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EFFECT OF ACETYLCHOLINE ON IN VITRO IL-2 PRODUCTION AND NK CELL CYTOTOXICITY OF RATS

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ABSTRACT

In the present study, we explored the effect of acetylcholine (ACh) on lymphocyte function and the receptor mechanisms mediating the effect. Concanavalin A (Con A)-induced interleukin-2 (IL-2) production and natural killer (NK) cell cytotoxicity were used to assess function of the T lymphocytes and the NK cells from rat spleens. Muscarinic ACh receptors (mAChRs) agonist pilocarpine and antagonist atropine, as well as nicotinic ACh receptors (nAChRs) agonist nicotine and antagonist tubocurarine were used to determine the action pathways of ACh on T and NK cells. ACh at the concentrations of 10^{-10} to 10^{-8} M exerted an enhancing effect on Con A-induced IL-2 production and an inhibitory effect on NK cell cytotoxicity. Both pilocarpine and nicotine at the same doses as ACh could mimic these effects of ACh. The enhancing effect of ACh on IL-2 production could be blocked by either atropine or tubocurarine. But the inhibitory effect of ACh on NK cell cytotoxicity was abolished only by atropine, not by tubocurarine. These results suggest that ACh, which is a neurotransmitter of peripheral parasympathetic nervous system, can regulate function of T and NK cells, and the different regulatory effects of ACh on the two types of lymphocytes may be mediated by the different receptor mechanisms.

Acetylcholine (ACh) is a classical neurotransmitter in central and peripheral nervous systems and performs an important regulatory role in almost all tissues of the body. The early findings that lymphoid tissues were richly innervated by parasympathetic nerve fibers (1-3) and that muscarinic ACh receptors (mAChRs) and nicotinic ACh receptors (nAChRs) existed on lymphocytes (4-5) provided evidence that the parasympathetic nervous system could modulate lymphocyte function through ACh and its receptors. Although over the recent more than twenty years, sympathetic and parasympathetic nervous systems have been demonstrated, by a large number of experiments, to regulate immune function (6-19), the reports concerning the immunoregulatory role of parasympathetic nerves and their transmitter ACh were not as numerous as those about sympathetic nerves and their transmitter norepinephrine (NE).

In general, the sympathetic and parasympathetic nervous systems, as well as their transmitters NE and ACh, modulate various functions of the body in an antagonistic or a coordinated way. In our recent experiments (submitted for publication), NE was found to suppress both the concanavalin A (Con A)-induced interleukin-2 (IL-2) production and the natural killer (NK) cell cytotoxicity. Thus, so far as lymphocytes are

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concerned, whether ACh also exerts a contrary regulatory role to NE is an interesting question to answer.

T cells and NK cells are two different types of lymphocytes. Therefore, in the present study, we measured Con A-induced IL-2 production and NK cell cytotoxicity, which are the major functional indexes for T cells and NK cells respectively. In our previous work through directly testing T cell proliferation, we showed a contrary effect of ACh to NE and clarified the effective action times of ACh and NE (15,16). Since IL-2 production by activated T cells is an essential step of the cascade for carrying out their function of cellular immunity, we presently explored the effect of ACh on the production of IL-2 by proliferating T cells to better understand the role of ACh in the regulation of T cell function. In addition, we were interested in the modulation of NK cells by ACh, a less recognized phenomenon.

Recently, molecular biologists from other laboratories have demonstrated that lymphocytes could express nAChRs and mAChRs (20-22). Therefore, we investigated the respective role of these two types of receptors in mediating the effects of ACh on the T and NK cells.

MATERIALS AND METHODS

Cell Culture Medium

RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2.5×10^{-2} M HEPES (Sigma), 5×10^{-5} M 2-mercaptoethanol, 1×10^{-3} M sodium pyruvate and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin) was used as complete culture medium.

Cell Suspensions

Single lymphocyte suspensions were prepared from spleens of Sprague-Dawley rats anesthetized with urethane (1 g/kg, i.p.). Erythrocytes were lysed with sterilized distilled water. Final concentration of the lymphocytes in the complete culture medium was 2×10^6 or 5×10^6 cells/ml depending on experimental aim.

Colorimetric MTT Assay for Con A-induced IL-2 Production

MTT (3-(4,5-dimethyl-z-thiazol)-2,5-diphenyl tetrazolium bromide) assay was quantitatively measured as described by Mosmann (22). Briefly, single splenocytes suspended in complete culture medium at the concentration of 5×10^6 cells/ml were incubated with 5 µg/ml Con A (Sigma) in an ESPEC incubator (BNA-311, Japan) with 5% CO_2 at 37°C for 24 h. The supernatants containing IL-2 were added to the other splenocyte cultures of 2×10^6 cells/ml at the 25% concentration. Before this process, the 2×10^6 cells/ml splenocyte cultures were incubated with 2 µg/ml Con A for 48 h and washed with methyl-α-D-mannopyranoside (Fluka). And then, together with methyl-α-D-mannopyranoside (10 mg/ml), they were incubated in 5% CO_2 at 37°C for 48 h. The MTT (Fluka) solution of 5 mg/ml was added to the cultures containing IL-2 (10 µl MTT solution per 100 µl medium), followed by incubation with 5% CO_2 at 37°C for 4 h. Sodium dodecyl sulfate (SDS, 20%) containing 50% N'N-dimethylformamide was added to the cultures, which were then incubated for 20 h at 37°C in 5% CO_2. Lastly, their optical density (OD) was read on a Universal Microplate Reader (Elx 800, Bio-TEK instruments, Inc., USA) using a test wavelength of 570 nm.

The experimental drug (ACh, pilocarpine, nicotine, atropine or tubocurarine–all from Sigma) was first incubated with the splenocyte suspensions (5×10^6 cells/ml) at a dose of 10^{-10}, 10^{-9}, or 10^{-8} M. One hour later, Con A was added to the suspensions and cultured for 24 h. The subsequent processes were the same as described above. The control experiments were conducted simultaneously but without any drug.

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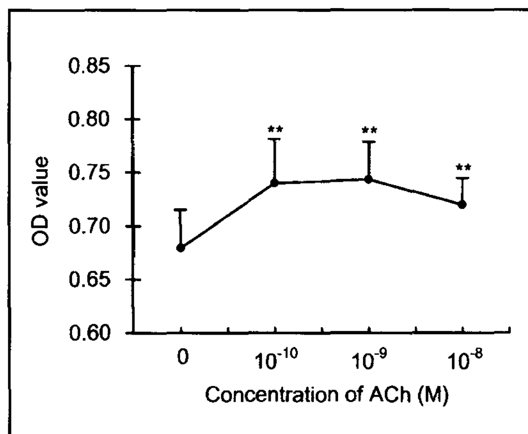

*Fig. 1. Effect of ACh on Con A-induced IL-2 production. The splenocytes were exposed to ACh at the concentrations of 10^{-10}, 10^{-9} and 10^{-8} M. Each point is the mean and standard deviation of eight repeated experiments. ** P < 0.01, compared with the control (0 concentration of ACh).*

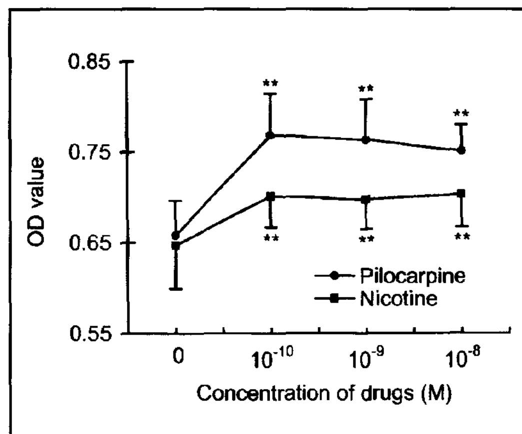

*Fig. 2. Effect of mAChRs and nAChRs agonists on the Con A-induced IL-2 production. Pilocarpine and nicotine were used to stimulate the mAChRs and nAChRs on the T cells, respectively, with concentrations of 10^{-10}, 10^{-9} and 10^{-8} M. Each point is the mean and standard deviation of eight repeated experiments. ** P < 0.01, compared with the control (0 concentration of drugs).*

Lactate Dehydrogenase (LDH) Release Assay for Evaluation of NK Cell Cytotoxicity

The Yac-1 cell line, a Moloney leukemia virus induced mouse lymphoma with noted sensitivity to NK cells, was used as target cells (obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). The target cells were maintained in continuous suspension culture at 37°C in a 5% CO_2 incubator. Their final concentration in the complete culture medium was 5×10^5 cells/ml. Effector cells (NK cells), from the spleens of rats anesthetized with urethane (1 g/kg, i.p.), were isolated as described by Konjevic et al. (23). Non-adherent cells were collected and resuspended in the complete culture medium at the concentration of 5×10^6 cells/ml. The subsequent processes were conducted according to the following formula:

$$\frac{LDH_{experimental} - LDH_{spontaneous}}{LDH_{maximal} - LDH_{spontaneous}} \times 100\%$$

where $LDH_{experimental}$ was 100 µl target cells (5×10^5 cells/ml) plus 100 µl NK cells (5×10^6 cells/ml); $LDH_{spontaneous}$ was 100 µl target cells plus 100 µl complete medium; and $LDH_{maximal}$ was 100 µl target cells plus 100 µl 1% nonidet P-40 (Fluka). They were incubated in 5% CO_2 at 37°C for 2 h, followed by centrifugation at 500 g for 5 min. The supernatants were read as OD on the Universal Microplate Reader using the test wavelength of 570 nm. Lastly, NK cell cytotoxicity was calculated as percentage according to the above formula.

In the $LDH_{experimental}$, each drug at the three doses of 10^{-10}, 10^{-9}, or 10^{-8} M was first incubated with the NK cells for 1 h, and then the NK cells interacted with the target cells for 2 h, as described above. The control experiments were conducted similarly but without any drug.

STATISTICAL ANALYSIS

The data were expressed as the mean ± standard deviation (M±SD). Statistical

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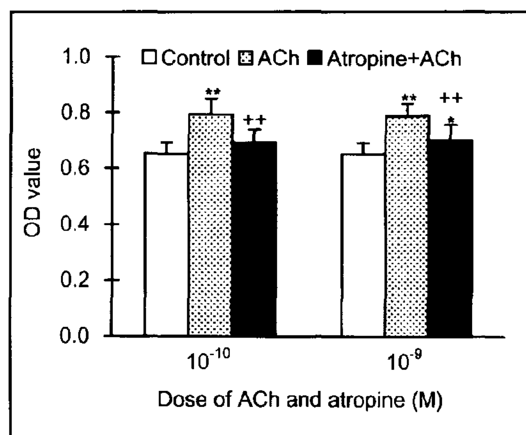

*Fig. 3. Blocking action of mAChRs antagonist on ACh enhancement of the Con A-induced IL-2 production. The splenocytes were treated with atropine plus ACh or ACh alone at 10^{-10} and 10^{-9} M concentrations. This experiment was repeated eight times. * P < 0.05, ** P < 0.01, compared with the control; ++ P < 0.01, compared with the ACh group.*

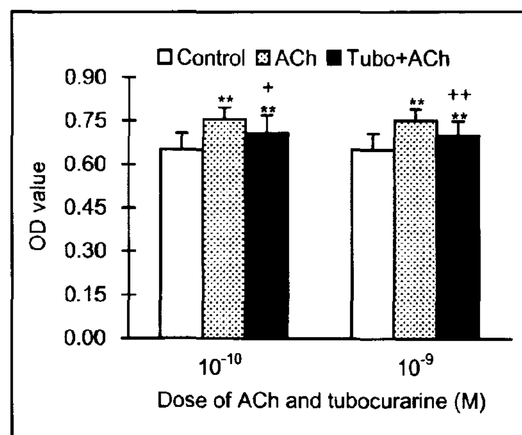

*Fig. 4. Blocking action of nAChRs antagonist on ACh enhancement of the Con A-induced IL-2. The experimental parameters were the same as Fig. 3, except tubocurarine (Tubo) was used instead of atropine. ** P < 0.01, compared with the control; + P < 0.05, ++ P < 0.01, compared with the ACh group.*

analysis was carried out with Stata software (Computer Resource Center 7.0, USA). The data were submitted to the two-way analysis of variance (ANOVA). The Student-Newman-Keul's test was also used to compare the data of all groups with each other. Differences were considered statistically significant at $P<0.05$.

RESULTS

Enhancing Effect of ACh on the Con A-induced IL-2 Production

ACh was added to the splenocyte suspensions with the three concentrations, 10^{-10}, 10^{-9}, or 10^{-8} M. The OD values, which indirectly reflected the quantity of the Con A-induced IL-2 production, were remarkably increased in all the drug groups when compared with the control group without any drug *(Fig. 1)*. The data showed that ACh had an enhancing effect on the Con A-induced IL-2 production.

Enhancing Effect of mAChRs and nAChRs Agonists on the Con A-induced IL-2 Production

Pilocarpine (mAChRs agonist) or nicotine (nAChRs agonist) was exposed to the splenocyte suspensions at the three concentrations of 10^{-10}, 10^{-9}, or 10^{-8} M. The OD value of each drug group was higher than that of the control group, although the elevation of the OD value in the nicotine group was less than that in the same dose of pilocarpine group *(Fig. 2)*. These results indicated that both the mAChRs and nAChRs agonists could increase the Con A-induced IL-2 production.

Blocking Effect of mAChRs Antagonist on ACh Enhancement of the Con A-induced IL-2 Production

The splenocytes were exposed to ACh or atropine (mAChRs antagonist) plus ACh, with their concentrations being 10^{-10}, 10^{-9} M. The OD value of ACh alone, as described above, was notably increased in comparison

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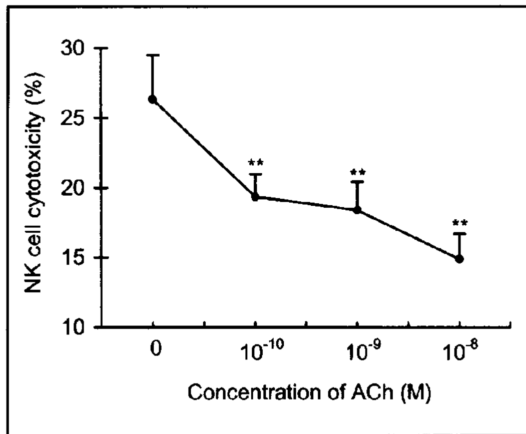

*Fig. 5. Effect of ACh on the NK cell cytotoxicity. The NK cells from spleens of rats were exposed to ACh at concentrations of 10^{-10}, 10^{-9} and 10^{-8} M. Each data point represents the mean and standard deviation of five repeated experiments. ** $P < 0.01$, compared with the control (0 concentration of ACh).*

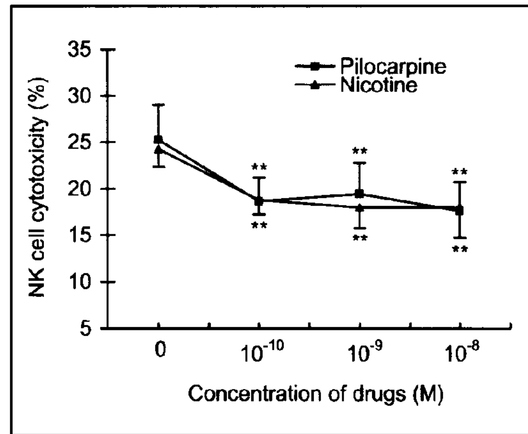

*Fig. 6. Effect of mAChRs and nAChRs agonists on the NK cell cytotoxicity. Pilocarpine and nicotine were used to stimulate the mAChRs and nAChRs on the NK cells, respectively, and their concentrations in the splenocyte suspensions were 10^{-10}, 10^{-9} and 10^{-8} M. The experiments were repeated six times for pilocarpine and seven times for nicotine. ** $P < 0.01$, compared with the control (0 concentration of drugs).*

with that of control. The OD values of the two concentrations of atropine plus ACh dropped to a level close to that of control groups, with significant difference from the same concentration of the ACh group *(Fig. 3)*. These data revealed that atropine could mostly block the enhancing effect of ACh on the Con A-induced IL-2 production.

Blocking Effect of nAChRs Antagonist on ACh Enhancement of the Con A-induced IL-2 Production

Similarly, two doses of ACh or tubocurarine (nAChRs antagonist) plus ACh were used in the experiment. The OD value of either dose of ACh was higher than that of control. After either dose of tubocurarine was applied, the OD value fell to a level that was significantly different from both the same dose of ACh and control group *(Fig. 4)*. These results showed that tubocurarine could partly block the enhancing effect of ACh on Con A-induced IL-2 production.

Inhibitory Effect of ACh on the NK Cell Cytotoxicity

The cytotoxicity of the NK cells that were pretreated with 10^{-10}, 10^{-9}, or 10^{-8} M of ACh was significantly decreased in comparison to the control without treatment of ACh *(Fig. 5)*. The results indicated that ACh could suppress NK cell cytotoxicity.

Inhibitory Effect of mAChRs and nAChRs Agonists on the NK cell Cytotoxicity

The results *(Fig. 6)* show that the cytotoxicity of the NK cells was weakened after these cells were pretreated with pilocarpine or nicotine at concentrations of 10^{-10}, 10^{-9}, or 10^{-8} M, and there was similar inhibition with the two kinds of ACh receptor agonists.

Blocking Effect of mAChRs Antagonist on ACh Suppression of the NK cell Cytotoxicity

After the NK cells were pretreated with atropine plus ACh (10^{-10} and 10^{-9} M), their

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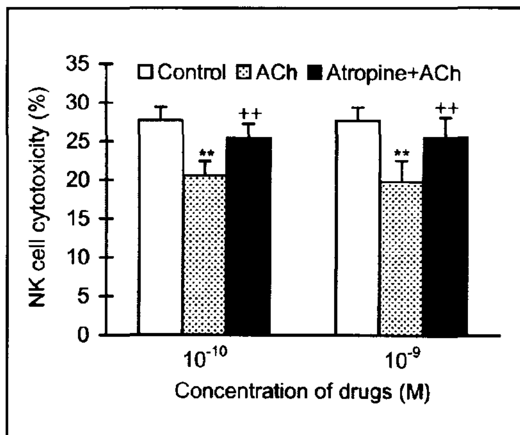

*Fig. 7. Blocking action of mAChRs antagonist on ACh suppression of the NK cell cytotoxicity. Atropine plus ACh or ACh alone was added to the NK cell suspensions at 10^{-10} and 10^{-9} M concentrations. Each bar represents the mean and standard deviation of six repeated experiments. ** $P < 0.01$, compared with the control; ++ $P < 0.01$, compared with the ACh group.*

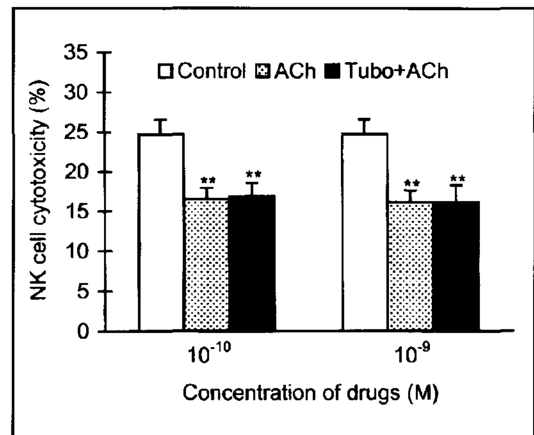

*Fig. 8. Effect of nAChRs antagonist on ACh suppression of the NK cell cytotoxicity. The experimental parameters were the same as Fig. 7, except tubocurarine (Tubo) was used instead of atropine and experimental repetition of eight times. ** $P < 0.01$, compared with the control.*

cytotoxicity rose to the level not different from the control and significantly different from ACh alone *(Fig. 7)*. Thus, atropine could completely abolish the suppressive effect of ACh on the NK cell cytotoxicity.

Effect of nAChRs Antagonist on ACh Suppression of the NK cell Cytotoxicity

The cytotoxicity of NK cells that were pretreated with tubocurarine plus ACh (10^{-10} or 10^{-9} M) was still significantly reduced in comparison to that of the control group and similar to that of the ACh alone pretreated group *(Fig. 8)*. This result suggested that tubocurarine could not reverse the suppressive effect of ACh on the NK cell cytotoxicity.

DISCUSSION

It has been known that many immune tasks performed by T lymphocytes rely on IL-2 production, and IL-2 is an important cytokine for facilitating cellular immunity. In the present study, ACh in the concentrations used ranging from 10^{-10} to 10^{-8} M was found to enhance Con A-induced IL-2 production *(Fig. 1)*. In combination with our previous observation that ACh could increase T cell proliferation induced by Con A stimulation (15), these findings indicate that ACh not only facilitates T cell proliferation but also enhances the production of IL-2 by the proliferating T cells. Thus, the modulation of T cell functions by ACh are significant and comprehensive. Contrary to the effect of ACh, NE could attenuate the Con A-induced IL-2 production in our recent study (submitted for publication). This kind of coordinated and contrary regulation of lymphocytes by ACh and NE is one of the essential mechanisms for maintaining the homeostasis of immune and physiological functions.

Other investigators focusing on the role of mAChRs or nAChRs have shown that these two types of receptors participate in the modulation of lymphocytes (14,24). We used the agonists of mAChRs and nAChRs in the same experiments, and found that the

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stimulation of the receptors respectively by pilocarpine and nicotine could mimic the enhancing effect of ACh on the Con A-induced IL-2 production (slightly stronger effect on the stimulation of mAChRs) *(Fig. 2)*. These findings suggest that both mAChRs and nAChRs may mediate the modulation of T cell function by ACh. An additional experiment using mAChRs and nAChRs antagonists further confirmed the results. Atropine at 10^{-9} M could not completely block the facilitating effect of the same dose of ACh on the Con A-induced IL-2 production *(Fig. 3)*, suggesting the possibility of mediating the effect of ACh via another type of receptors (nAChRs). Similarly, the finding that tubocurarine at concentrations of 10^{-10} and 10^{-9} M could only partly reverse the enhancing effect of the same dose of ACh on the Con A-induced IL-2 production was consistent with another type of receptor, mAChRs, that was not blocked and continued to act *(Fig. 4)*.

NK cells are another type of lymphocytes with the main activity of cytotoxicity killing their target cells, such as virus and tumor cells. However, whether the parasympathetic nervous system modulates NK cell cytotoxicity is less known. In this study, we found that ACh in the concentration range of 10^{-10} to 10^{-8} M inhibited NK cell cytotoxicity *(Fig. 5)*, suggesting that parasympathetic nerves may influence NK cell function and play a suppressive role. The contrary effect of ACh on Con A-induced IL-2 production and NK cell cytotoxity shows the variety and complexity of regulation of lymphocytes by the nervous system.

The fact that both pilocarpine and nicotine in the same concentration range with ACh could suppress the NK cell cytotoxicity and produce similar inhibitory tendency revealed that mAChRs and nAChRs were both involved in the regulation of the NK cell cytotoxicity *(Fig. 6)*. Interestingly, atropine at the two concentrations used of 10^{-10} and 10^{-9} M could abolish the suppressive effect of the relevant dose of ACh on the NK cell cytotoxicity *(Fig. 7)*, whereas neither 10^{-10} nor 10^{-9} M of tubocurarine could antagonize the inhibitory effect of corresponding doses of ACh on the NK cell cytotoxicity *(Fig. 8)*. A possible mechanism for these phenomena is that mAChRs play a crucial role in mediating the inhibitory effect of ACh on the NK cell cytotoxicity, so that when the mAChRs on the NK cells were blocked by atropine, the suppressive effect of ACh on the NK cells could not occur, but when tubocurarine blocked the nAChRs on the NK cells, ACh could still achieve its effect through the mAChRs.

CONCLUSION

In conclusion, our work demonstrated that ACh enhanced the Con A-induced IL-2 production and inhibited NK cell cytotoxicity, with the former effect through the mAChRs and nAChRs, and the latter mainly via mAChRs. In addition, our results also provided evidence that the parasympathetic nervous system, together with the sympathetic nervous system, modulated lymphocyte functions to maintain the homeostasis of immune and other systems of the body.

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