M-acetylcholine Receptors Regulate Inflammatory Responses Through *Arginases 1/2* in Zebrafish

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Abstract

Muscarinic acetylcholine receptors (mAChRs) are widely expressed in parasympathetic effector cells and have been proved to play vital roles in synaptic transmission, smooth muscle contraction, digestive secretion. However, there are relatively few literatures revealing the roles of mAChRs in inflammatory process, and its underlying regulatory mechanisms have not been elucidated. Taking the advantages of live imaging of zebrafish, we found that inhibition of mAChRs resulted in increased neutrophils recruitment and proinflammatory cytokines expression, whereas activation of mAChRs led to opposite outcome. Subsequently, we found that mAChRs regulated the expression of arginases (*args*), and pharmacological intervention of *args* level could reverse the effects of mAChRs on neutrophils migration and cytokines expression, suggesting that *args* are important downstream proteins of mAChRs that mediate its regulation of inflammatory response. In this study, we identified Args as novel downstream proteins of mAChRs in the inflammatory process, provided additional evidence for peripheral immune regulation of cholinergic receptors.

1. Introduction

Neural activity has been shown to dominate multiple central and peripheral inflammatory responses and is essential for maintaining immune homeostasis [1, 2]. As an important neurotransmitter in the neuronal system, acetylcholine (Ach) is synthesized from choline and acetyl-CoA by choline acetyl transferase (ChAT), then is transported and released by vesicular acetylcholine transporter (VAChT) to bind to acetylcholine receptors (AChRs) to transmit neural signals [3]. AChRs have been identified to be expressed in both neuronal and non-neuronal cells, mainly including muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs) [4, 5].

The mAChRs, composed of five main subtypes, M1, M2, M3, M4, and M5, are widely expressed in effector cells innervated by parasympathetic ganglionic fibers, including pancreatic cells [6], keratinocytes [7], intestinal epithelium [8] and most immune cells, such as T cells, B cells and monocytes [9]. These finding suggested that non-neuronal cholinergic systems were involved in the regulation of immune cell function [10]. A few studies have shown that compared with wild type, the expression of pro-inflammatory cytokines such as tumor necrosis factor (*Tnf-α*), *Ifn-γ* and *Il-6* in splenocytes in mAChRs M1/M5 knockout mice were significantly changed [11, 12] and more antigen-specific antibodies were produced in M1/M5 mutant group [12]. Based on static or *in vitro* experiments, these studies suggested that mAChRs could regulate immune responses at the cytokines level, but roles of mAChRs in the recruitment of immune cells, especially neutrophils, have rarely been reported, and its potential regulatory mechanisms were not been elucidated. Neutrophils are the most important member type of innate immune cells, accounting for about 40%-60% of the total number of leukocytes [13, 14], which is essential for regulating the inflammatory responses [15, 16]. As a model animal, the optical transparency of juveniles makes zebrafish an ideal model for *in vivo* imaging to study the neutrophils [17, 18]. Hence, this study intends to explore the effects of mAChRs on the recruitment of neutrophils and also the expression of cytokines in zebrafish.

There are two subtypes of arginases, arginase 1 (Arg1) and arginase 2 (Arg2), which are mainly distributed in organs such as liver, kidney and testes [19]. Recent studies also showed that Arg1 and Arg2 were widely expressed in immune cells [20, 21] and found that the expression of Arg1 could be induced by inflammatory signals including interferons and transforming growth factor-beta (Tgf-β) [19]. Given the role of Args in inflammatory response, we speculated that Args might be involved in the regulation of mAChRs on inflammation.

In this study, we used pharmacological methods to inhibit and activate mAChRs, and found that mAChRs could regulate neutrophils recruitment and cytokines expression using *in vivo* imaging in zebrafish. We further proved that Args, as a downstream protein of mAChRs, could participate in the regulation of inflammatory response in zebrafish. Here, we used zebrafish to provide additional evidence that cholinergic receptors regulate peripheral immune responses, deepening the link between nervous system and immune functions.

2. Materials And Methods

2.1 Experimental animals

Feeding and mating were carried out according to the operating rules of zebrafish BOOK [22]. Wild-type (WT) and transgenic Tg (*lyz*:EGFP) zebrafish embryos were cultured with Hank's solution at a constant temperature of 28.5 ℃, pH of 6.5–7.5, and a day-night light cycle of 14H: 10H environment (light at 8:30 in the morning and darkness at 10:30 at night) [23]. We strictly followed the guidelines and regulations of the Animal Resource Center of Anhui Agricultural University (SYXK (Anhui) 2016-007). At the same time, all protocols conformed to the guidelines of the China National Institute for Food and Drug Control for laboratory animals.

2.2 Pharmacological treatment

In the experiment, atropine (HY-B1205, MCE) and bethanechol (HY-B0406, MCE) were used to antagonize and agonize the functions of mAChR, respectively, according to previous studies [24, 25]. After the toxicity was evaluated by zebrafish hatchability, body length and motor activity, atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L) were selected for the subsequent inflammation experiment. Zebrafish larvae at 5 dpf (days post fertilization) were treated with atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L) for 3 h respectively to *in vivo* evaluate their effects on cytokines expression and neutrophils migration. CB1158 (HY-101979, MCE) was used to inhibit the activity of *arg1* and *arg2* [26]. To verify whether mAChR affects the inflammatory responses through the *arg* signaling, 5 dpf zebrafish larval were soaked with atropine (100 μg/L), bethanechol (10 μM/L) and CB-1158 (10 μM/L) for 3 h, respectively.

2.3 Morphological observation

Zebrafish embryos of 24 hpf (hours post fertilization) were continuously soaked with atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L) and the solutions were changed every day. The tail

wagging rate (1 min) at 30 hpf; heart rate (20 s) at 48 hpf and 72 hpf; body length at 96 hpf, survival rate during 4 days of larvae were detected using a stereomicroscope. About fifty zebrafish in each group were used in the experiments.

2.4 Behavior test

The motor behavior of larval zebrafish at 5 dpf was assessed by Viewpoint (France) using the photo motor response (PMR) model [17]. The control, atropine (10 μg/L, 100 μg/L), bethanechol (1 μM/L, 10 μM/L) groups were arranged in a 48 wells plate in different experiments, with 16 fish in each group. The experimental procedure was set up as follows: 30 minutes of darkness for dark adaptation, followed by 3 cycles of 5 minutes of light and 5 minutes of dark to monitor the activity of larval zebrafish.

2.5 Tail fin damage and imaging

After larvae of 5–7 dpf were anesthetized with 0.1 g/mL MS-222 solution (Sigma, E10521), the tail fin of the zebrafish was damaged by a surgical blade at the tail pigmented end using a stereo microscope. After the caudal fin injury zebrafish larvae were treated with atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L). About 30 zebrafish larvae were used in each group. Three hours post injury, the migration of neutrophils in the caudal fin was observed under a fluorescence microscope, and the number of neurophils was analyzed by ImageJ software.

2.6 Lipopolysaccharide (LPS) micro-injection

Zebrafish larvae of 3 dpf were anesthetized with a small amount of 0.1g/mL MS-222 solution, and 50 nl of the prepared LPS (0.2 μg/L, S11060, yuanye) solution was injected into the intraperitoneal of zebrafish larvae with a micro-injection apparatus. After the injection, zebrafish larvae were treated with atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L). Each group contained 30 zebrafish larvae.

2.7 QPCR

After zebrafish of 5 dpf were continuously soaked in atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L) for 3 h, 30 zebrafish larvae in each group were collected into EP tubes with 500 μl TRIZOL (Takara, 9108). After sonication of the tissue, total RNAs were extracted using chloroform and isopropanol. Then, the extracted total RNAs were reversely converted to cDNA using a reverse transcription kit and Q-PCR was performed using a commercial kit. Specific primers (Table 1) were used to detect gene expression by RT-qPCR with TB Green® Fast qPCR Mix (Takara, RR430S). Each experiment was carried out 3 times. The experimental data were calculated with $2^{-\Delta\Delta ct}$ according to previous study [27].

Table 1

Gene name	Sequence (5’-3’)	Application
q*tnf-α*-F	GCGCTTTTCTGAATCCTACG	Expression analysis
q*tnf-α*-R	TGCCCAGTCTGTCTCCTTCT	Expression analysis
q*il-1β*-F	GTACTCAAGGAGATCAGCGG	Expression analysis
q*il-1β*-R	CTCGGTGTCTTTCCTGTCCA	Expression analysis
q*il-6*-F	GCTATTCCTGTCTGCTACACTGG	Expression analysis
q*il-6*-R	TGAGGAGAGGAGTGCTGATCC	Expression analysis
q*il-8*-F	CCACACACACTCCACACACA	Expression analysis
q*il-8*-R	CCACTGAATTGTCCTTTCATCA	Expression analysis
q*arg1*-F	TCCGTTCTCCAAAGGACAGC	Expression analysis
q*arg1*-R	CTTCACCACACAACCTTGCC	Expression analysis
q*arg2*-F	GGGGAGATCACAGCTTAGCG	Expression analysis
q*arg2*-R	AAGGTGAAGTCAGAGGCGTG	Expression analysis
q*β-actin*-F	ACGAACGACCAACCTAAACTCT	Expression analysis
q*β-actin*-R	TTAGACAACTACCTCCCTTTGC	Expression analysis
Primer designed for cloning and expression analysis in this study.		

2.8 Statistical analysis

The GraphPad Prim 9.0 was used to analyze the experiment data. The data were analyzed with one-way ANOVA, t-test and displayed as mean ± SD.

3. Results

3.1 Atropine and bethanechol showed no obvious effects on the growth and activity of larvae.

In this experiment, we used atropine (mAChRs antagonist) and bethanechol (mAChR agonist) to inhibit or activate the functions of mAChRs to study the effect of mAChRs on the inflammatory responses [28, 29]. In order to determine whether the concentrations of atropine and bethanechol used in the experiments are pharmacologically toxic to zebrafish larvae. Zebrafish of 24 hpf were continuously soaked in atropine (10 μg/L, 100 μg/L) and bethanechol (1 μM/L, 10 μM/L). We assessed growth and development status of larvae by monitoring a series of data such as heart rate, tail wagging, body length, survival rate and activity within 96 hpf of larvae.

Compared with control group, 10 μg/L atropine had no significant effect on the survival rate (96 hpf), tail wagging (30 hpf), heart rate (48 hpf, 72 hpf) and body length (96 hpf) of zebrafish larvae (Fig. 1A-D); 100 μg/L atropine had only a slight increase in heart rate (48 hpf, 72 hpf) (Fig. 1B); The overall morpha of the juveniles in atropine group observed by stereomicroscope was not significantly different from the control group (Fig. 1E). Compared with control group, 1 μM/L bethanechol also had no significant effect on the survival rate (96 hpf), tail wagging (30 hpf), heart rate (48 hpf, 72 hpf), and body length (96 hpf) of the larvae (Fig. 2A-D); 10 μM/L bethanechol also only slightly increased the heart rate (Fig. 2B); The overall morpha of larvave observed by stereomicroscope in bethanechol group was not significantly different from control group (Fig. 2E). In addition, we detected the activity of zebrafish larvave by PMR experiments using a behavioral monitor. Compared with control group, atropine and bethanechol treatment had no significant effect on the activity of zebrafish larval under both light and dark conditions (Fig. 1F and 2F).

Together, these results indicated that concentrations of atropine (10 μg/L and 100 μg/L) and bethanechol (1 μM/L, 10 μM/L) we used in the study had no significant effect on the basic physiological functions of zebrafish larvae.

3.2 Inhibition of mAChRs increased neutrophils recruitment and proinflammatory cytokine levels

Neutrophils are important members of the innate immune system and are essential for host immune defense. We induced acute inflammation by damaging the caudal fin of transgenic zebrafish Tg(lyz:EGFP) (5 dpf) labeled with fluorescent neutrophils, and assessed whether inhibition of mAChRs affected neutrophils migration towards the inflammatory site by *in vivo* imaging. The results showed that 10 μg/L atropine treatment had no significant effect on the migration of neutrophils to injury, but 100 μg/L atropine treatment significantly increased this process (Fig. 3A left and 3B). Meanwhile, we found that there was no statistical difference in the number of neutrophils in the statistical area between the treatment group and the control group without caudal fin injury (Fig. 3A right and 3C), indicating that the increase in neutrophils recruitment caused by mAChRs inhibition is indeed due to the enhancement of its inflammatory response ability, rather than the differences in the initial number of neutrophils in the caudal fins between groups.

Expression of pro-inflammatory cytokines is a key event in the inflammatory process and is often used to assess inflammation levels. After LPS microinjection, zebrafish larvae were treated with atropine (10 μg/L, 100 μg/L) for 3h, and then the cytokines expression were detected. The results showed that the expression of pro-inflammatory cytokines *il-1β* and *il-8* were significantly increased after 10 μg/L atropine treatment (Fig. 3D). Compared with the control group, *tnf-α*, *il-1β, il-6* and *il-8* levels in 100 μg/L atropine group were significantly increased (Fig. 3D). These results suggested that mAChRs suppression significantly up-regulated the levels of pro-inflammatory cytokines in zebrafish.

Overall, we demonstrated that inhibition of mAChRs in zebrafish up-regulates inflammatory responses by up-regulating neutrophils migration and increasing proinflammatory cytokines levels.

3.3 Activation of mAChRs decreased neutrophils migration and proinflammatory cytokines levels.

According to the above experimental design, we explored whether activation of mAChRs could down-regulated neutrophils migration and pro-inflammatory cytokines levels. There was no significant difference in the number of neutrophils in a certain area of the uninjured caudal fin between the treated and control groups (Fig. 4A right and 4C). However, the number of neutrophils migrating at the site of caudal fin injury in 1 μM/L and 10 μM/L bethanechol groups was significantly decreased compared with control group (Fig. 4A left and 4B). Compared with control group, the 1 μM/L bethanechol treatment down-regulated the levels of *tnf-α*, *il-1β*, *il-6* (Fig. 4D), but had no significant effect on *il-8* (Fig. 4D), and the levels of *tnf-α*, *il-1β*, *il-6* and *il-8* were all significantly decreased in the 10 μM/L bethanechol group (Fig. 4D).

In conclusion, we demonstrate that mAChR activation in zebrafish down-regulated inflammatory responses by reducing neutrophils migration and decreasing proinflammatory cytokines levels.

3.4 The mAChRs regulated the level of *arg1* and *arg2* in zebrafish larvae.

The above results indicated that inhibition or activation of mAChRs regulated the expression of cytokines, and Args have also been shown to be involved in the regulation of inflammatory processes in mice. Therefore, we wondered whether mAChRs have effects on the expression of *arg1/2* in zebrafish.

In the experiment, we found that LPS injection significantly increased the expression of *arg1* and *arg2* in zebrafish. Compared with the LPS group, the levels of *arg1* and *arg2* in 10 μg/L atropine group were not significantly changed (Fig. 5A), but the levels of *arg1* and*arg2* in the 100 μg/L atropine group were significantly up-regulated (Fig. 5A); In addition, we found that 1 μM/L and 10 μM/L bethanechol significantly down-regulated the expression of *arg1* and *arg2* induced by LPS (Fig. 5B).

3.5 The *args* reversed the inflammatory increase caused by inhibition of mAChRs.

Here, we aim to prove whether *args* was involved in the regulation of mAChRs on inflammatory responses in zebrafish. In the experiment, we used an inhibitor, CB-1158, to inhibit *args* expression and found that CB-1158 (10 μM/L) could significantly decrease the level of *args* induced by inhibition of mAChRs in larvae (Fig. 5A). Then, we found that inhibition of *args* significantly down-regulated the increase in neutrophils migration towards the injured fin caused by atropine treatment, but inhibition of *args* could not further decrease the reduction in neutrophils recruitment induced by bethanechol treatment (Fig. 6A left and 7A left). There was no significant difference in the number of neutrophils in statistical area of the uninjured caudal fin between the treated and control groups (Fig. 6A right, 6C and 7A right, 7C). Similarly, the levels of *tnf-α*, *il-1β*, *il-6* and *il-8* were significantly decreased in the atropine and CB-1158 dual

treatment groups compared with the atropine group (Fig. 6D). However, CB-1158 treatment only further significantly decreased the reduction of *il-1β* levels induced by bethanechol treatment (Fig. 7D), while *tnf-a, il-6* and *il-8* levels did not change significantly (Fig. 7D).

In conclusion, inhibition of *args* could reverse the increase of neutrophils migration and cytokines expression caused by inhibition of mAChRs to a certain extent in zebrafish, but the inflammatory response induced by activation of mAChRs could not be significantly altered.

4. Discussion

As a G protein-coupled receptor, mAchRs, composed of multiple subunits (M1, M2, M3, M4, M5), can participate in a variety of life activities such as nerve signal transduction [30]. Recent studies showed that five subtypes of mAchRs were expressed in T cells, B cells and other immune cells [9]; In M1/M5 receptor knockout mice, the secretion levels of pro-inflammatory cytokines were changed [31]. These results demonstrated that the non-neuronal cholinergic system was involved in the regulation of immune cell function. In general, there are relatively few reports on the role of mAchRs in systemic immune response, and almost no reports on the role of mAchRs in the dynamic behavior of peripheral blood neutrophils and other immune cells, and its potential regulatory mechanism is still unclear. In this study, we took advantage of zebrafish larval transparency and *in vivo* imaging to explore the role of mAchRs in inflammatory response and investigate whether mAchRs regulate inflammation through arginase in zebrafish.

We used atropine (an antagonist of mAChRs) and bethanechol (an agonist of mAChRs) to inhibit or activate mAChRs to study the roles of mAChRs in inflammatory response. The results showed that inhibition of mAChRs up-regulated neutrophils migration and cytokines expression (Fig. 3A and 3D), while activation of mAChRs down-regulated neutrophils recruitment and cytokines expression (Fig. 4A and 4D) in the caudal fin injury model of zebrafish larvae. Taken together, these results suggested that pharmacological interventions of mAchRs could regulate inflammatory responses in zebrafish. We know that there are nine kinds of subtypes of mAChRs in zebrafish, and in this part of the study, we only interfered with the function of mAChRs by pharmacological means. Therefore, we can not provide evidence for which receptor subtypes play a more important role in inflammation, especially neutrophil migration, although we did experiment with RNA interference of some subtypes (data not shown). We will investigate this question further by CRISPR/Cas9 knockdown of specific receptor subtypes.

Arginase is widely expressed in a variety of immune cells and has been shown to play important roles in immune response [19]. Therefore, we speculated that mAchRs might affect the inflammatory process by regulating Args in zebrafish. Our results showed that LPS injection induced increased expression of *arg1* and *arg2*, suggesting that *arg1* and *arg2* were positively correlated with inflammatory response, and inhibition of mAChRs could further promote the up-regulation of *arg1* and *arg2* levels during this process, while activation of mAchRs reduced the expression of *arg1* and *arg2* (Fig. 5A and 5B). The regulatory effect of mAchRs on *arg1* and *arg2* is basically similar to the effect on cytokines expression and

neutrophils migration. Next, we wondered if mAChRs could modulate the inflammatory response by modulating *arg1* and *arg2* in zebrafish. We inhibited the expression of *arg1* and *arg2* by CB-1158, and found that inhibition of *arg1* and *arg2* significantly reversed the increase of neutrophils migration and cytokines expression induced by supression of mAChRs (Fig. 6A and 6D). However, inhibition of *arg1* and *arg2* could not further down-regulate the reduction of neutrophils migration and cytokines expression induced by activation of mAChRs (Fig. 7A and 7D), which might be due to the fact that *arg1* and *arg2* were already at a very low expression level during activation of mAChRs. Our study for the first time found the association between mAchRs and Args, and concluded that mAChRs regulate inflammatory responses in zebrafish by regulating *arg1* and *arg2* levels. Admittedly, in this study, we have not proved how mAchRs regulate *arg1* and *arg2* expression, and it is a worthwhile question to find out which other proteins are involved in this regulation process.

Our study shows that mAchRs, as an important component of cholinergic anti-inflammatory pathway, played roles in *in vivo* recruitment of neutrophils and secretion of pro-inflammatory cytokines in zebrafish, providing new theoretical evidence for the immune research of mAchRs.

5. Conclusion

We conclude that mAchRs significantly affect neutrophils migration and pro-inflammatory cytokines expression by regulating *arg1* and *arg2* levels in zebrafish.

Declarations

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Author's Contribution

All authors participated in the experimental design, experimental research, analysis of experimental results, data analysis, and the writing of the first draft of the paper. Da-long Ren is the creator and director of the project, directing experimental design, data analysis, thesis writing and revision. All authors read and agree to the final text.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

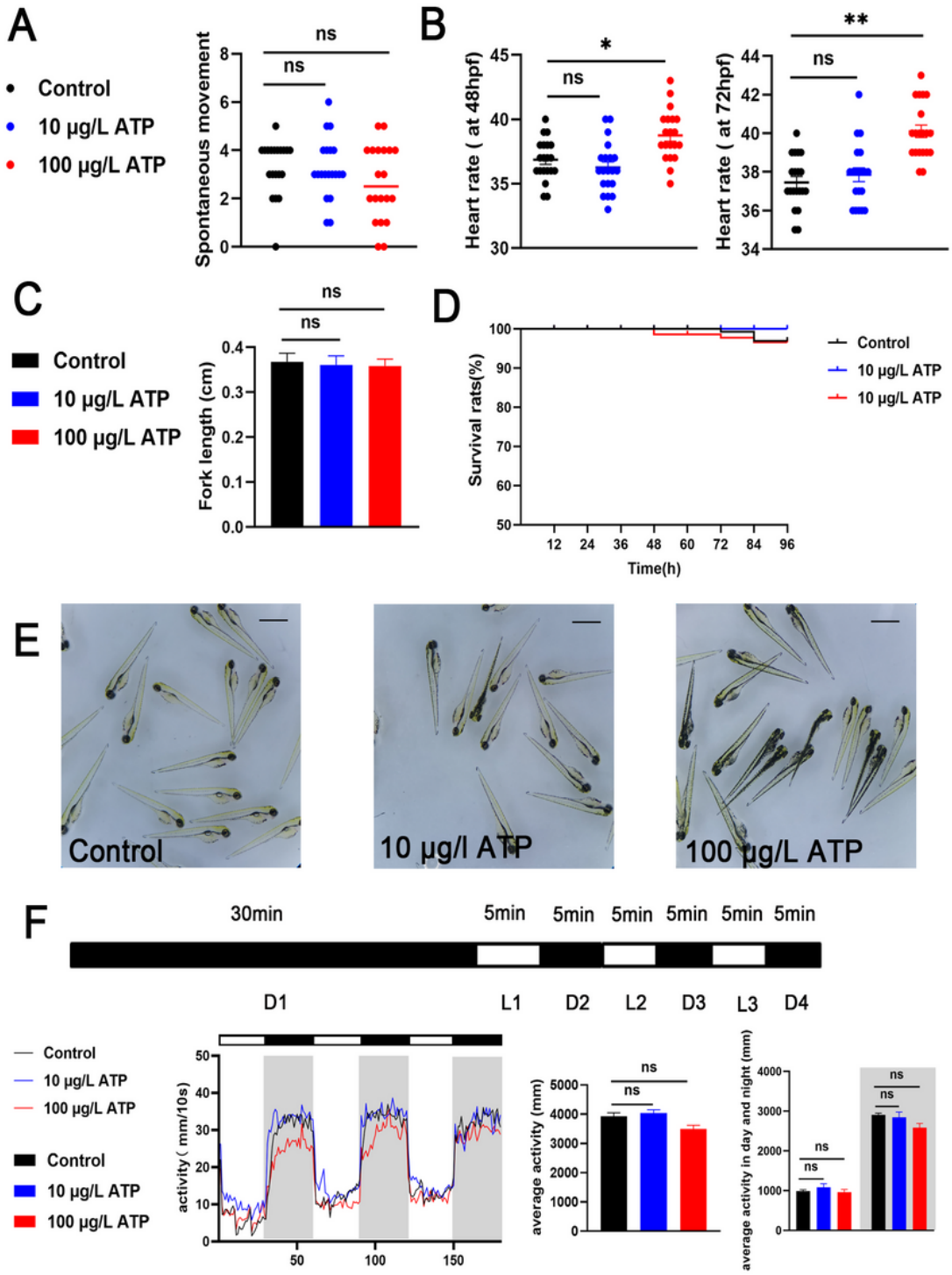

Figure 1

Atropine had no significant effect on the growth, development and activity of zebrafish larvae.

(A) Times of tail wagging of 30 hpf larval zebrafish (times/min, n=15). (B) Heart rate of 48 hpf, 72 hpf larval zebrafish (times/20s, n=15). (C) Body length of 96 hpf zebrafish larvae (cm, n=15). (D) 0-96 hpf larvae survival curve (%, n=15). (E) Morpha of 96 hpf zebrafish under a stereomicroscope. (F) PMR

behavior of zebrafish larvae monitored by Viewpoint (n=16). ATP in the figure represents atropine, The analysis showed that there was no obvious difference between atropine and control group. (*P<0.05, ** P< 0.01, ***P<0.001, t-test and ANOVA analysis).

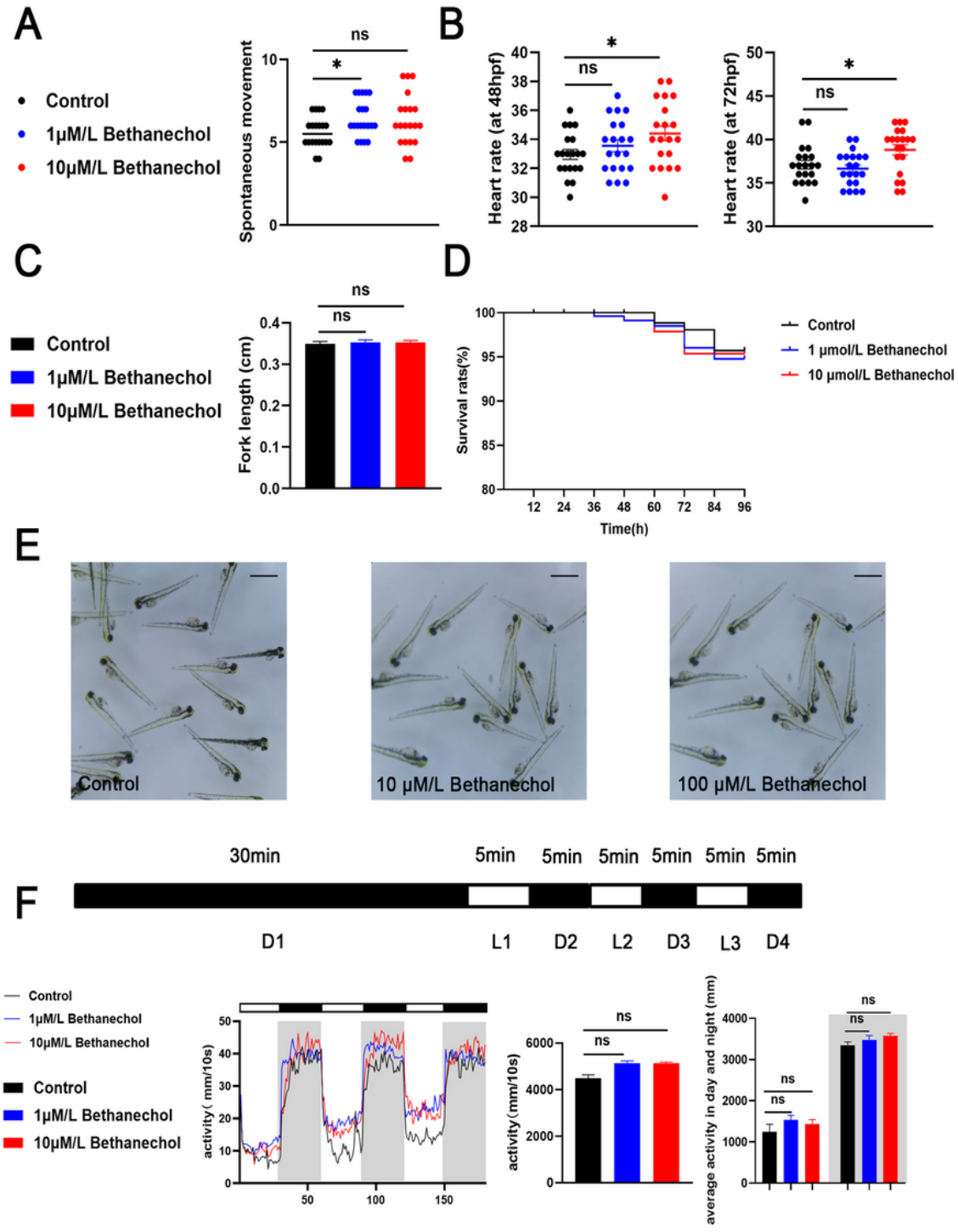

Figure 2

Bethanechol treatment showed no significant effect on the growth, development and activity of zebrafish larvae.

(A) Times of tail wagging of 30 hpf larval zebrafish (times/min, n=15). (B) Heart rate of 48 hpf, 72 hpf larval zebrafish (times/20s, n=15). (C) Body length of 96 hpf zebrafish larvae (cm, n=15). (D) 0-96 hpf zebrafish survival curve (%, n=15). (E) Morpha of 96 hpf zebrafish under a stereomicroscope. (F) PMR behavior of zebrafish larvae monitored by Viewpoint (n=16). Bet in the figure represents bethanechol. The results showed that there was no obvious difference between bethanechol and control group. (*P<0.05, ** P< 0.01, ***P<0.001, t-test and ANOVA analysis).

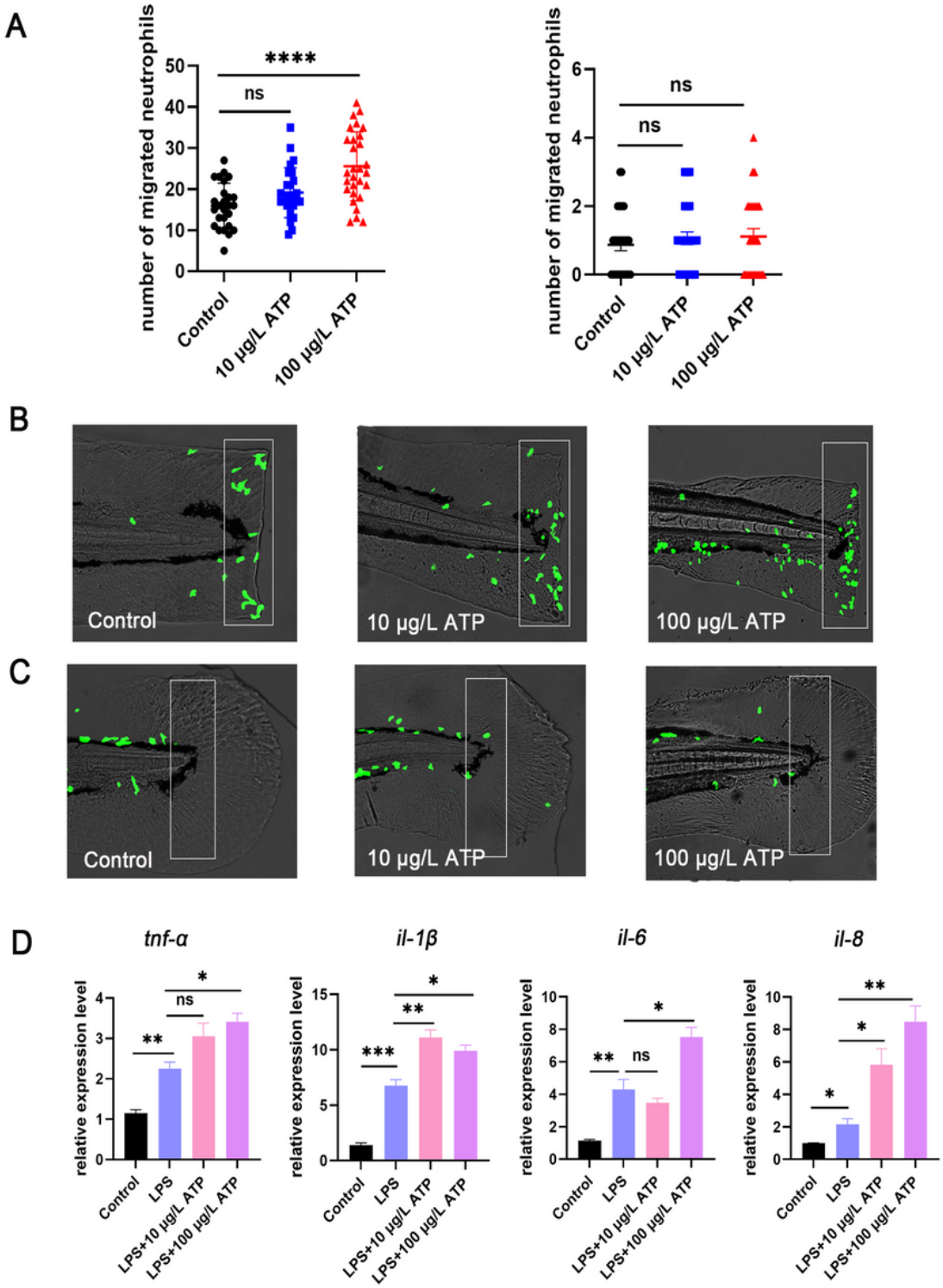

Figure 3

Inhibition of mAChRs up-regulated neutrophils migration and increased proinflammatory cytokines level.

(A) Neutrophils were photographed with a fluorescence microscope (n=25), and neutrophils migration increased after atropine (10 μg/L, 100 μg/L) treatment in the statistical area. (B) Model of caudal fin injury: atropine (10 μg/L, 100 μg/L) treated or untreated caudal fins of zebrafish were cut with a scalpel

blade (white rectangles indicate statistical areas). (C) When the zebrafish caudal fin was not damaged, there was no difference in the number of neutrophils in the statistical area between atropine (10 μg/L, 100 μg/L) and the control group (the white rectangles indicate the statistical area). (D) The expression levels of *tnf-α*, *il-1β*, *il-6* and *il-8* in zebrafish treated with atropine (10 μg/L, 100 μg/L) were increased compared with the LPS group (n=30). (*P<0.05、** P< 0.01, ***P<0.001, ****P<0.0001, t-test and ANOVA analysis).

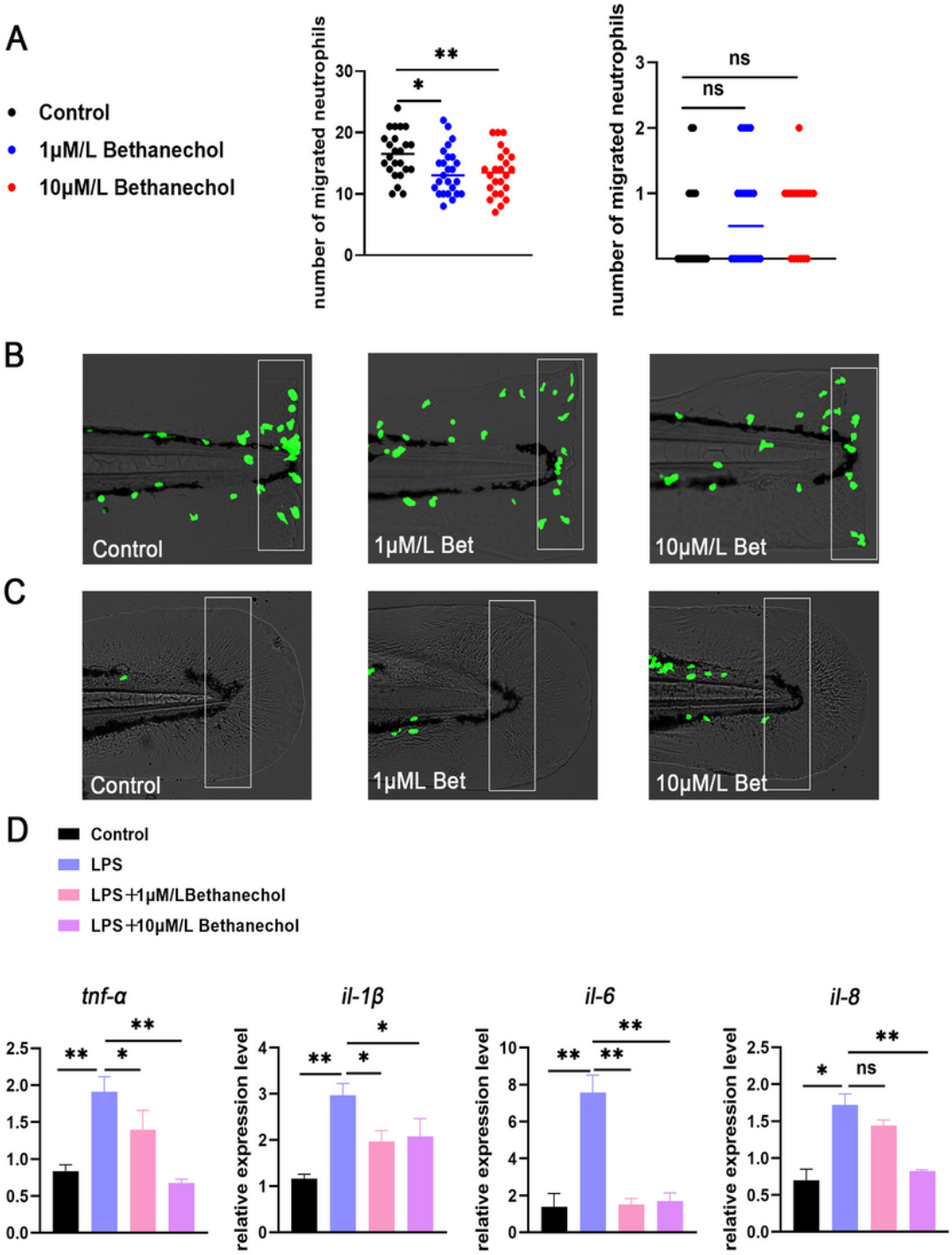

Figure 4

Activation of mAChRs down-regulated neutrophils migration and reduced proinflammatory cytokines level.

(A) Neutrophils were photographed with a fluorescence microscope (n=25), and neutrophils migration was reduced after treatment with bethanechol (1 μM/L, 10 μM/L) in the statistical area. (B) Model of caudal fin injury: The bethanechol (1 μM/L, 10 μM/L) treated or untreated caudal fin was cut with a scalpel blade (white rectangles indicate statistical areas). (C) When the zebrafish caudal fin was not damaged, there was no difference in the number of neutrophils in the statistical area between bethanechol (1 μM/L, 10 μM/L) and the control group (the white rectangles indicate the statistical area). (D) The expression levels of *tnf-α*, *il-1β*, *il-6* and *il-8* in zebrafish treated with bethanechol (1 μM/L, 10 μM/L) were decreased compared with the LPS group (n=30). (*P<0.05、** P< 0.01, ***P<0.001, ****P<0.0001, t-test and ANOVA analysis).

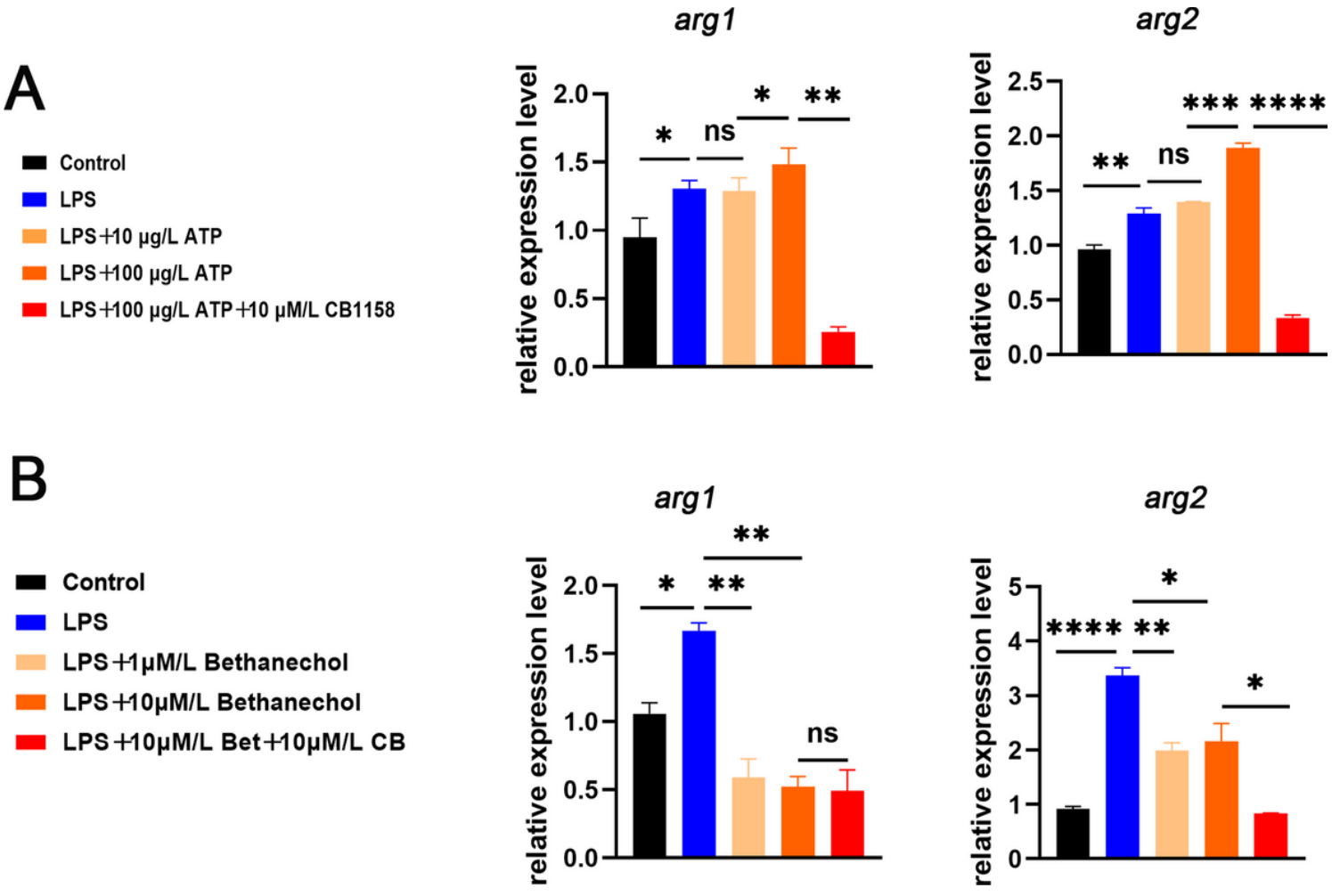

Figure 5

The mAChRs regulated the expressions of *arg1* and *arg2* in zebrafish.

(A) In LPS induced inflammation model, zebrafish were treated with atropine (10 μg/L, 100 μg/L) to detect the levels of *arg1* and *arg2*. Treated with atropine (100 μg/L), the expression of *arg1* and *arg2* was significantly increased which could be inhibited by CB-1158. (B) In LPS induced inflammation model, zebrafish were treated with bethanechol (1 μM/L, 10 μM/L) to detect the expression of *arg1* and *arg2*, and after treatment with bethanechol (10 μM/L), the *arg2* level significantly decreased. (*P<0.05 ** P< 0.01, ***P<0.001, ****P<0.0001, t-test and ANOVA analysis).

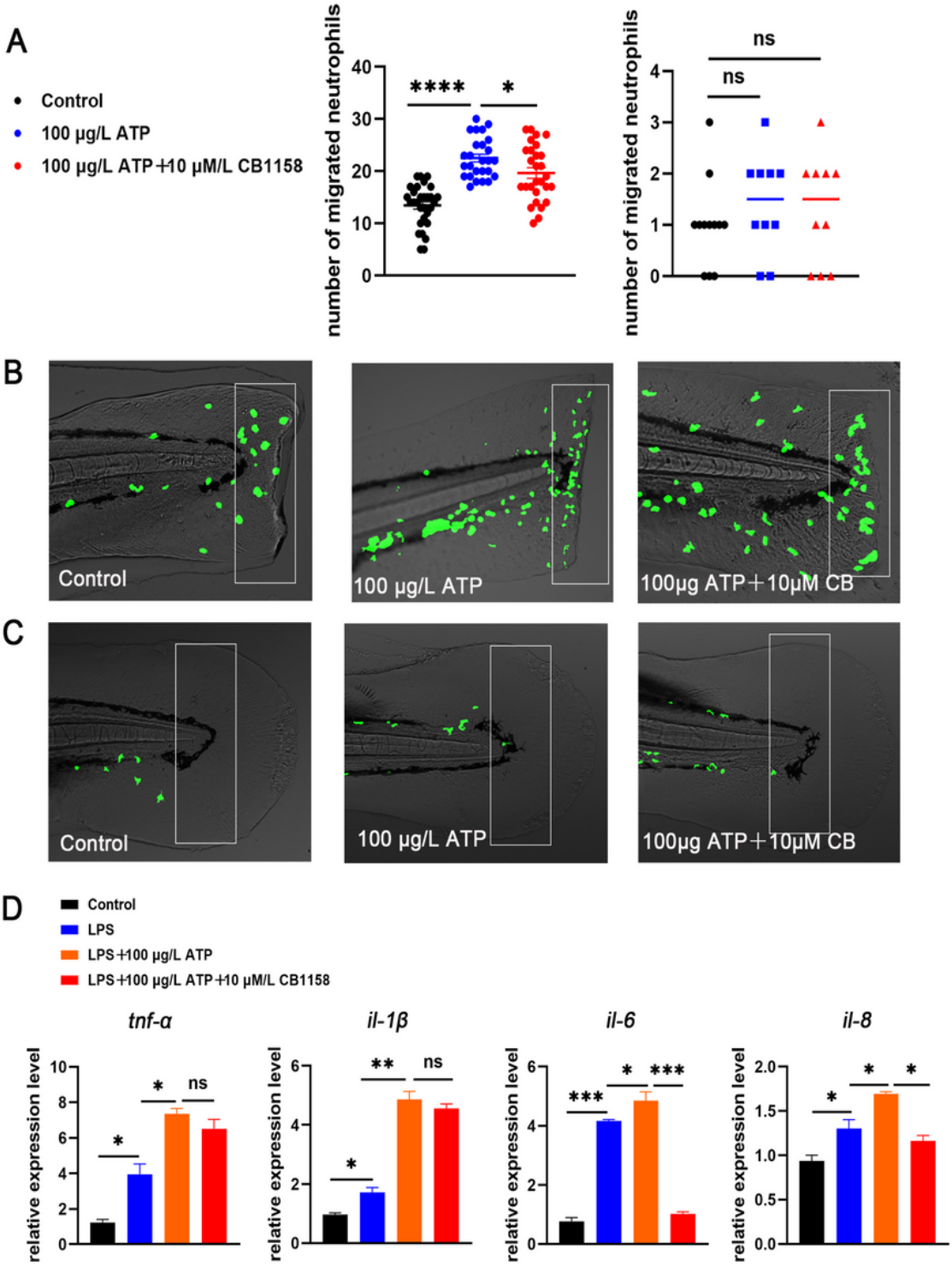

Figure 6

Inhibition of *arg1/2* down-regulated the increase in neutrophils migration and cytokines level caused by mAChR supression.

(A) Neutrophils were photographed with a fluorescence microscope, and neutrophils migration increased after treatment with atropine (100 μg/L) in the statistical area, which could be reversed by CB-1158 (10 μM/L). (B) Model of caudal fin injury: atropine (100 μg/L), atropine (100 μg/L) and CB-1158 (10 μM/L) treated or untreated caudal fins of zebrafish were cut with a scalpel blade (white rectangles indicate statistical areas). (C) When the zebrafish caudal fin was not damaged, there was no difference in the number of neutrophils in the statistical area between atropine (100 μg/L), atropine (100 μg/L) and CB-1158 (10 μM/L) treatment and the control group (white rectangles indicate statistical area). (D) Treatment with atropine (100 μg/L), atropine (100 μg/L) and CB-1158 (10 μM/L) in LPS induced inflammation model to detect the expression levels of *tnf-α*, *il-1β*, *il-6* and *il-8* (n=30). (*P<0.05 ** P< 0.01, ***P<0.001, ****P<0.0001, t-test and ANOVA analysis).

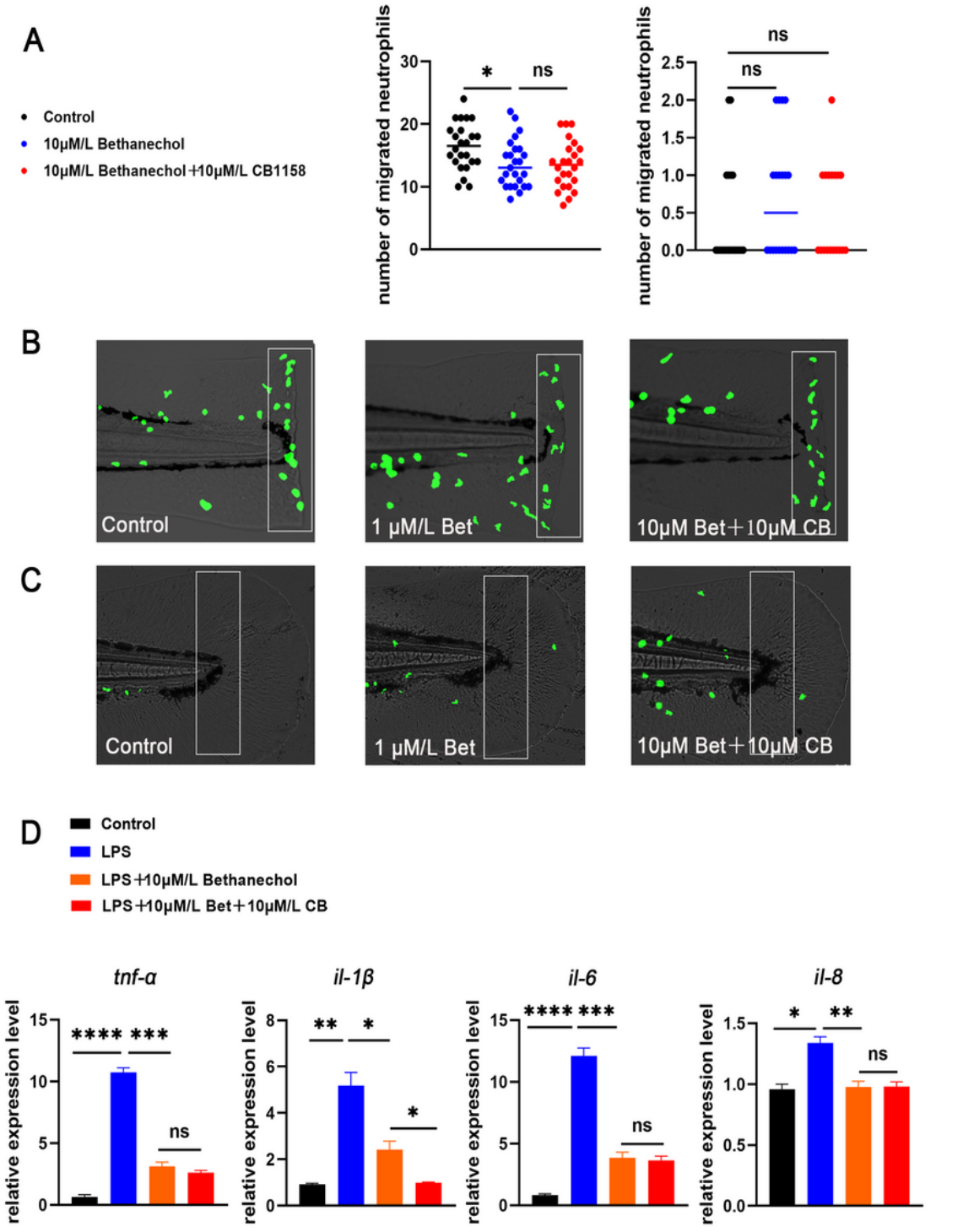

Figure 7

Inhibition of *arg1/2* could not further down-regulated the reduction in neutrophils migration and cytokines level caused by mAChR activation.

(A) Neutrophils were photographed with a fluorescence microscope, and neutrophils migration was reduced after treatment with bethanechol (10 μM/L) in the statistical area, and treated with bethanechol

(10 μM/L) and CB-1158 (10 μM/L), neutrophils migration in the statistical area was unchanged compared to the bethanechol (10 μM/L) treatment alone. (B) Model of caudal fin injury: caudal fins of zebrafish treated or untreated with bethanechol (10 μM/L), bethanechol (10 μM/L) and CB-1158 (10 μM/L) were cut with a scalpel blade (white rectangles indicate statistical areas). (C) When no damage to the caudal fin, bethanechol (10 μM/L) treatment, bethanechol (10 μM/L) and CB-1158 (10 μM/L) treatment showed no difference in the number of neutrophils in the statistical area (white rectangles indicate statistical area). (D) Treatment with bethanechol (10 μM/L), bethanechol (10 μM/L) and CB-1158 (10 μM/L) in LPS induced inflammation model to detect the expression levels of *tnf-α*, *il-1β*, *il-6* and *il-8* (n=30). (*P<0.05** P< 0.01, ***P<0.001, ****P<0.0001).