one type of saturated fatty acids) was obtained from Sigma-Aldrich (USA), and was dissolved using 0.1 mmol/l sodium hydroxide at 70℃ for 5 minutes. Then, the solution is mixed using 10% bovine serum albumin at 55℃ for another 10 minutes, and was adjusted to a series of concentrations (0, 400, 800, 1200, 1600 μmol/l) using specific neuronal medium (NM, Cat. #1521).

TAK-242 (one type of TLR4 blocker) was purchased from MedChemExpress (USA), and was dissolved using 0.1% dimethylsulfoxide (DMSO) to produce several concentrations of the reagent (0, 50, 100, 200, 400, 800 nmol/l).

After that, as shown in Fig. 1, the prepared cells were separately treated with different concentrations of PA and TAK-242 for 12 hours. Cell activity was determined using CCK8 assay to assess the safety of the reagents. Expression of miR-124 and TLR4 was measured separately using qPCR and western blotting to evaluate the effectiveness of the reagents. As a result, 800 μmol/l of PA and 200 nmol/l of TAK-242 were determined to be the optimal concentrations for further experiments.

2.3 Grouping and cell transfection

The BV2 cells were divided into control group, PA group, PA + mir_mimic group, PA + mir_inhibitor group, PA + mir_ctl group, PA + TAK group, PA + nlrp_siR group and PA + nlrp_ctl group. Each group contained ten wells.

MiR-124 mimic, miR-124 inhibitor and negative control were mixed with Lipofectamine 2000 transfection reagent (RiboBio, Guangzhou, China), and were adopted for cell transfection separately in the PA + mir_mimic, PA + mir_inhibitor and PA + mir_ctl groups [20]. Specific experimental steps referred to the specification of the commercial kit. Cell transfection was performed in in 37℃, 5% CO2 incubator for 48 hours.

The cells in the PA + nlrp_siR and PA + nlrp_ctl groups were separately transfected with NLRP3 siRNA and control siRNA using Lipofectamine LTX transfection reagent (RiboBio, Guangzhou, China) according to the manufacturer's protocols. [21]. Sense and anti-sense of NLRP3 siRNA were 5'-GCUUCAGCCACAUGACUUUTT-3' and 5'-AAAGUCAUGUGGCUGAAGCTT-3'. Sense and anti-sense of control siRNA were 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAG -AATT-3'. The cells were also incubated for 48 hours before further measurement.

2.4 Modeling and intervention

After cell transfection, all the BV2 cells except those in the control group were treated with 800 μmol/l PA for 12 hours to construct an in vitro model of high-fat diet [22]. Meanwhile, the BV2 cells in the PA + TAK group were treated with 200 nmol/l TAK-242 for 12 hours to inhibit the expression of TLR4.

2.5 Cell viability assay

After the intervention, cell viability was measured using a commercial CCK8 assay kit (#ab228554, Abcam, UK). Briefly, all of the wells to be tested were incubated with CCK-8 solution (10 μl) at 37℃ for 2 hours. Then, the absorbance of the specimens at 450 nm was determined using a Thermo Scientific microplate reader (Waltham, MA, US).

2.6 qPCR assay

After the intervention, expression of miR-124 was measured using qPCR assay. First, total RNA extraction was adopted using a commercial RNAeasy™ animal RNA isolation kit (centrifugal column type) (Beyotime, Shanghai, China). Second, the obtained RNA was reverse-transcribed into complementary DNA using a commercial PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio, JPN). Third, the qPCR process was performed using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, JPN) and Applied Biosystems 7500 Real-Time PCR System (Thermo Scientific, Waltham, MA, US). The PCR cycling stages (parameters) were listed as followed: denaturation (95˚C, 30 seconds), annealing (60˚C, 30 seconds) and elongation (72˚C, 30 seconds). Expression of mRNA was normalized by U6, and the latter was adopted as an endogenous control. Primer sequences of miR-124 were 5'-GCTAAGGCACGCGGTG-3' and 5'-GTGCAGGGTCCGAGGT-3'. Primer sequences of U6 were: 5'-ATTGGAACGATACAGAGAAGATT-3' and 5'-GGAAC -GCTTCACGAATTTG-3'.

2.7 Western blotting

Expression of several proteins was analyzed using western blotting. First, the cells were treated with RIPA Lysis buffer containing protease and phosphatase inhibitors. Second, the lysates were incubated at 0℃ for 12 minutes and centrifuged at 10,000 rpm for 5 minutes. Third, protein amounts of the obtained supernatants were measured using a commercial BCA assay kit (Abcam, Cambridge, UK). Fourth, 50 μg of proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then moved to polyvinylidene fluoride (PVDF) membranes for 60 minutes. Fifth, the membranes were blocked using 5% non-fat milk for 2 hours, and were incubated at 4℃ for 16 hours with TLR4 (#14358, 1:1000), MyD88 (#4283, 1:1000), NF-κB p65 (#8242, 1:1000), NLRP3 (#15101, 1:1000), IL-1β (#31202, 1:1000), CD206 (#24595, 1:1000), Arg-1 (#93668, 1:1000), CD86 (#19589, 1:1000), iNOS (#13120, 1:1000) primary antibodies (Cell Signaling, USA). Sixth, the membrane were washed three times using PBS, and were incubated with anti-rabbit HRP-conjugated IgG secondary antibodies for 60 minutes (Cell Signaling, USA). Seventh, protein bands were visualized using enhanced chemiluminescence (Immolilon Western, USA), and the intensities of them were measured using image J software.

2.8 Flow cytometry

Pyroptosis rate was determined by a FAM-FLICA in vitro caspase-1 detection kit (ImmunoChemistry, USA) [23]. First, the cells were mixed with trypsin. Second, the cells were washed several times using phosphate buffer saline. Third, the cells were stained with 10 μl FAM-FLICA and 5 μl PI for 20 minutes in a dark environment at room temperature. Fourth, fluorescence intensity was measured using a Coulter Epics XL

flow cytometer. Pyroptosis rate was calculated using a formula: number of double-positive cells / number of total cells × 100%.

2.9 Statistical analysis

Continuous variables in the study were expressed in the form of mean ± standard deviation. Difference of two continuous variables was measured using independent sample t test, and difference of more than two continuous variables was measured using ANOVA with LSD test. If P value was less than 0.05, the difference was statistically significant.

Results

3.1 Determination of concentrations for PA and TAK-242

In Figure 1, prepared BV2 cells were treated with different concentrations of PA or TAK-242. As a results, the study did not find any abnormality in cell activity when the cells were treated with 800 μmol/l of PA or 200 nmol/l of TAK-242 (P = 0.344, P = 0.237, respectively). However, when the concentration of PA or TAK-242 exceed the boundary, the cell activity began to decline (P = 0.023, P = 0.005, respectively). The study also revealed that 800 μmol/l of PA and 200 nmol/l of TAK-242 can separately affect the expression of miR-21 and TLR4 (P ≪ 0.001, P ≪ 0.001, respectively), which proved that the reagents at these concentrations can play an effective biological role. Therefore, 800 μmol/l of PA and 200 nmol/l of TAK-242 were adopted to conduct following experiments.

3.2 Effect of miR-124 on TLR4/MyD88/NF-κB p65 signaling in BV2 cells

In Figure 2, PA treatment significantly increased the expression of miR-124 and TLR4/MyD88/NF-κB p65 proteins (P ≪ 0.001, P ≪ 0.001, P ≪ 0.001, P = 0.010, respectively). Meanwhile, up-regulation of miR-124 significantly inhibited the expression of TLR4/MyD88/NF-κB p65 proteins (P ≪ 0.001, P = 0.001, P = 0.030, respectively), and down-regulation of miR-124 may re-increased the expression of these three signaling proteins (P ≪ 0.001, P = 0.016, P = 0.018, respectively). These findings indicated that miR-124 can inhibit the up-regulation of TLR4/MyD88/NF-κB p65 signaling induced by PA.

3.3 Effect of miR-124 on NLRP3 inflammasome and pyroptosis in BV2 cells

In Figure 3, compared with the control group, PA treatment in the PA group elevated the expression of NLRP3 and IL-1β (P = 0.001, P ≪ 0.001, respectively), and also increased the rate of pyroptosis (P = 0.003). Meanwhile, up-regulation and down-regulation of miR-124 separately inhibited and re-elevated the expression of the proteins and the rate of pyroptosis (up-regulation: P = 0.011, P ≪ 0.001, P = 0.015, respectively; down-regulation: P ≪ 0.001, P ≪ 0.001, P = 0.011, respectively). These findings indicated that miR-124 inhibited the activity of NLRP3 inflammasome and rate of pyroptosis in the PA-treated model.

3.4 Effect of miR-124 on microglial M2 polarization in BV2 cells

In Figure 4, PA treatment decreased the expression of CD206 and Arg-1 (P = 0.022, P = 0.020, respectively), and increased the expression of CD86 and iNOS (P ≤ 0.001, P ≤ 0.001, respectively). More importantly, up-regulation of miR-124 reversed the effect of PA on the expression of CD206, Arg-1, CD86 and iNOS (P ≤ 0.001, P ≤ 0.001, P ≤ 0.001, P = 0.001, respectively). And, down-regulation of miR-124 aggravated the effect of PA on such proteins (P ≤ 0.001, P = 0.003, P = 0.003, P ≤ 0.001, respectively). These findings indicated that miR-124 inhibited the microglial M1 polarization and promoted the microglial M2 polarization in the PA-treated model.

3.5 Effect of TLR4 on MyD88/NF-κB p65 signaling, NLRP3 inflammasome and pyroptosis in BV2 cells

In Figure 5, TLR-4 blocker TAK-242 significantly inhibited the expression of TLR4/MyD88/NF-κB p65 proteins (P ≤ 0.05, P ≤ 0.05, P ≤ 0.05, respectively), but can not affect the level of miR-124 (P ≤ 0.05). Meanwhile, the blocker inhibited the expression of NLRP3 and IL-1β (P ≤ 0.05, P ≤ 0.05, respectively), and also decreased the rate of pyroptosis (P = 0.014). These findings indicated that inactivity of TLR4/MyD88/NF-κB p65 signaling inhibited the activity of NLRP3 inflammasome and rate of pyroptosis in PA-treated BV2 cells.

3.6 Effect of TLR4 on microglial M2 polarization in BV2 cells

In Figure 6, TAK-242 was able to increase the expression of CD206 and Arg-1 (P ≤ 0.05, P ≤ 0.05, respectively), and decrease the expression of CD86 and iNOS in the PA-treated cells (P ≤ 0.05, P ≤ 0.05, respectively). These findings indicated that down-regulation of TLR4 expression inhibited the microglial M1 polarization and promoted the microglial M2 polarization in PA-treated BV2 cells.

3.7 Relationship between NLRP3 inflammasome activation and microglial M2 polarization in BV2 cells

In Figure 7, down-regulation of NLRP3 elevated the expression of CD206 and Arg-1 (P ≤ 0.001, P ≤ 0.001, respectively), and inhibited the expression of CD86 and iNOS (P = 0.001, P ≤ 0.001, respectively). These findings indicated that down-regulation of NLRP3 expression inhibited the microglial M1 polarization and promoted the microglial M2 polarization.

Discussion

High-fat diet and obesity contributed to cognitive impairment, which was related to inflammation in central nervous system. PA was one type of saturated fatty acids. High level of PA in peripheral circulation not only triggered inflammation in brain, but also enhanced susceptibility to develop a neurodegenerative disease [24]. So, in the present study, the BV2 cells were treated with the reagent to establish an in vitro model of high-fat diet. In the process, we first confirmed a optimal concentration of PA (800 μmol/l) for 12-hour treatment, which was largely consistent with a previous study [25]. Second, in the main part of the experiment, we discovered that PA treatment significantly increased the activity of NLPR3 inflammasome and M1 polarization in microglia, and both promoted the development of

neuroinflammation. Therefore, we believed that such in vitro model can simulate the state of microglia after high-fat diet.

Previous study revealed that miR-124 was an important regulator of triglycerides metabolism in liver tissue [26]. Its expression can be up-regulated by high-fat diet, and its known function was to promote cellular triglyceride accumulation through inhibition of triglyceride and fatty acid catabolism, in parallel with decreased inflammatory factors [26]. And, in vascular endothelial tissue, over-expression of miR-124 inhibited macrophage (one common inflammatory cell) proliferation and promoted macrophage apoptosis, which attenuated vascular local inflammation and atherosclerosis [27]. As mentioned above, miR-124 was highly expressed in brain tissue, and was involved in regulation of neuroinflammation in brain trauma and Parkinson's disease [15, 16]. Actually, high-fat diet or obesity also triggered neuroinflammation in brain tissue and caused diseases. However, regulative effect of miR-124 on inflammation in such condition has not been clarified. So, the present study focused this topic, and first revealed that PA treatment was able to increase the expression of miR-124 in microglia, and up-regulated miR-124 in turn exerted an anti-inflammatory neuroprotective effect via promoting microglial M2 polarization and inhibiting NLRP3 inflammasome activity.

TLR4 played an special role in both acquired and innate immunity. More importantly, TLR4 was highly expressed in microglia, and the TLR4/MyD88/NF-κB p65 signaling pathway had been proved that it can regulate the microglial polarization in other models [28, 29]. The present study also revealed that PA treatment activated the signaling pathway and inhibited microglial M2 polarization, which can be reversed by up-regulation of miR-124. In addition, the study confirmed the regulatory effect of the TLR4/MyD88/NF-κB p65 signaling pathway on NLRP3 inflammasome. All of these findings may partly explained the anti-inflammatory mechanism of miR-124 in high-fat treated BV2 cells.

Both microglial polarization and NLRP3 inflammasome were regarded as crucial mechanisms for regulating neuroinflammation. However, potential relationship between them were unclear. Only one study from Cui et al. reported that inhibition of TLR4 might induce microglial M2 polarization and exert neuroprotection through NLRP3 signaling in Alzheimer's disease [13]. The present study further explored such relationship in high-fat model, and provided some evidences that NLRP3 may indeed be an upstream signal molecule for microglial polarization. And, detailed mechanism of NLRP3 on regulating microglial polarization should be fully explored in the future.

In conclusion, the study revealed that PA treatment induced significant neuroinflammation partly through inhibiting microglial M2 polarization. The study also reported that up-regulation of miR-124 may reverse the harmful effect of PA mentioned above through regulation of TLR4/MyD88/NF-κB p65/NLRP3 signaling pathway.

Declarations

Funding

Not applicable.

Conflicts of interest

Not applicable.

Ethics approval

The study was approved by the ethics committees of Tianjin Medical University General Hospital and Tianjin Anding Hospital (Tianjin, China).

Consent to participate

Not applicable.

Consent for publication

All authors have confirmed and agreed with this article to submit to your journal.

Availability of data and material

The data can not be shared due to this is an ongoing study.

Code availability (software application or custom code)

All these analyses were performed using SPSS 18.0 software (US).

Acknowledgments

We want to thank Prof. JQ from TIANJIN UNIVERSITY (China) for the funding and laboratory support for this project.

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Figures

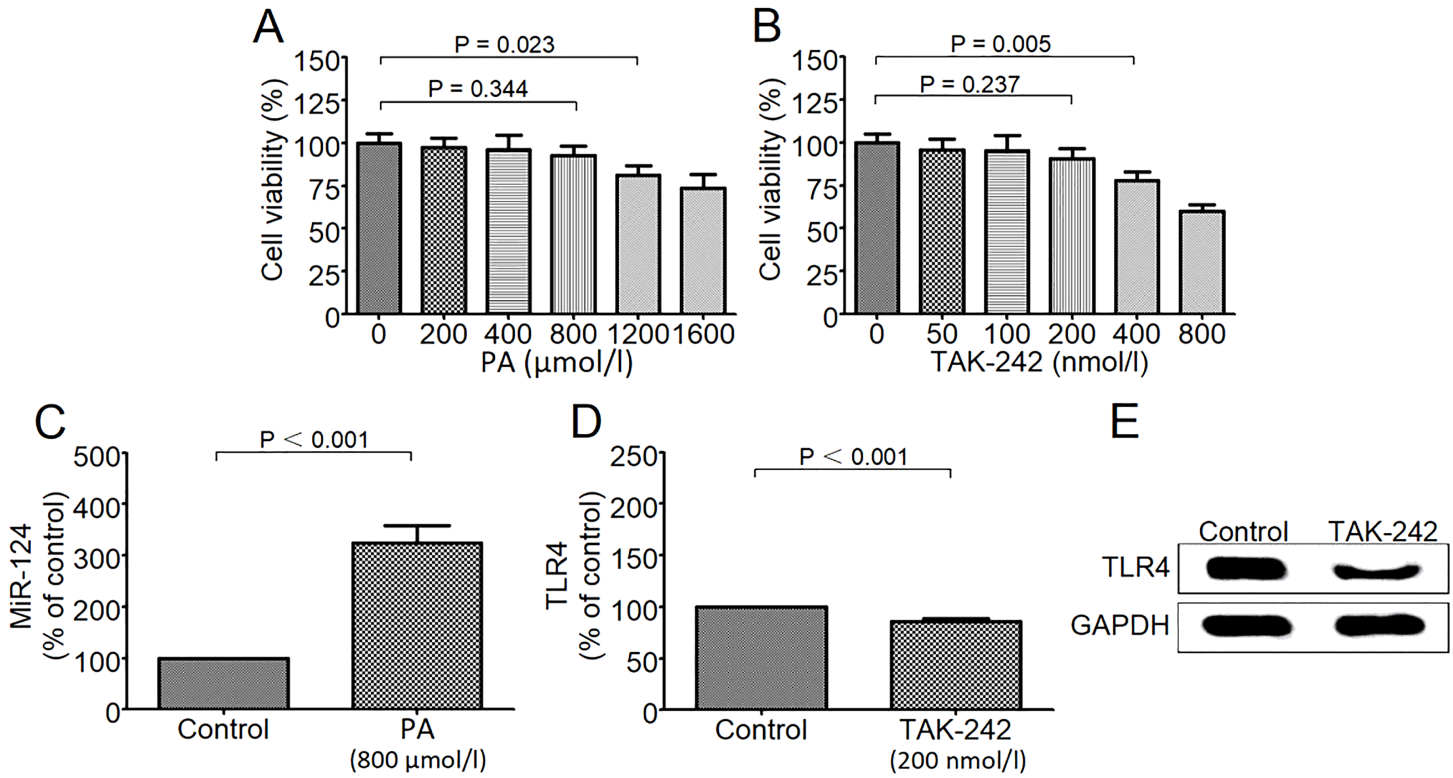

Figure 1

Determination of experimental concentrations for PA and TAK-242 Prepared BV-2 cells were treated with different concentrations of PA or TAK-242. And, 800 μmol/l of PA and 200 nmol/l of TAK-242 were the highest concentrations of the reagents, which did not affect the cell activity. Meanwhile, the reagents of these concentrations were separately able to affect the expression of miR-124 and TLR4. So, 800 μmol/l of PA and 200 nmol/l of TAK-242 were adopted to conduct following experiments. Difference of two variables was measured using independent sample t test, and difference of more than two variables was measured using ANOVA with LSD test. P□0.05 indicated statistically significant.

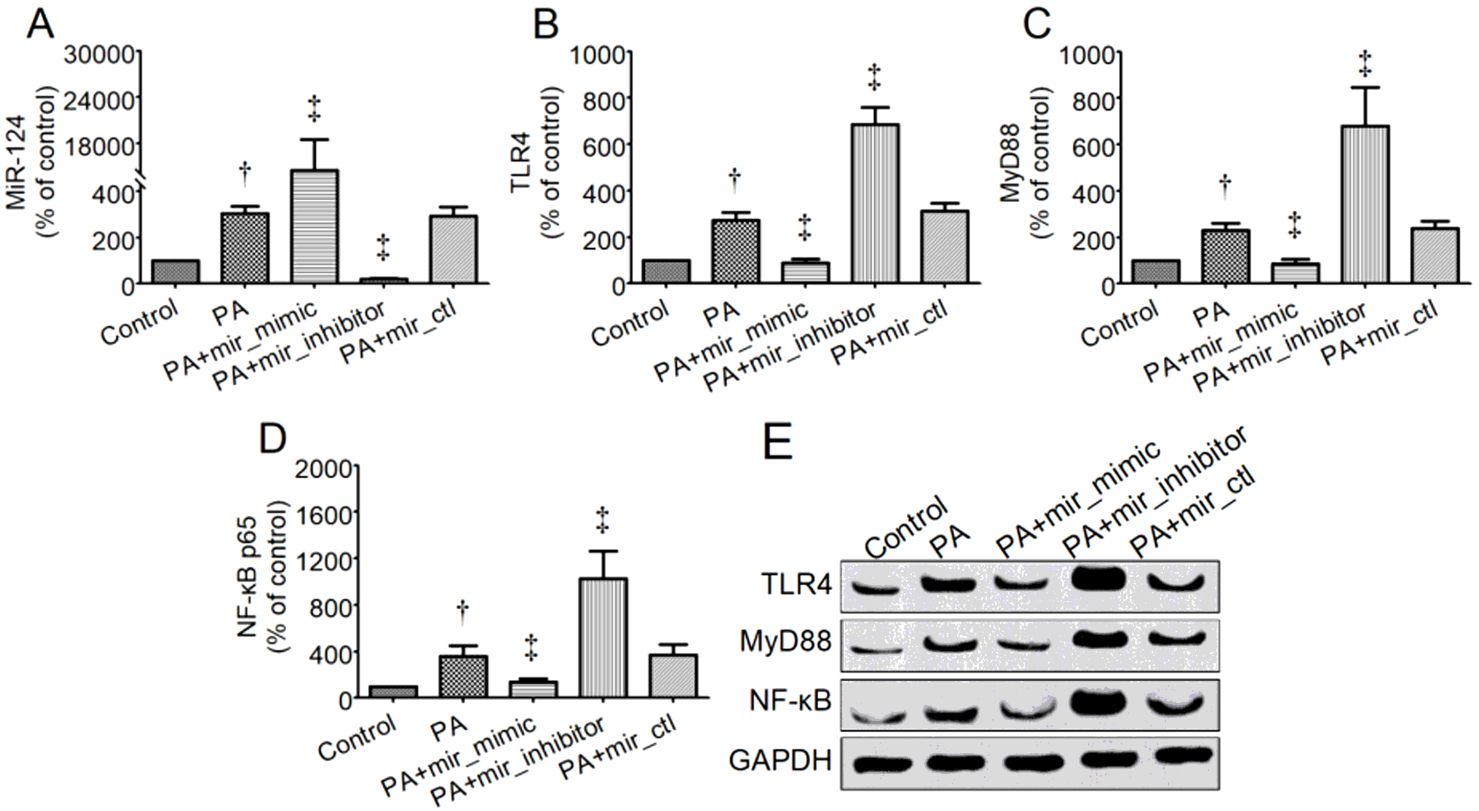

Figure 2

Effect of miR-124 on TLR4/MyD88/NF-κB p65 signaling pathway in BV2 cells Prepared BV-2 cells were separately transfected with miR-124 mimic and inhibitor to up-regulate and down-regulate the expression of the nucleic acid. Then, PA was adopted to establish an in vitro model of high-fat. After that, expression of TLR4/MyD88/NF-κB p65 proteins was determined using western blotting. Difference of more than two variables was measured using ANOVA with LSD test. P□0.05 indicated statistically significant. “†” indicated P □ 0.05 compared to control group; “‡” indicated P □ 0.05 compared to PA group.

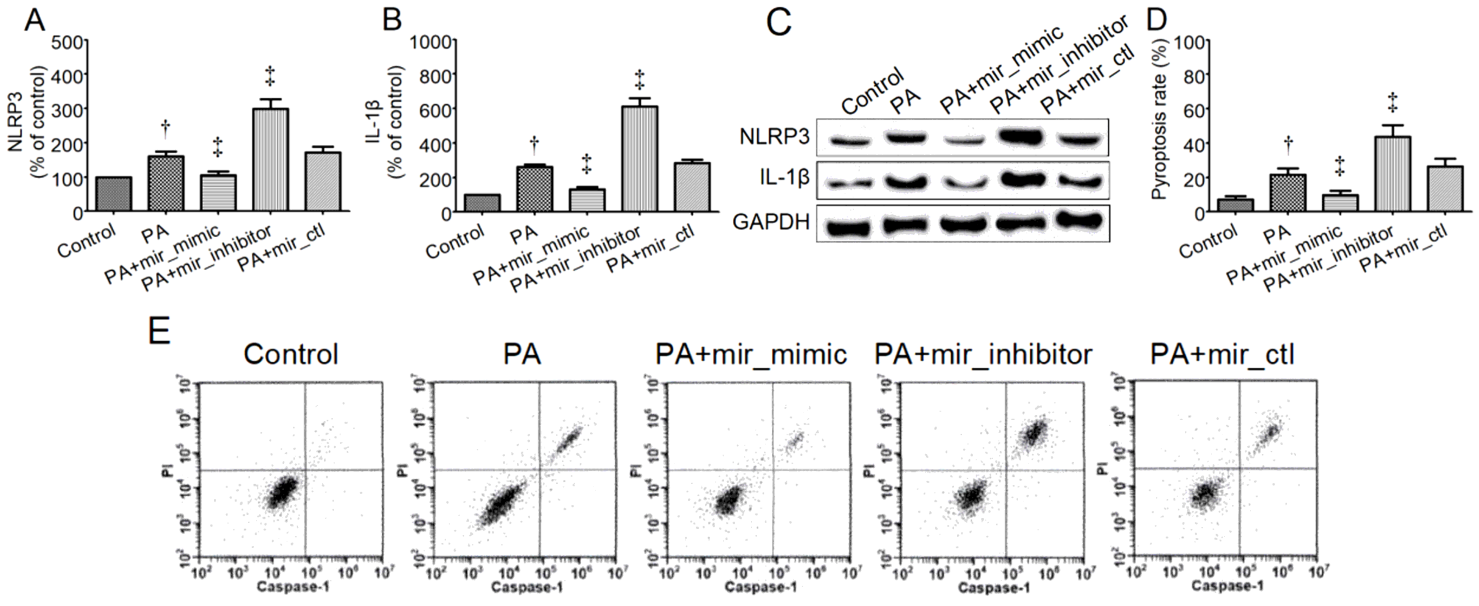

Figure 3

Effect of miR-124 on NLRP3 inflammasome and pyroptosis in BV2 cells Prepared BV-2 cells were separately transfected with miR-124 mimic and inhibitor to up-regulate and down-regulate the expression of the nucleic acid. Then, PA was adopted to establish an in vitro model of high-fat. After that, expression of NLRP3 and IL-1β proteins was determined using western blotting. Pyroptosis rate was determined using flow cytometry. Difference of more than two variables was measured using ANOVA with LSD test. P 0.05 indicated statistically significant. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group.

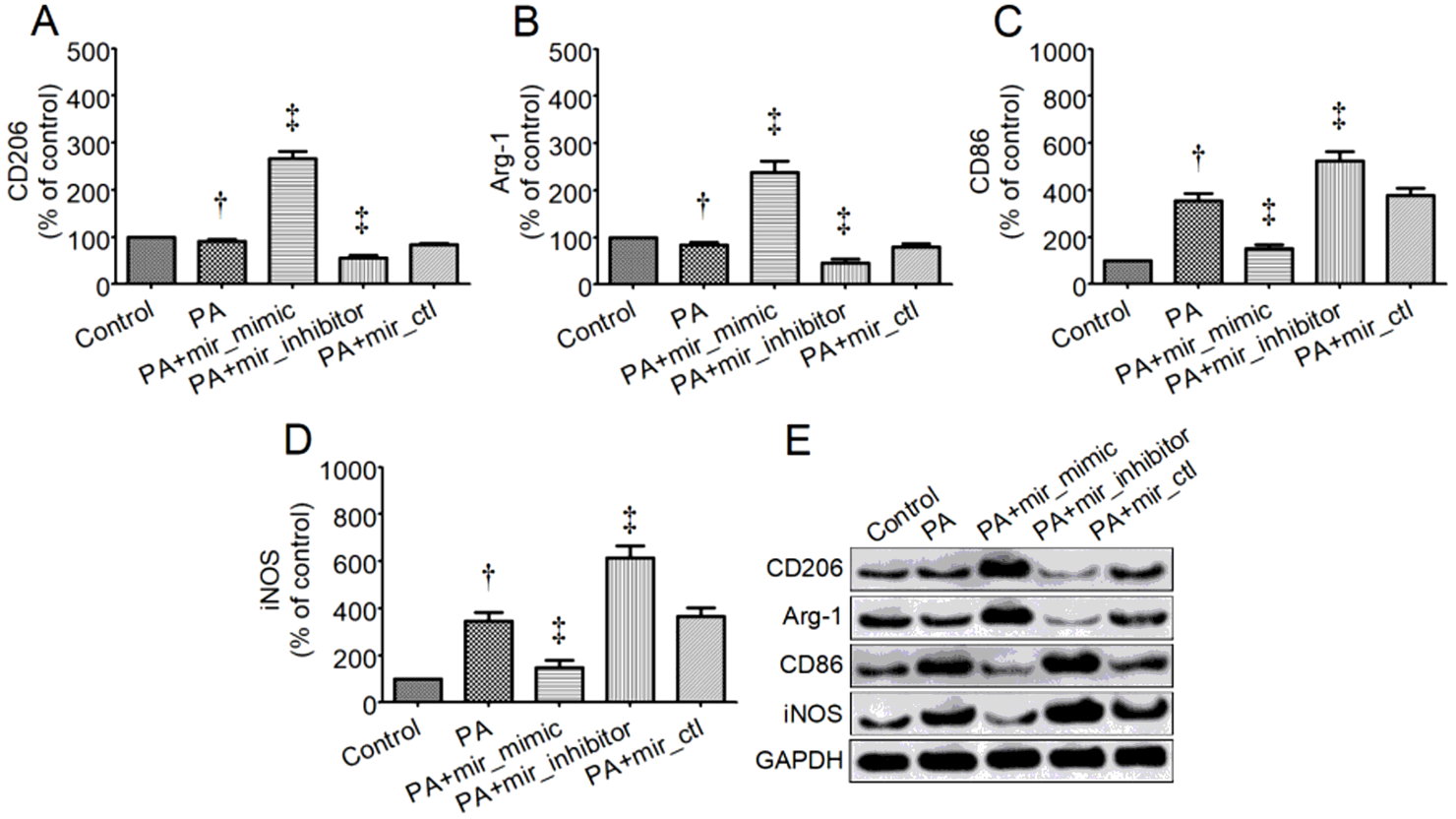

Figure 4

Effect of miR-124 on microglial M2 polarization in BV2 cells Prepared BV-2 cells were separately transfected with miR-124 mimic and inhibitor to up-regulate and down-regulate the expression of the nucleic acid. Then, PA was adopted to establish an in vitro model of high-fat. After that, expression of CD206, Arg-1, CD86 and iNOS proteins was determined using western blotting. Difference of more than two variables was measured using ANOVA with LSD test. P0.05 indicated statistically significant. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group.

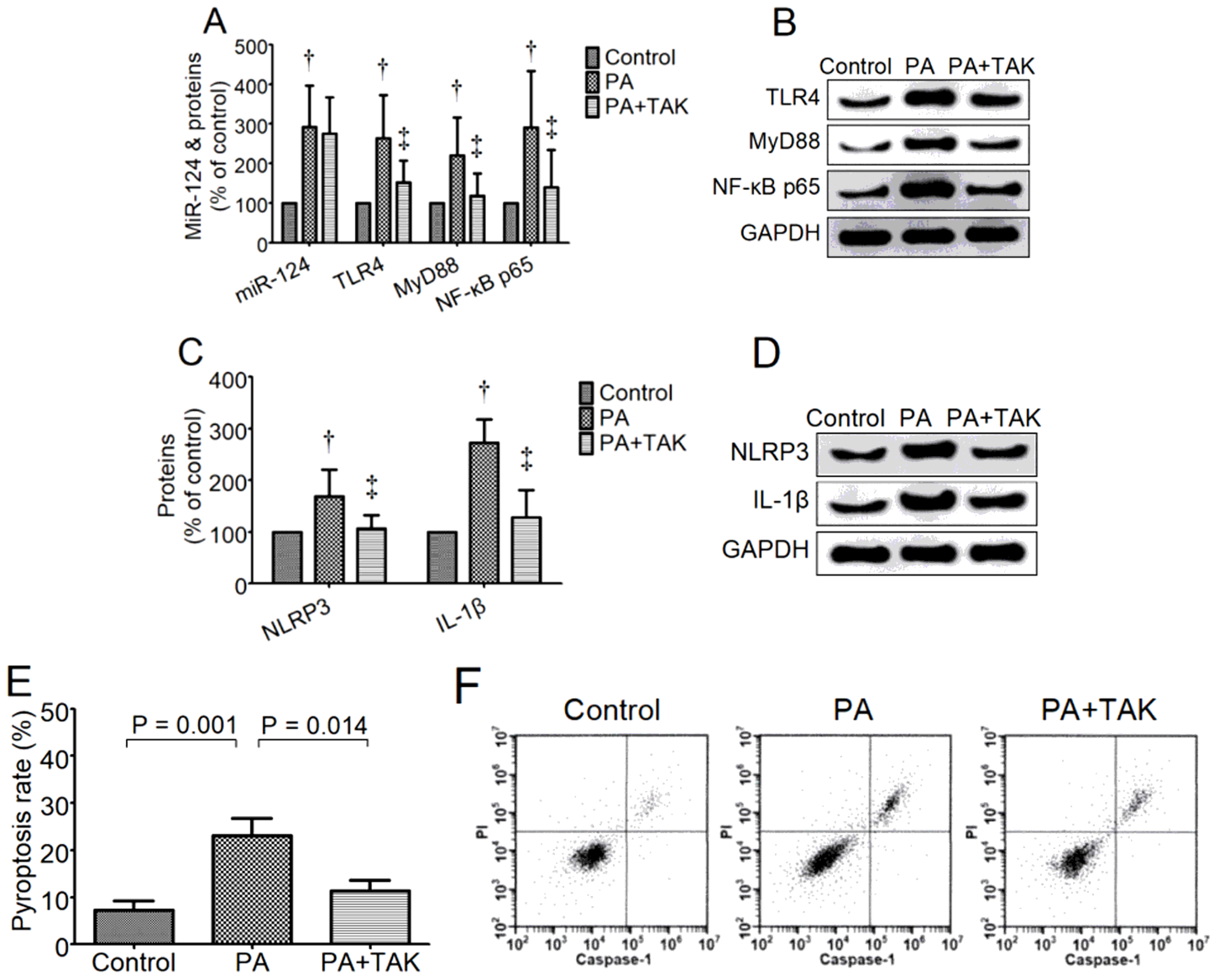

Figure 5

Effect of TLR4 on TLR4/MyD88/NF-κB p65 signaling pathway and pyroptosis in BV2 cells PA was adopted to establish an in vitro model of high-fat. Meanwhile, the BV-2 cells were treated with TAK-242 to regulate the expression of TLR4. After that, expression of TLR4, MyD88, NF-κB, NLRP3 and IL-1β proteins was determined using western blotting. Pyroptosis rate was determined using flow cytometry. Difference of more than two variables was measured using ANOVA with LSD test. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group.

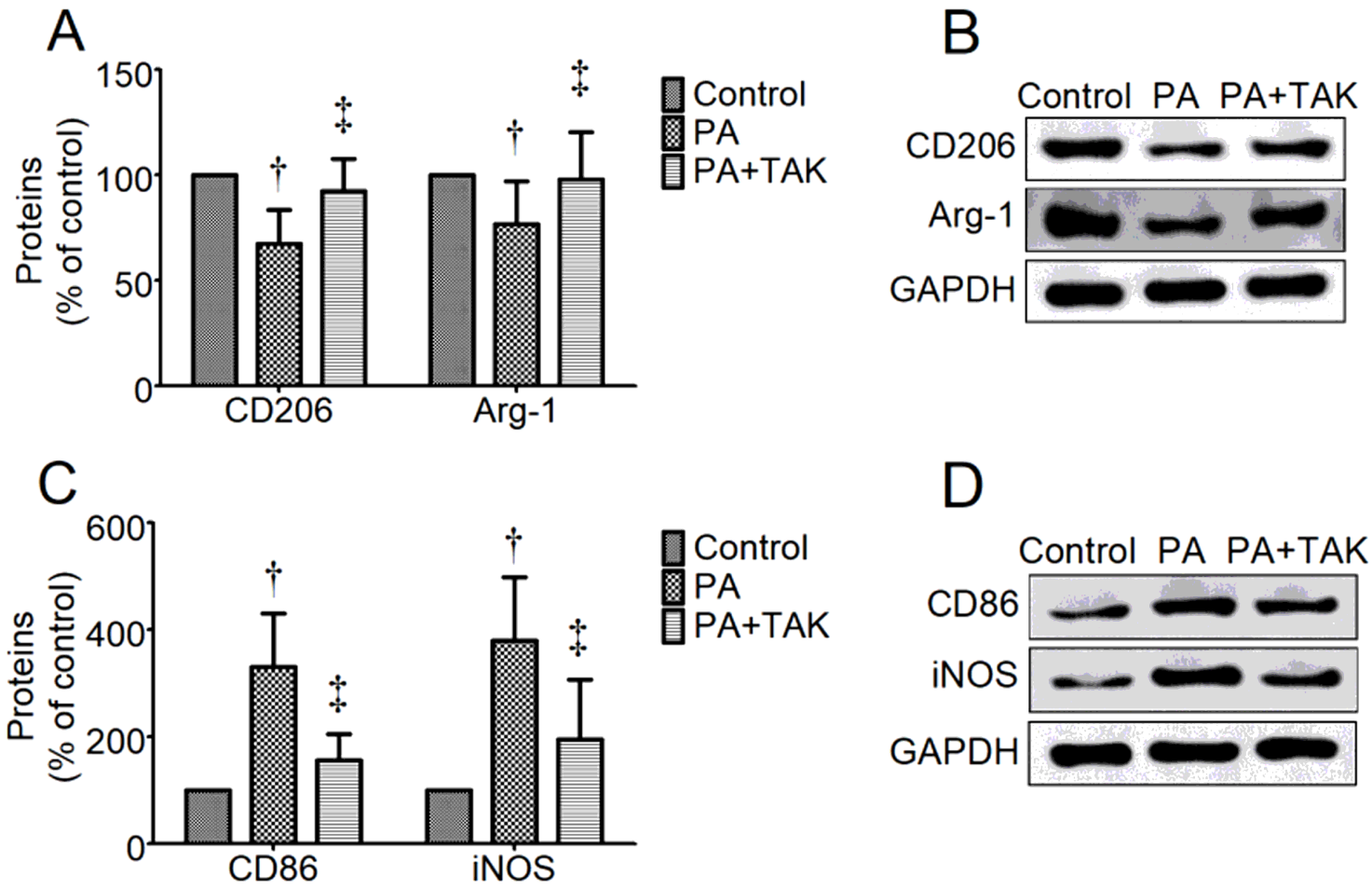

Figure 6

Effect of TLR4 on microglial M2 polarization in BV2 cells PA was adopted to establish an in vitro model of high-fat. Meanwhile, the BV-2 cells were treated with TAK-242 to regulate the expression of TLR4. After that, expression of CD206, Arg-1, CD86 and iNOS proteins was determined using western blotting. Pyroptosis rate was determined using flow cytometry. Difference of more than two variables was measured using ANOVA with LSD test. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group. “†” indicated P 0.05 compared to control group; “‡” indicated P 0.05 compared to PA group.

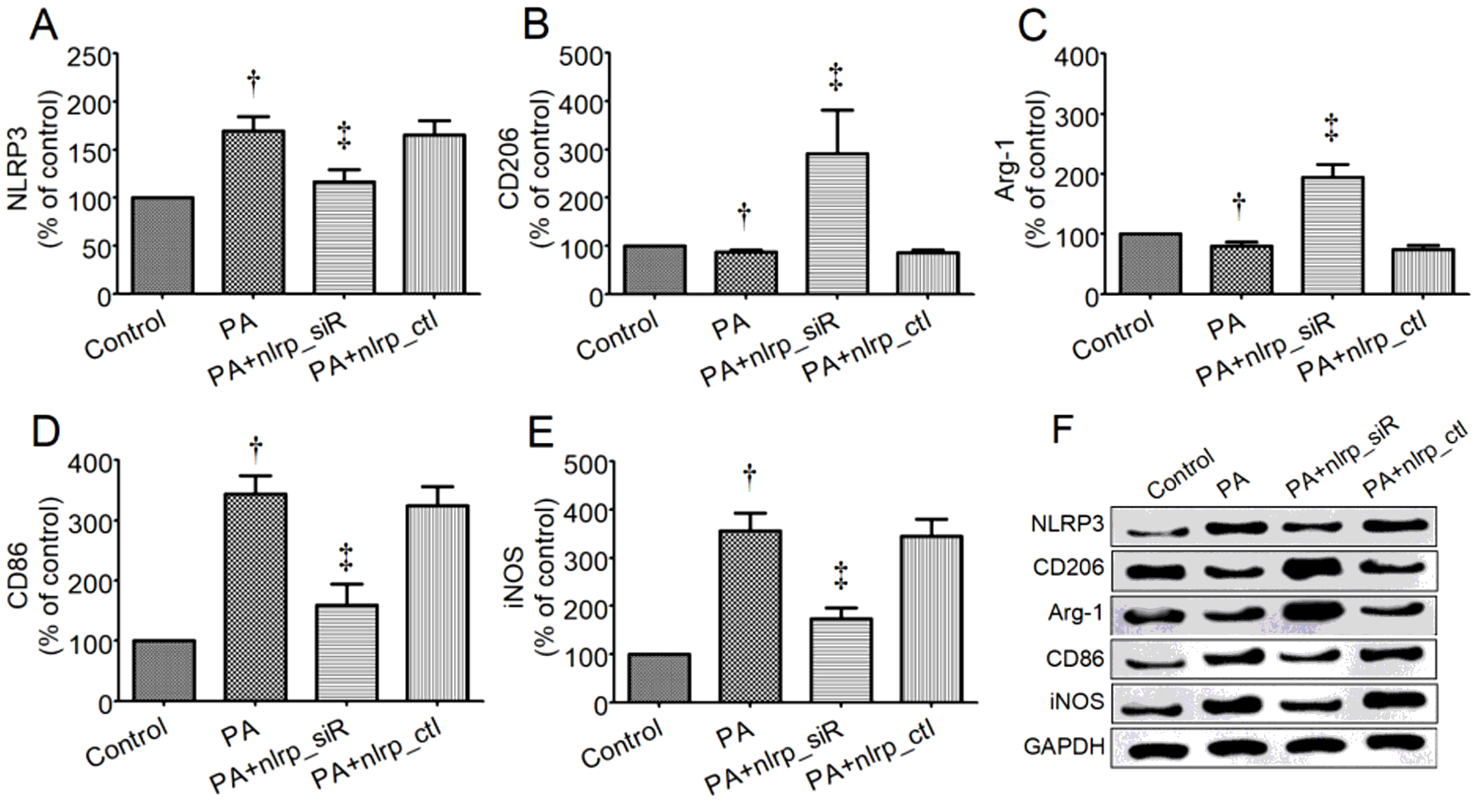

Figure 7

Relationship between NLRP3 inflammasome activation and microglial M2 polarization in BV2 cells Prepared BV-2 cells were transfected with NLRP3 siRNA to down-regulate the expression of NLRP3. Then, PA was adopted to establish an in vitro model of high-fat. After that, expression of NLRP3, CD206, Arg-1, CD86 and iNOS proteins was determined using western blotting. Difference of more than two variables was measured using ANOVA with LSD test. P□0.05 indicated statistically significant. “†” indicated P □ 0.05 compared to control group; “‡” indicated P □ 0.05 compared to PA group.