

# PACAP Improves Macrophage M1 Polarization and Inflammatory Response by Regulating NF- $\kappa$ B and MAPKs Pathways

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**Abstract:** Inflammation is an important defense mechanism evolved by the host to defend against external threats. But an excessive inflammatory response can lead to a variety of inflammatory diseases. Macrophage M1 polarization is a key factor in inducing inflammatory response, and inhibiting macrophage M1 polarization is of great significance for regulating inflammatory diseases. PACAP is an anti-inflammatory neuropeptide, but its mechanism of action on macrophage polarization and inflammatory responses is unclear. The macrophage M1 polarization and inflammation model was constructed using lipopolysaccharide (LPS), and then treated with PACAP. The results showed that PACAP significantly inhibited the LPS-induced expression of M1 polarization marker genes and inflammatory factors in macrophages, and down-regulated the phosphorylation of IKK2/I $\kappa$ B $\alpha$ /NF- $\kappa$ B, while PACAP also inhibited the phosphorylation of JNK/p38. Thus, PACAP improved LPS-induced macrophage M1 polarization and inflammatory response by regulating NF- $\kappa$ B and MAPKs signaling pathways.

**Keywords:** PACAP, Macrophage M1 polarization, NF- $\kappa$ B, MAPKs.

## 1. Introduction

Inflammation is an adaptive defense mechanism evolved by the body [1]. Inflammatory signals such as endotoxin activate immune cells, initiate immune responses, and maintain system homeostasis [2, 3]. However, excessive inflammatory response will damage the structure and function of tissues, gradually form chronic inflammation characterized by macrophage infiltration, and further lead to various inflammatory diseases such as obesity [4]. Lipopolysaccharide (LPS) is a classic bacterial endotoxin and an efficient inducer of M1 polarization of macrophages [5], which can induce the body to produce a non-specific immune response, synthesize a large number of inflammatory mediators, and trigger a systemic inflammatory response. LPS can activate TLR4 on the surface of macrophages and promote NF- $\kappa$ B signaling [6, 7]. In the NF- $\kappa$ B signaling pathway, the IKK complex can regulate the activity of downstream NF- $\kappa$ B inhibitory molecules (Inhibitor of NF- $\kappa$ B, I $\kappa$ B) [8, 9]. In the non-stimulated state, NF- $\kappa$ B binds to I $\kappa$ B in an inactive form and binds to the cytoplasm; after stimulation, I $\kappa$ B is phosphorylated and degraded through the proteasome pathway, releasing the inhibitory effect on NF- $\kappa$ B, NF- $\kappa$ B p65/p50 heterodimer translocates into the nucleus and binds to the corresponding site of the target gene DNA, regulates the mRNA transcription of the target gene, induces the expression of pro-inflammatory factors, and then triggers the M1 polarization of macrophages [10, 11]. In addition, TLR4 also mediates the activation of downstream mitogen-activated protein kinase (MAPK) family (JNK, p38, etc.) [12]. Activated JNK and p38 can mediate the activity regulation of the downstream key transcription factor Activating protein-1 (AP-1) [13, 14], which induces the expression of various pro-inflammatory factors at the gene level and promotes M1-type

polarization of macrophages [15]. This suggests that targeting NF- $\kappa$ B/MAPKs may improve macrophage-mediated inflammatory responses.

Most of the anti-inflammatory drugs currently in use have obvious side effects, which may lead to general gastrointestinal toxicity and increase the risk of cardiovascular disease [16, 17]. Many drugs have been reduced or even banned from clinical use due to obvious toxic side effects [18–21]. The quantity and safety of anti-inflammatory drugs are still limited, so it is necessary to develop more safe and effective new anti-inflammatory drugs. PACAP, a neuropeptide isolated from the ovine hypothalamus [22], has general anti-inflammatory activity, but the molecular mechanism of its inhibition of macrophage M1 polarization is unclear. Therefore, this study verifies the anti-inflammatory effect of PACAP, clarifies the mechanism of PACAP in improving lipopolysaccharide-induced macrophage M1 polarization and inflammatory response, and provides new ideas for the development of new anti-inflammatory drugs.

## 2. Materials and Methods

### 2.1. Cell Culture

Mouse RAW264.7 cells were obtained from Wuhan Cell Bank and cultured in Dulbecco's modified Eagle's medium (Thermo, MA, USA) containing 10% fetal bovine serum (Thermo, MA, USA), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and 2 mM L-glutamine. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### 2.2. Cell Viability Assay

The effect of PACAP on the viability of RAW264.7 cells was evaluated by CCK-8 method. The cells were seeded in a

96-well plate at a density of  $8 \times 10^3$  cells/well, treated with different concentrations of PACAP for 24 h, and the culture medium was discarded, and 100  $\mu$ l of working solution (containing 10  $\mu$ l of CCK-8 reagent) was added to each well. After incubation at 37°C for 60-120 min, the absorbance at 450 nm was detected with a microplate reader (BIO-RAD, CA, USA).

### 2.3. NO Assay

The operation was carried out according to the protocol of Biyuntian Total Nitric Oxide Detection Kit (Biyuntian, Shanghai, China).

### 2.4. RNA Extraction and Real-time PCR

RNAiso Plus (TaKaRa, Kyoto, Japan), PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan) were used to extract and reverse transcribe total mRNA in RAW264.7 cells. and RT-qPCR was performed. The primer sequences for each gene are shown below: Arg1: Forward 5-CCACAGTCTGGCAGTTGGAAG-3 and Reverse 5-GGTTGTCAGGGGAGTGTGATG-3; Fizz1: Forward 5-CCTGCTGGGATGACTGCTA-3 and Reverse 5-TGGTTCTCCACCTCTTCAT-3; TNF- $\alpha$ : Forward 5-AATGGCCTCCCTCTCATCAGTT-3 and Reverse 5-CCACTTGGTGGTTTGCTACGA-3; NOS-2: Forward 5-AATCTTGGAGCGAGTTGTGG-3 and Reverse 5-CAGGAAGTAGGTGAGGGCTTG-3;  $\beta$ -actin: Forward 5-TGTCCACCTTCCAGCAGATGT and Reverse 5-AGCTCAGTAACAGTCCGCCTAGA-3. A real-time PCR machine was used and data were normalized with  $\beta$ -actin according to the  $2^{-\Delta\Delta t}$  method.

### 2.5. Total Protein Extraction and Western Blot

$2 \times 10^6$  cells were lysed in RIPA lysis buffer (Biyuntian, Shanghai, China) containing protease inhibitors and phosphatase inhibitors. Proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Blocked in

blocking solution containing 5% bovine serum albumin and 1% Tween-20 for 2 h at room temperature, incubated with primary antibody diluted in 2% TBST at 4°C overnight, and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Blots were visualized by ECL chemiluminescent solution (Yeasen, Shanghai, China). Blot densities were quantified with the software ImageJ, normalized to  $\beta$ -actin. The primary antibodies used in this study include: anti-IL-6, anti-MCP-1, anti-IL-1 $\beta$ , anti-TNF- $\alpha$ , anti-TLR4, anti-IKK2, anti-p-IKK2, anti-I $\kappa$ B $\alpha$ , anti-p-I $\kappa$ B $\alpha$ , anti-p-NF- $\kappa$ B, anti-JNK, anti-p-JNK, anti-p38, anti-p-p38, anti- $\beta$ -actin were obtained from CST and Santa cruz.

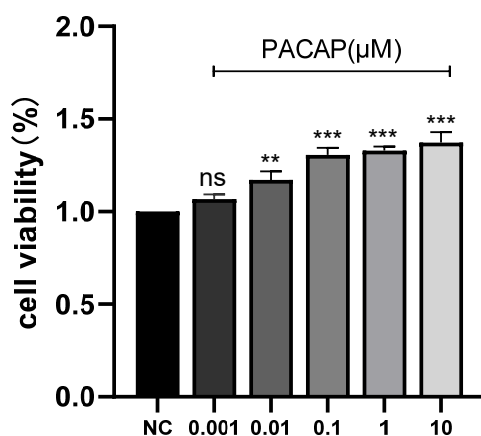
### 2.6. Statistics

All data were statistically analyzed by GraphPad Prism 8 software, and the results are expressed as mean  $\pm$  SEM. Data analysis was performed using one-way ANOVA and independent samples t-test. Differences were considered statistically significant when  $P < 0.05$ .

## 3. Results

### 3.1. PACAP Does Not Impair the Viability of Mouse RAW264.7 Cells

PACAP is a multifunctional neuropeptide with broad anti-inflammatory effects. Firstly, the effect of different concentrations of PACAP on the viability of RAW264.7 cells was detected by CCK-8 method. The results showed that at the concentration of 0.01  $\mu$ M, the cell viability was 117%, which was significantly different from the control group (Fig. 1) ( $P < 0.05$ ). With the increase of PACAP concentration, the viability of macrophages increased significantly compared with the normal control group ( $P < 0.001$ ), suggesting that PACAP does not produce cytotoxicity and has a significant effect on promoting cell proliferation in the concentration range of 0.01-10  $\mu$ M.



**Figure 1.** Cytotoxicity of different concentrations of PACAP on RAW264.7 cells. RAW264.7 cells were treated with 0.001-10  $\mu$ M PACAP for 24 h, and cell viability was determined by CCK-8 method. Normal control. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NC group vs different concentrations of PACAP treatment groups. ns, no significant difference. Data are presented as mean  $\pm$  SEM.

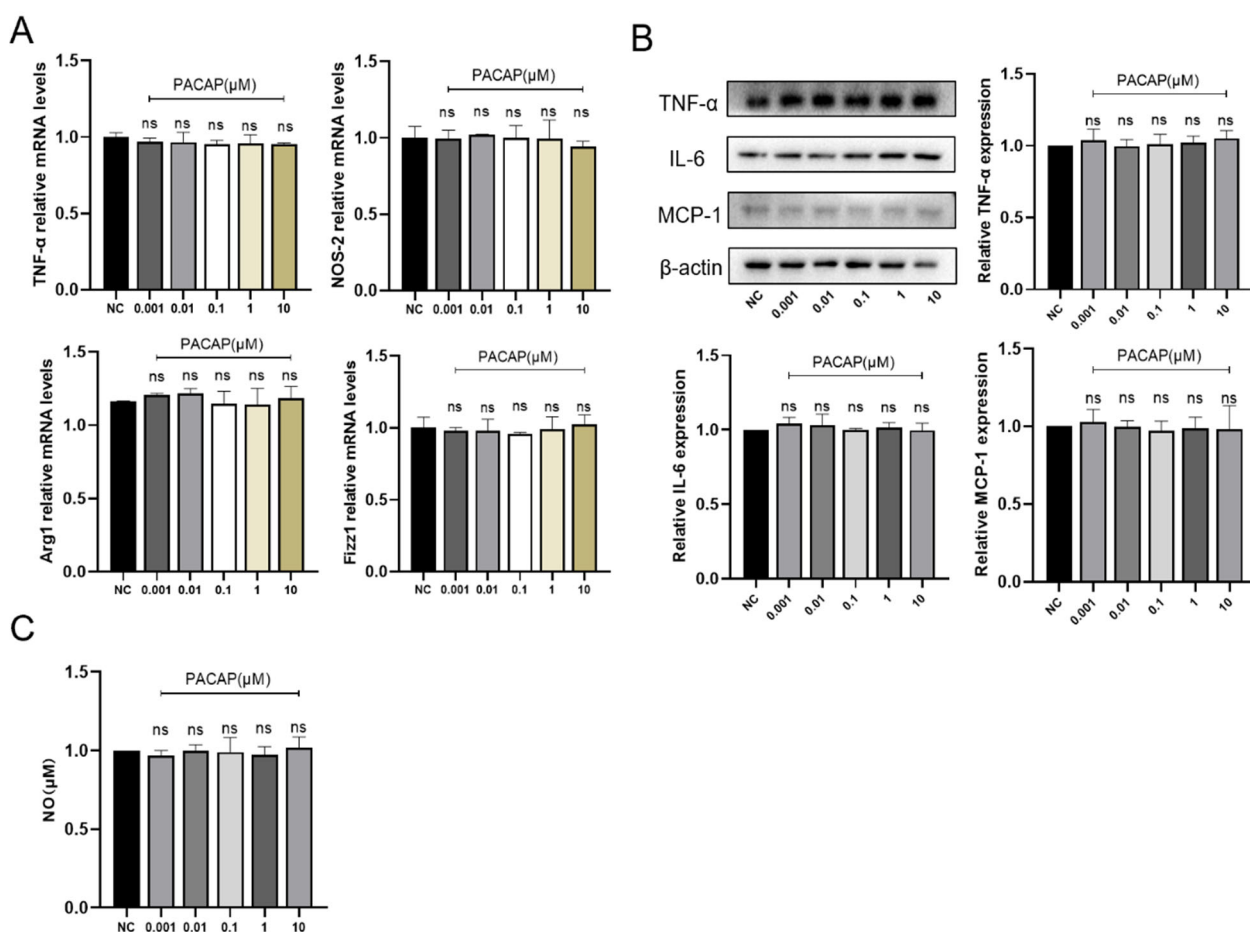
### 3.2. PACAP Does Not Directly Cause RAW264.7 Macrophage Polarization

In order to explore whether PACAP can directly induce M1 or M2 polarization of RAW264.7 cells, after adding different concentrations of PACAP for 24 h, the transcription levels of

M1 and M2 marker genes in macrophages were detected by RT-qPCR (Fig. 2A). The effect of PACAP on the expression of inflammatory factors was detected by Western Blot (Fig. 2B) and NO content in cell culture medium (Fig. 2C). The results showed that compared with the control group, there were no significant differences in the transcription levels of

M1 and M2 marker genes, the expression of inflammatory factors and the NO content in the cell culture medium of macrophages treated with different concentrations of PACAP.

It indicated that the M1 or M2 polarization did not occur after PACAP directly acted on RAW264.7 cells.



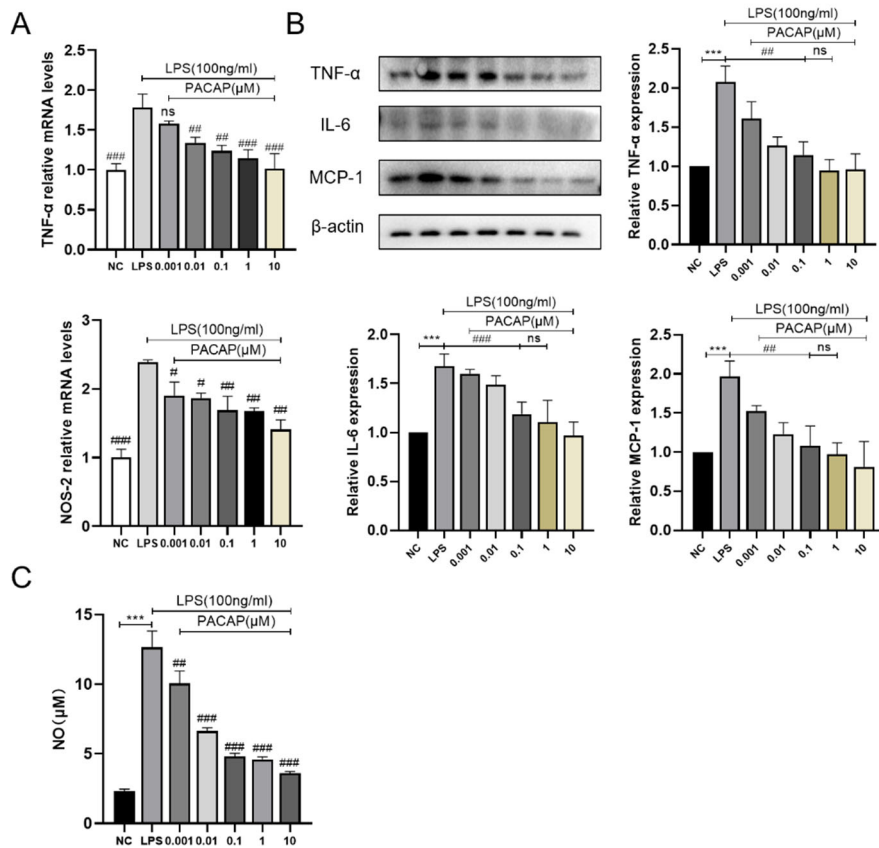
**Figure 2.** PACAP does not directly cause RAW264.7 macrophage polarization. RAW264.7 cells were directly incubated with 0.001-10 $\mu$ M PACAP for 24h and (A) M1 and M2 marker gene transcription levels were detected by RT-qPCR. (B) the expression of inflammatory factors were detected by Western Blot. (C) NO content in cell culture medium was detected. NC group vs different concentrations of PACAP treatment groups. Data are presented as mean  $\pm$  SEM.

### 3.3. PACAP Improves LPS-induced M1 Polarization and Inflammatory Mediator Expression in RAW264.7 Cells

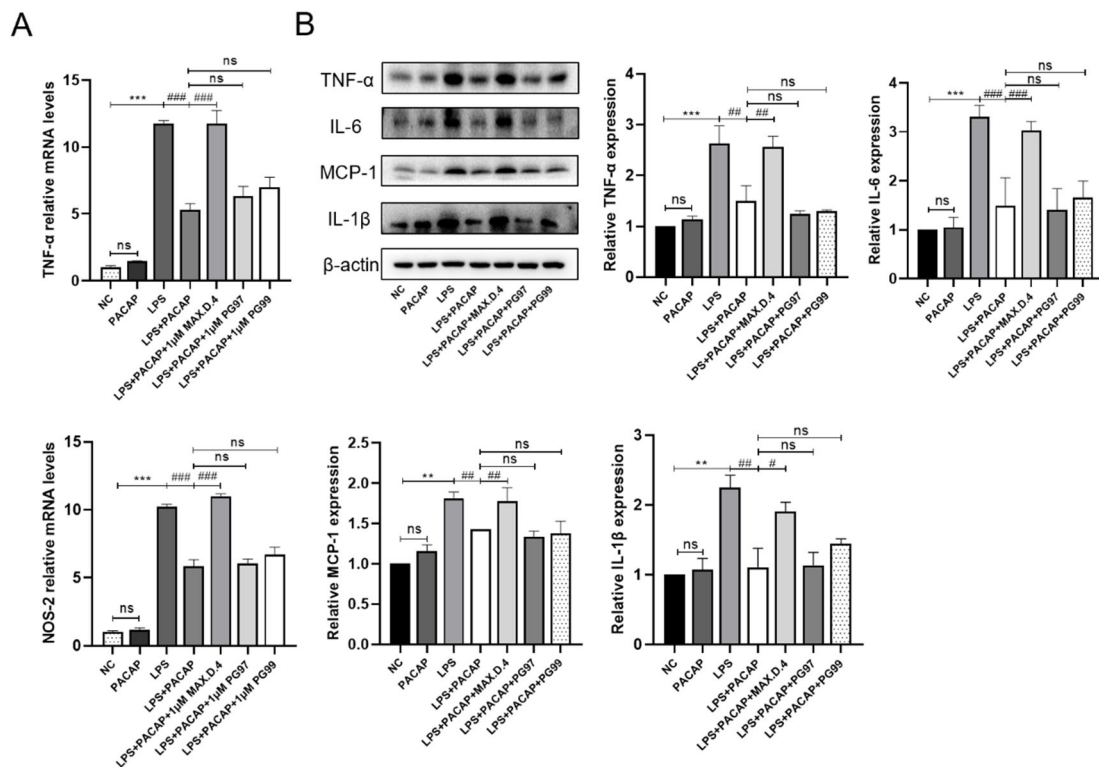
To explore the effect of PACAP on macrophage M1 polarization, LPS was used to construct a model of macrophage M1 polarization and inflammation. The effects of 0.001-10  $\mu$ M PACAP on the transcription of M1 polarization marker genes TNF- $\alpha$  and NOS-2 in RAW264.7 cells were detected by RT-qPCR (Figure 3A), and Western blot was used to detect the expression levels of intracellular inflammatory factors such as TNF- $\alpha$ , IL-6, and MCP-1 (Fig. 3B) and NO content in cell cultures (Fig. 3C). The results showed that, at the concentration of 0.1  $\mu$ M, the inhibitory effect of PACAP on M1 marker gene and inflammatory mediators such as TNF- $\alpha$ , IL-6, MCP-1, and NO was significantly different from that of the modeling group, and there were no significant differences between 0.1  $\mu$ M PACAP treatment group and 1  $\mu$ M PACAP treatment group. Therefore 0.1  $\mu$ M was chosen as the PACAP concentration for subsequent experiments.

### 3.4. PACAP Inhibits Macrophage M1 Polarization and Inflammatory Response by Specifically Activating PAC1 Receptors

PACAP has three receptors, PAC1, VPAC1 and VPAC2. The type of receptors in which PACAP exerts its anti-inflammatory effect is determined by using receptor-specific inhibitors. After LPS stimulation of cells, 1  $\mu$ M PG97-269, 1  $\mu$ M PG99-465, and 1  $\mu$ M MAX.D4 were added to RAW264.7 cells for pre-incubation for 30 min, and then 0.1  $\mu$ M PACAP was added to continue the culture for 24 h, the transcription levels of M1 polarization marker genes TNF- $\alpha$  and NOS-2 in RAW264.7 cells were detected by RT-qPCR (Fig 4A), and Western blot was used to detect the expression levels of intracellular inflammatory factors such as TNF- $\alpha$ , IL-6, MCP-1, and IL-1 $\beta$  (Figure 4B). The results showed that PACAP could not inhibit the M1 polarization and inflammatory response in RAW264.7 cells after adding PAC1 receptor specific inhibitor MAX.D4, indicating that PACAP exerts anti-inflammatory effect through PAC1 receptor in RAW264.7 cells.



**Figure 3.** PACAP inhibited LPS-induced M1 polarization and inflammatory mediator expression in RAW264.7 cells. After LPS stimulation 12 h, 0.001-10 μM PACAP was treated for 24h. (A) M1 and M2 marker gene transcription levels were detected by RT-qPCR. (B) the expression of inflammatory factors were detected by Western Blot. (C) NO content in cell culture medium was detected.  $^{\#}P<0.05$ ,  $^{\#\#}P<0.01$ ,  $^{\#\#\#}P<0.001$ , LPS treatment group vs different concentrations of PACAP treatment groups Data are presented as mean  $\pm$  SEM.

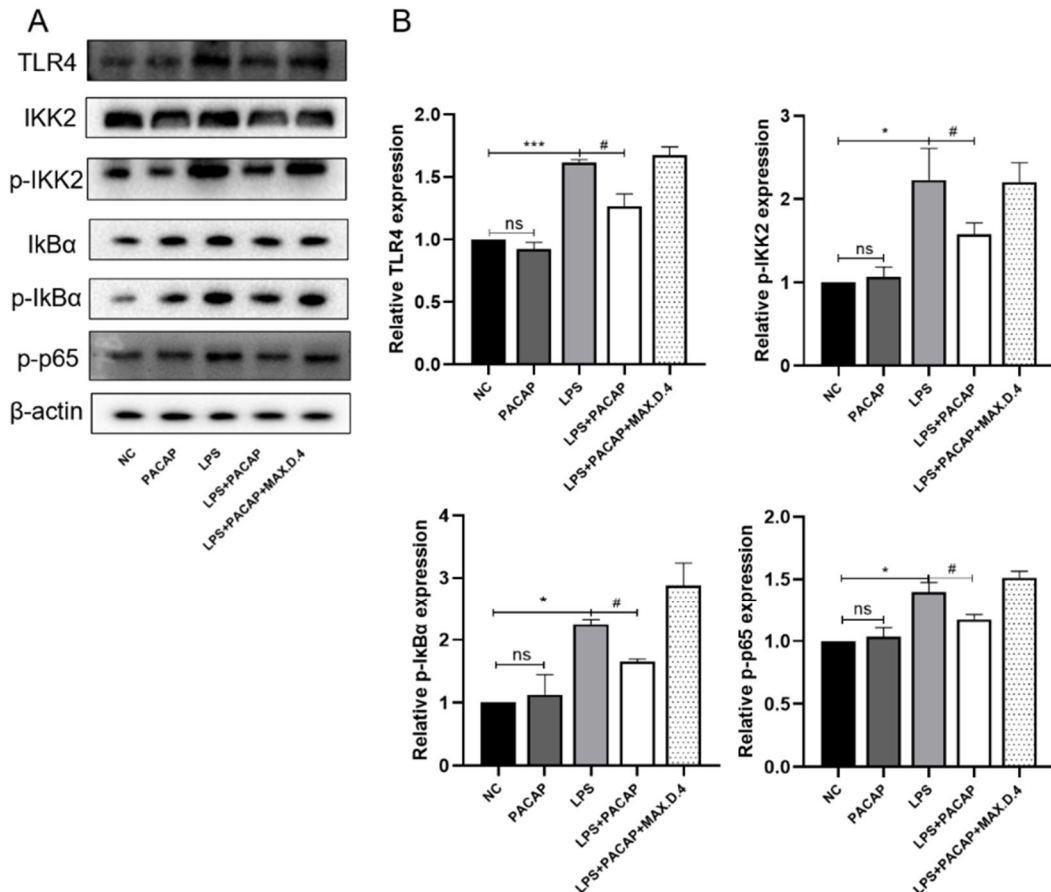


**Figure 4.** PACAP inhibits macrophage M1 polarization and inflammatory response by specifically activating PAC1 receptors. After LPS stimulated cells 12 h, receptor-specific inhibitors were added for pre-incubation for 30 min, and then 0.1 μM PACAP was added to continue to culture for 24 h. (A) M1 and M2 marker gene transcription levels were detected by RT-qPCR. (B) the expression of inflammatory factors were detected by Western Blot.  $^{**}P<0.01$ ,  $^{***}P<0.001$ , NC vs LPS;  $^{\#\#}P<0.01$ ,  $^{\#\#\#}P<0.001$ , LPS+PACAP vs LPS or LPS+PACAP+MAX.D.4. Data are presented as mean  $\pm$  SEM.

### 3.5. PACAP Inhibits NF- $\kappa$ B Signaling Pathway in RAW264.7 Cells

The NF- $\kappa$ B signaling pathway is one of the classic pathways activated by LPS and plays an important role in mediating inflammatory responses, so we further investigated whether PACAP exerts anti-inflammatory activity by regulating NF- $\kappa$ B signaling. Studies have shown that NF- $\kappa$ B activity is regulated by the upstream IKK complex and I $\kappa$ B $\alpha$ . When the inflammatory reaction occurs, the IKK complex activates and phosphorylates I $\kappa$ B $\alpha$ , which relieves the inhibition of NF- $\kappa$ B, and the phosphorylation of NF- $\kappa$ B p65

is incorporated into the nucleus to initiate the transcription of target genes. Therefore, we detected the phosphorylation levels of IKK2, I $\kappa$ B $\alpha$ , and NF- $\kappa$ B, and the results showed that the phosphorylation of IKK2, I $\kappa$ B $\alpha$ , and NF- $\kappa$ B was enhanced after LPS stimulation, and PACAP significantly reduced the phosphorylation levels of IKK2, I $\kappa$ B $\alpha$ , and NF- $\kappa$ B, suggesting that the anti-inflammatory activity of PACAP depends on the regulation of NF- $\kappa$ B signaling pathway (Fig 5). PACAP may improve LPS-induced inflammatory response by regulating TLR4/IKK2/I $\kappa$ B $\alpha$ /NF- $\kappa$ B signaling pathway.

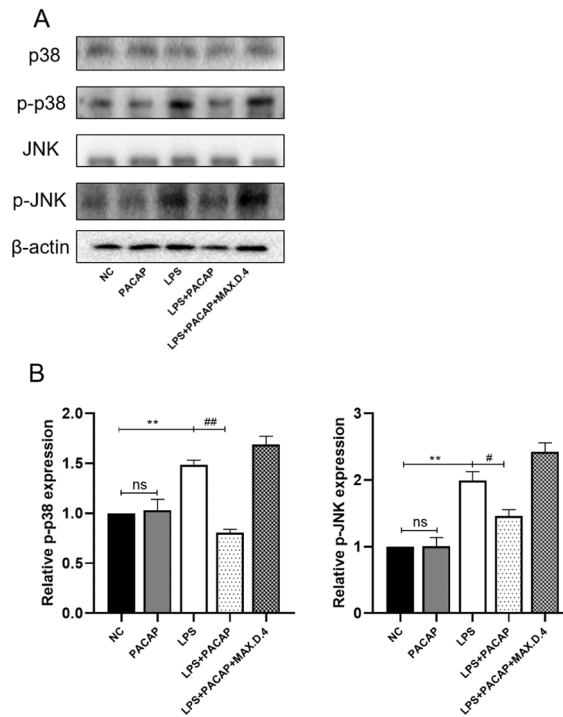


**Figure 5.** PACAP inhibited LPS-induced NF- $\kappa$ B signaling pathway. After LPS stimulation 12 h, the protein expressions of IKK2/p-IKK2, I $\kappa$ B $\alpha$ /p-I $\kappa$ B $\alpha$ , and p-p65 were detected by Western blot. (A) Western blot, (B) Blot quantitative analysis. Normalized by relative intensities of  $\beta$ -actin. \*\* $P$ <0.01, \*\*\* $P$ <0.001, NC vs LPS; # $P$ <0.01, ### $P$ <0.001, LPS+PACAP vs LPS or LPS+PACAP+MAX.D.4. Data are presented as mean  $\pm$  SEM.

### 3.6. PACAP Inhibits the Activation of MAPKs Components in RAW264.7 Cells

Similarly, mitogen-activated protein kinase family members are also involved in inflammatory responses. We evaluated the effect of PACAP on LPS-induced activity of MAPKs in RAW264.7 cells. As shown in the figure, the

phosphorylation levels of p38 and JNK in RAW264.7 cells were significantly increased after LPS treatment, and the phosphorylation of JNK and p38 was down-regulated after PACAP treatment, but the phosphorylation of ERK was not down-regulated (data not shown). It is suggested that PACAP exerts anti-inflammatory activity to a certain extent by selectively inhibiting the phosphorylation of JNK and p38.

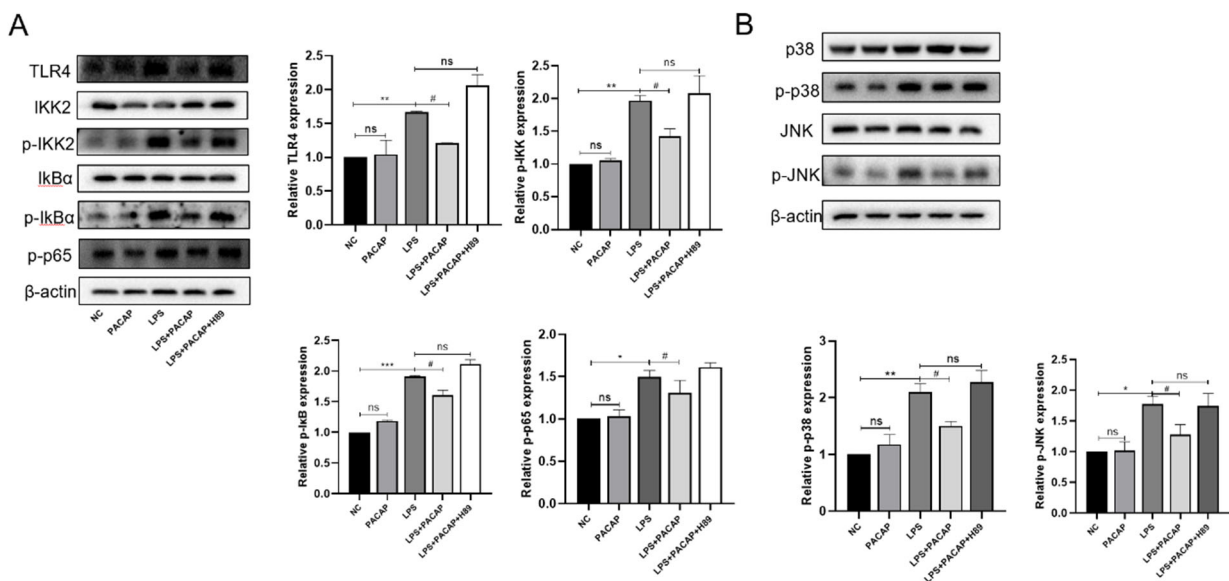


**Figure 6.** PACAP inhibits the phosphorylation of JNK and p38 in RAW264.7 cells. After LPS stimulation 12 h, Western blot was used to detect the expression of p38/p-p38 and JNK/p-JNK. **(A)** Western blot, **(B)** Western blot quantitative analysis. Normalized by relative intensities of  $\beta$ -actin. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NC vs LPS; ## $P < 0.01$ , ### $P < 0.001$ , LPS+PACAP vs LPS or LPS+PACAP+MAX.D4. Data are presented as mean  $\pm$  SEM.

### 3.7. PACAP Mediates Anti-inflammatory Effects Through the PAC1/cAMP/PKA Pathway

PACAP can exert different biological effects by activating different signal transduction pathways. Therefore, in order to further clarify the mechanism of PACAP inhibiting the LPS-induced NF- $\kappa$ B and MAPKs pathways, RAW264.7 cells were

treated with 0.1  $\mu$ g/ml LPS for 12 h, and the cells were preincubated with 30  $\mu$ M H89 (cAMP/PKA pathway specific inhibitor) for 30 minutes. As shown, the inhibitory effect of PACAP on LPS-induced TLR4 expression and phosphorylation of IKK2, I $\kappa$ B $\alpha$ , p65, JNK, and p38 was counteracted by the addition of H89 (Fig 7). It indicated that PACAP inhibited the M1 polarization of macrophages and improved the inflammatory response through the PAC1/cAMP/PKA pathway.



**Figure 7.** PACAP mediates anti-inflammatory effects through the PAC1/cAMP/PKA pathway. After H89 preincubation, Western blot was used to detect the phosphorylation levels of NF- $\kappa$ B and MAPKs pathway proteins. **(A)** NF- $\kappa$ B pathway protein bands and their blotting quantitative analysis **(B)** MAPK pathway protein (JNK, p38) bands and their blotting quantitative analysis. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NC vs LPS; ## $P < 0.01$ , ### $P < 0.001$ , LPS+PACAP vs LPS. Data are presented as mean  $\pm$  SEM.

## 4. Discussion

Inflammation is an important defense mechanism for the host to resist pathogens, but excessive inflammatory responses can cause damage to tissue structure and function, gradually trigger chronic inflammation, and eventually develop into inflammatory diseases [2]. The M1 polarization of macrophages is an important cause of many inflammatory diseases, so inhibiting the M1 polarization of macrophages and reducing the inflammatory response is a potential strategy for the treatment of inflammatory diseases. LPS can induce M1 polarization of macrophages, up-regulate the expression of inflammatory mediators such as TNF- $\alpha$ , IL-6, NO, etc., triggering a significant inflammatory response [23], and pro-inflammatory mediators can further promote TLR4 signaling and aggravate the inflammatory response .

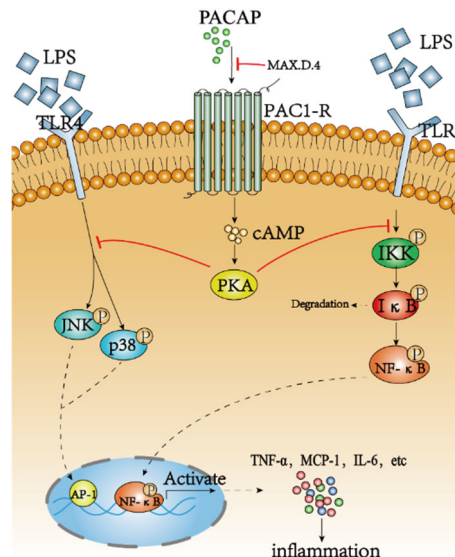
Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with good anti-inflammatory effect, widely expressed in mammals, and its amino acid sequence is still highly conserved in long-term evolution [24–26]. The previous study of our group showed that exogenous administration of PACAP could inhibit the inflammatory response in mouse GC-2 cells induced by palmitic acid (PA), and showed a significant inhibitory effect on inflammatory factors such as TNF- $\alpha$  and IL-6, improved the reproductive impairment of obese mice; in addition, PACAP could improve insulin resistance and reduce the levels of inflammatory factors in mouse tissue in obese mice fed with high-fat diet. Our study found that 0.1  $\mu$ M PACAP treatment can significantly inhibit the transcription level of LPS-induced macrophage M1 polarization markers TNF- $\alpha$  and NOS2 gene, significantly down-regulate the expression of inflammatory factors such as IL-6, IL-1 $\beta$ , MCP-1, and TNF- $\alpha$  (Fig 3).The above results were consistent with the ones our group previously found, confirming that PACAP has significant anti-inflammatory activity.

Then, the mechanism of PACAP in inhibiting M1 polarization and inflammatory response was further explored. PACAP receptor-specific inhibitors were used to block receptor function to identify the type of receptor that PACAP exerts anti-inflammatory effects in RAW264.7 cells. The experimental results showed that after the addition of

MAX.D4, the PAC1 receptor was specifically bound, so that PACAP could no longer bind to the receptor, blocking the signal transduction of PACAP, its inhibitory effect on LPS-induced expression of inflammatory genes and inflammatory factors was abolished(Fig 4B). This suggests that PACAP inhibits M1 polarization of macrophages by specifically activating PAC1 receptors and improves the inflammatory response.

Studies have shown that the activation of NF- $\kappa$ B and MAPKs pathways are both key events in the inflammatory response, and they play a key role in the regulation of macrophage behavior and amplifying inflammatory signals [27]. Interfering with NF- $\kappa$ B and MAPKs pathways can improve inflammation to varying degrees [28, 29]. Therefore, we further explored the effect of PACAP on the signaling of NF- $\kappa$ B and MAPKs pathways in the inflammatory response. The results showed that PACAP could significantly down-regulate the expression of TLR4 and the phosphorylation of IKK2, I $\kappa$ B $\alpha$ , and NF- $\kappa$ B (Fig. 5A). Similarly, studies have shown that the activation of IKK and NF- $\kappa$ B in obese mice is inhibited, and tissue inflammation is improved when salicylic acid is administered exogenously [30]. The phosphorylation of JNK, p38 and ERK was strongly induced by LPS in RAW264.7 cells, which was consistent with the mainstream research results. PACAP can inhibit the phosphorylation of JNK and p38, but has no inhibitory effect on the phosphorylation of ERK, which is consistent with the results of Dziarski [31]. It indicated that PACAP exerts anti-inflammatory effect by selectively inhibiting JNK and p38.

PACAP can trigger a dramatic increase in cAMP levels. Studies have shown that the increase of cAMP level has an important effect on regulating the expression of iNOS and its product NO in mouse macrophages [32, 33]. Therefore, in order to clarify the pathway by which the downstream components of PACAP inhibit NF- $\kappa$ B and MAPKs, we pretreated the cells with the cAMP/PKA-specific pathway inhibitor H89. The results showed that the inhibitory effect of PACAP on NF- $\kappa$ B and MAPKs signaling pathway was affected Attenuated, indicating that PACAP acts on RAW264.7 macrophages by activating the PAC1/cAMP/PKA pathway to inhibit the NF- $\kappa$ B and MAPKs pathways, thereby inhibiting M1 polarization and inflammation.



**Fig 8** PACAP inhibiting macrophage M1 polarization and inflammatory response by regulating the NF- $\kappa$ B and MAPKs signaling pathways.

## 5. Conclusion

Previously, the anti-inflammatory ability of PACAP was mostly reported in hepatocytes, nerve cells and other cells, and the anti-inflammatory effect and mechanism of PACAP in LPS-induced RAW264.7 cells were not clear. In this study, we report the anti-inflammatory effect and possible mechanism of PACAP on LPS-induced M1 polarization and inflammatory response in RAW264.7 cells. As a highly safe neuropeptide, PACAP can target key molecules of the inflammatory pathway, regulate macrophage M1 polarization and inflammatory response, and provide an effective strategy for the treatment of inflammatory diseases and the development of new multi-targeted anti-inflammatory drugs.

## Acknowledgment

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