Cholinergic neuron-like D-U87 cells promote polarization of allergic rhinitis T-helper 2 cells

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Background: **Parasympathetic nerve hypersensitivity contributes to the severity of allergic rhinitis (AR), but the precise mechanism underlying neuroimmune regulation in patients with AR remains unclear. This study investigated the effect of cholinergic nerve inhibition on AR $CD4^+$ T-helper (Th)2-cell polarization and the underlying regulatory mechanism in vitro.**

Methods: **An in-vitro neuroimmune coculture model of D-U87 cells and $CD4^+$ T cells was established. D-U87 cells with cholinergic neuron characteristics were used as cholinergic neuron models. $CD4^+$ T cells were derived from peripheral blood monocytes from AR patients (n = 60) and control subjects (n = 40). Th1- and Th2-cell percentages were measured by flow cytometry. Proteins involved in related signaling pathways were analyzed by protein chip assay and Western blotting.**

Results: **The Th2-cell percentage among $CD4^+$ T cells from AR patients was significantly increased after coculture with D-U87 cells and was decreased by ipratropium bromide (IB) treatment. In contrast, the Th1-cell percentage among control $CD4^+$ T cells was significantly increased after coculture with D-U87 cells, but was unaltered by IB treatment. Furthermore, phosphorylated Akt (p-Akt) protein levels increased in $CD4^+$ T cells from both controls and AR patients after coculture with D-U87 cells and decreased after IB treatment. However, higher p-Akt levels were observed in cells from AR patients than in cells from control subjects. Moreover, Akt inhibition decreased Th2-cell percentage in AR patients.**

Conclusion: **In-vitro cholinergic nerve inhibition with IB decreased AR $CD4^+$ T-cell polarization into Th2 cells partially through an Akt-dependent mechanism.** © 2019 ARS-AAOA, LLC.

Key Words:
Akt; allergic rhinitis; ipratropium bromide; neuroimmune regulation; T helper cells

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Allergic rhinitis (AR) is a chronic, noninfectious inflammatory disease that is very common worldwide, with a prevalence of approximately 10% to 40%.[1] It is usually characterized by nasal discharge, obstruction, itching, and sneezing. Nasal parasympathetic nerve hypersensitivity plays a pivotal role in exacerbating nasal symptoms and nasal hypersecretion is closely related to muscarinic receptors.[2–5] According to our previous retrospective study, nasal vidian neurectomy alleviates nasal symptoms and improves the quality of life of patients with refractory AR who failed to respond to various medical treatments, including immunotherapy.[6] In addition, based on accumulating evidence, treatment with a muscarinic antagonist improves the nasal hypersecretion symptoms of patients with AR.[7–9] Although these clinical studies strongly imply the efficacy of inhibiting nasal parasympathetic or cholinergic nerve hyperactivity in patients with AR, the mechanism underlying the effect of nasal nerve fibers on the immune function of patients with AR remains unclear.

A growing body of research has demonstrated a mutual regulation between the neural and immune systems. The components of the cholinergic system, including acetylcholine (ACh), muscarinic and nicotinic receptors,

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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acetylcholinesterase, and choline acetyltransferase, were found to be expressed by T and B cells.[10] Treatment of dendritic cells with ACh stimulated expression of the Th2-promoter OX40L and the synthesis of interleukin (IL)-4, IL-5, and IL-13 by T cells.[11] ACh induced polarization of naive $CD4^+$ T cells toward the Th1, Th2, and Th17 lineages by acting on cholinergic receptors.[12] Posterior nasal neurectomy has been shown to lead to decreases in IL-5 and mast cells in severe AR.[13] Our previous studies showed that inhibition of cholinergic nerve hyperactivity using ipratropium bromide (IB), a nonselective muscarinic cholinergic receptor inhibitor, decreased expression of IL-4 mRNA in the nasal mucosa of BALB/c mice.[14,15] However, in a recent study, Nishijima et al reported that the expression of IL-4 and interferon-γ (IFN-γ) mRNAs was not altered in the nasal mucosa of Sprague-Dawley rats after nasal posterior neurectomy.[16] The discrepancy between these studies may be explained by the use of different research methods. Thus, studies exploring reciprocal regulation between the neural and immune systems in patients with AR will provide important clarification of these controversial issues.

In this study, a coculture model of cholinergic neurons and T cells was established, and the polarization of $CD4^+$ T cells was observed after different treatments. Changes in signaling molecules were detected by protein chips during the process of $CD4^+$ T-cell polarization. The mechanism of cholinergic neuron regulation of T cells was investigated in patients with AR.

Patients and methods

Development of cholinergic neuron-like D-U87 cells

Cholinergic neuron-like D-U87 cells were generated as described elsewhere.[17] The human glioblastoma cell line U87 was purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Authentication of the U87 cell line through short tandem repeat (STR) profiling was performed by Genetic Testing Biotechnology Corp (Suzhou, China). Cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gemini, Woodland, CA, USA) and incubated in a humidified incubator at 37°C with a 5% CO_2 atmosphere. After 24 hours of growth, the medium was replaced with induction medium comprised of 2 mmol/L sodium butyrate (Sigma Co, St Louis, MO) in RPMI 1640 medium without FBS for differentiation. The cells were allowed to differentiate for 3 days. The levels of cholinergic nerve–related choline acetyltransferase (ChAT), muscarinic receptor 1 (M1), and M3 proteins in D-U87 cells treated with IB (Selleck, Houston, TX, USA) were examined using Western blotting. The induction medium was supplemented with 10^{-5} mol/L IB to inhibit the activation of muscarinic receptors. The differentiated cell line derived from U87 cells with characteristics of cholinergic neurons was named D-U87 cells.

Patients

This study was approved by the ethics committee of the Third Xiangya Hospital of Central South University. Written informed consent was obtained from all patients. The patients were recruited at the Department of Otolaryngology from November 1, 2016 through November 1, 2018. The AR group consisted of 28 males and 32 females, aged 18 to 45 years. All included patients with AR were diagnosed with moderate-to-severe, persistent AR, based on the Chinese guideline for AR.[18] Nasal symptoms included "nasal congestion," "rhinorrhea," "itching," and "sneezing." Each nasal symptom score was rated on a 4-point scale where: 0 = no symptoms; 1 = mild symptoms; 2 = moderate symptoms; and 3 = severe symptoms. The nasal symptoms that occurred within 1 year were evaluated. A skin-prick test (SPT) was conducted to screen for allergens using a standard protocol (ALK-Abello kit, ALK, Hørsholm, Denmark), and SPT positivity was defined as a wheal diameter ≥3 mm greater than the diameter of the negative control. All patients with AR demonstrated a positive reaction to *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. None of the individuals included had taken any medication for at least 1 month before the study. The control group consisted of 21 male and 19 female healthy volunteers with no nasal symptoms and a negative SPT reaction. Exclusion criteria were as follows: (1) AR diagnosed as seasonal AR, or AR not rated as moderate to severe; (2) drug treatment or immunotherapy within the past month; (3) pregnancy, or age <18 years or >60 years; or (4) AR accompanied by allergic diseases of other organs, such as asthma or skin allergies.

Isolation of $CD4^+$ T cells

First, peripheral blood monocytes (PBMCs) were prepared from the peripheral blood of healthy volunteers and patients with AR using the Ficoll-Hypaque method. $CD4^+$ T cells were then separated through negative selection using magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. A cocktail of antibodies against CD8, CD14, CD15, CD16, CD19, CD56, CD123, T-cell receptor (TCR)-γ/δ, and CD235α was used to label and deplete non-CD4 T cells. Purity was assessed with flow cytometry using an anti-CD4 monoclonal antibody, and only populations of $CD4^+$ T cells with a purity of >95% were used. Cell viability was assessed using trypan blue staining and cells displaying 95% viability were used in subsequent experiments.

In-vitro coculture model and treatment

First, 10^5 U87 cells were seeded in 24-well plates and differentiated for 3 days. The differentiated U87 cells (D-U87 cells) were used as a neuron model for coculture with $CD4^+$ T cells. Before coculture, the medium was removed from the D-U87 cells and replaced with RPMI 1640 medium. Then, 2×10^5 purified $CD4^+$ T cells, obtained from PBMCs,

were added to the 24-well plates with D-U87 cells and co-cultured for 24 hours with RPMI 1640 medium lacking FBS. In some cases, IB (10^{-5} mol/L; Selleck) or the Akt inhibitor MK-2206 (50 nmol/L; Selleck) was incubated with the D-U87 neuronal cultures 30 minutes before addition of $CD4^+$ T cells.

Flow cytometry

The percentages of Th1 and Th2 cells were determined using flow cytometry. Briefly, $CD4^+$ T cells were collected, suspended in 250 μL of RPMI 1640 medium, and then stimulated with 1 μL of a Phorbol-12-myristate-13-acetate (PMA)/ionomycin mixture and 1 μL of a Brefeldin A (BFA)/monensin mixture (MultiSciences [Lianke] Biotech) for 5 hours in a humidified incubator at 37°C in a 5% CO_2 atmosphere. After washing with phosphate-buffered saline (PBS), the cells were stained with 1 μL of antigen-presenting cell (APC)-conjugated mouse anti-human CD3 and 1 μL of PerCP/Cy5.5-conjugated mouse anti-human $CD8^+$ antibodies (BioLegend, San Diego, CA, USA) for 20 minutes. Subsequently, cells were fixed and permeabilized (Fix & Perm Kit, MultiSciences [Lianke] Biotech) for 15 minutes at room temperature. Cells were washed with PBS and then incubated with 1 μL of flourescein isothiocyanate–conjugated mouse anti-human interferon-gamma (IFN-γ) and 1 μL of phycoerythrin-conjugated mouse anti-human IL-4 antibodies (BioLegend) for 20 minutes at room temperature. After washing, cells were detected using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lake, NJ, USA), and the results analyzed with FlowJo version 7.6 software (Tree Star, Ashland, OR, USA). The gating strategy was based on fluorescence minus one (FMO) controls, and single live events were gated based on FSC and SSC. Lymphocytes within the region of $CD3^+CD8^-$ T cells were gated as $CD4^+$ T cells. Because the percentage of $CD4^+$ T cells was defined among $CD3^+CD8^-$ T cells, $CD3^+CD8^-IFN\gamma^+$ cells were defined as Th1 cells and $CD3^+CD8^-IL4^+$ cells were defined as Th2 cells.

Cell-signaling protein chip analysis

The details of cell-signaling protein chip analysis are presented in the supplementary material online (Methods S1).

Western blotting

The details of western blotting are presented in the supplementary material online (Methods S2).

Statistical analysis

GraphPad Prism 6 software (GraphPad, La Jolla, CA) was used to analyze the data. An independent-sample *t* test was used to compare data from the controls and patients with AR. The Kruskal-Wallis test and Dunn's multiple comparisons were used to determine the significance of differences between multiple groups. Values are presented as mean ± standard deviation (SD). $p < 0.05$ was considered statistically significant.

Results

Verification of cholinergic neuron characteristics in D-U87 cells

In our previous study, D-U87 cells demonstrated cholinergic neuron characteristics.[17] To further verify cholinergic functions in D-U87 cells, the levels of cholinergic nerve–related proteins in D-U87 cells treated with IB were observed. We found that the levels of ChAT, a specific marker of cholinergic neurons, as well as M1 and M3, were increased significantly in D-U87 cells and decreased after treatment with IB (Fig. 1). These data indicate that D-U87 cells exhibited cholinergic nerve function, which can be inhibited by IB treatment.

Patients' characteristics

Patients' characteristics are summarized in Table S1 online. No significant differences in gender or age were observed between the 2 groups. In contrast, the number of patients with a positive SPT for AR patients was greater than the number of individuals with a positive SPT in the control group. In addition, the AR group exhibited significantly higher nasal symptom scores than the control group. Furthermore, as shown in Figure S1 (online), patients with AR had a higher percentage of Th2 cells than the control individuals. The graphical gating strategy is described in Figure S2 (online).

Effect of D-U87 cells on $CD4^+$ T-cell polarization

We used an in-vitro coculture model of 10^5 D-U87 cells with 2×10^5 $CD4^+$ T cells to assess the effect of neuronal D-U87 cells on $CD4^+$ T-cell polarization. After 24 hours of coculture, $CD4^+$ T cells from patients with AR tended to polarize into Th2 cells, whereas cells from the healthy controls tended to polarize into Th1 cells (Fig. 2). In particular, among cells derived from patients with AR, the percentage of Th2 cells was significantly increased in the coculture group compared with that in the $CD4^+$ T-cell-only culture group, whereas the percentage of Th1 cells was not obviously different. In contrast, for cells derived from healthy controls, the percentage of Th1 cells increased significantly in the coculture group compared with the $CD4^+$ T-cell-only culture group, yet the percentage of Th2 cells did not exhibit an obvious change. Based on these data, D-U87 neuronal cells promoted the polarization of $CD4^+$ T cells from patients with AR into Th2 cells and promoted polarization of $CD4^+$ T cells from healthy controls toward the Th1-cell phenotype.

Effect of IB on polarization of $CD4^+$ T cells from patients with AR

The aforementioned in-vitro coculture model of D-U87 cells and $CD4^+$ T cells showed that D-U87 cells promoted polarization of $CD4^+$ T cells into completely different cell types, depending on whether the cells were obtained from healthy controls or patients with AR. Therefore, we sought

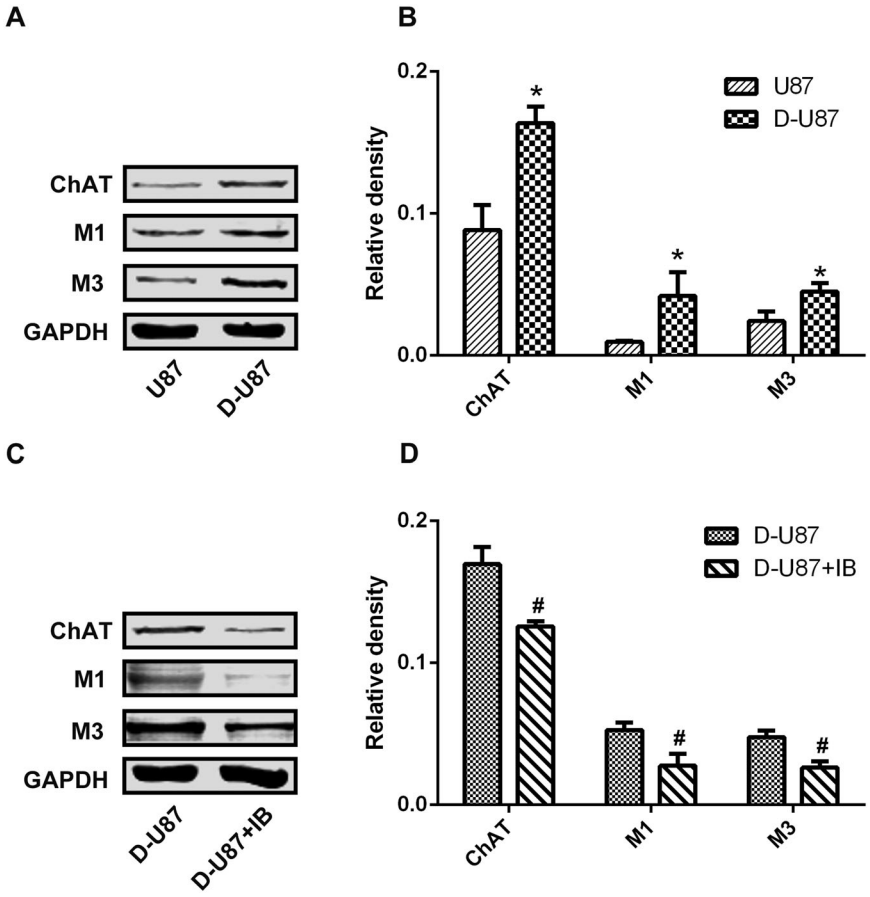

FIGURE 1. Levels of ChAT, M1, and M3 proteins in D-U87 cells treated with IB. (A) Representative Western blot bands and (B) densitometry of ChAT, M1, and M3 levels in D-U87 cells. (C) Representative Western blot bands and (D) densitometry showing changes in the levels of ChAT, M1, and M3 proteins in the D-U87 cells after IB treatment. The data represent results from at least 3 independent experiments (mean ± standard deviation). ChAT = choline acetyltransferase; D-U87 = U87 cells differentiated for 3 days; D-U87+IB = D-U87 cells treated with IB; IB = ipratropium bromide; M1 = muscarinic receptor 1; M3 = muscarinic receptor 3. $^{*}p < 0.05$ vs U87; $^{\#}p < 0.05$ vs D-U87.

to further investigate whether IB reversed the observed polarization trends. As shown in Figure 3, for cells derived from patients with AR, IB treatment significantly decreased the percentage of Th2 cells in $CD4^+$ T cells cocultured with D-U87 cells but did not change the percentage of Th2 cells in the $CD4^+$ T-cell-only cultures. On the other hand, IB did not exert a significant effect on the percentages of Th1 cells in both $CD4^+$ T-cell-only cultures and cocultures. In contrast, for cells derived from healthy controls, IB treatment did not exert a significant effect on the percentages of Th1 and Th2 cells in either $CD4^+$ T-cell-only cultures or cocultures. Thus, IB treatment reversed D-U87-cell-induced Th2 polarization of cells derived from patients with AR, but it did not exert a significant effect on the D-U87-cell-induced Th1 polarization of cells derived from healthy controls.

Changes in phosphorylated Akt expression in $CD4^+$ T cells after coculture or IB treatment

We intended to screen some signaling molecules using a protein chip to clarify the mechanism by which D-U87 cells induced Th2 polarization of cells from patients with AR. Nineteen signaling molecules related to the regulation of immune and inflammatory responses were identified. As shown in Figure 4A and B, phosphorylated Akt (p-Akt) levels were significantly increased in the coculture group and decreased after IB treatment in both the healthy control and AR groups. However, higher p-Akt expression was observed in the AR group than in the healthy control group. Western blot results (Fig. 4C and D) are similar to those from the protein chip analysis. For both the healthy control and AR group, the relative intensity of p-Akt in the coculture group increased significantly compared with that in the $CD4^+$ T-cell-only culture group, and decreased after IB treatment. However, significantly higher p-Akt levels were observed in the AR group than in the healthy control group.

Effect of Akt inhibition on Th2 polarization of cells from AR group

We analyzed the effect of an Akt inhibitor on the Th2 polarization of cells from patients with AR to verify that Akt participated in the mechanism regulating the process

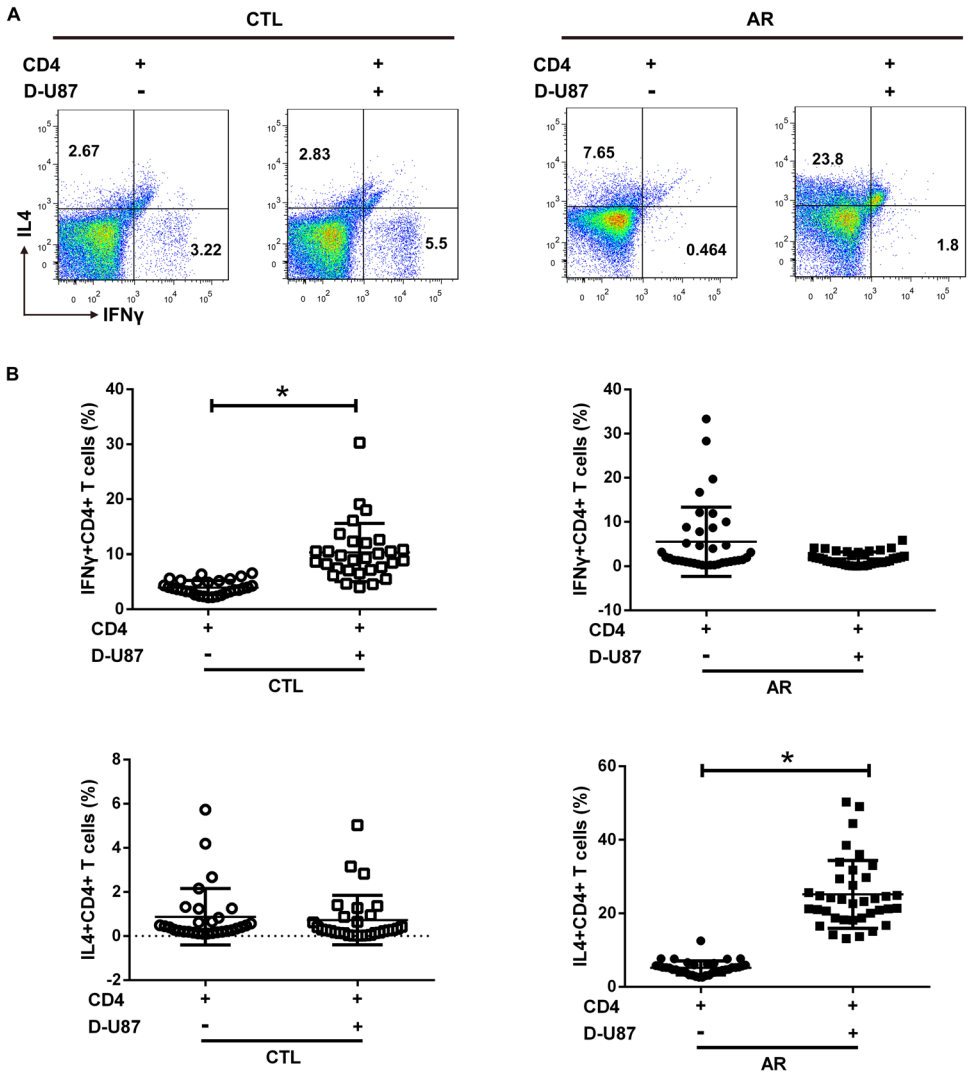

FIGURE 2. Polarization of CD4$^+$ T cells from patients with AR after coculture with D-U87 cells. (A) Representative dot plots comparing the percentages of Th1 and Th2 cells between CD4$^+$ T-cell-only cultures and cocultures of cells from healthy controls and patients with AR. (B) Percentages of Th1 and Th2 cells after CD4$^+$ T cells were cocultured with D-U87 cells compared with the culture of CD4$^+$ T cells alone (n = 30 for CTL, n = 37 for AR). Each symbol represents 1 donor. AR = allergic rhinitis; CD4 = CD4$^+$ T-cell-only culture; D-U87 = D-U87 neuronal cells; +/− = cultured with/without the indicated cells. $^*p < 0.05$ vs CD4$^+$ T-cell-only cultures.

just described. As shown in Figure 5, the Akt inhibitor significantly decreased the percentage of Th2 cells among CD4$^+$ T cells from the AR group that were cocultured with D-U87 cells. However, the Akt inhibitor did not exert significant effects on the percentage of Th2 cells among the CD4$^+$ T-cell-only cultures and the percentages of Th1 cells in the CD4$^+$ T-cell-only cultures and cocultures from the AR group. In contrast, in the healthy control group, the Akt inhibitor did not exert significant effects on the percentages of Th1 and Th2 cells in both the CD4$^+$ T-cell-only cultures and CD4$^+$ T-cell cocultures. Moreover, the effect of Akt inhibitor on CD4$^+$ T-cell viability was measured via live/dead staining. The results show that the percentage of live cells and the percentages of Th1 and Th2 cells in CD4$^+$ T cells were not significantly changed by the Akt inhibitor (see Fig. S3 online).

Discussion

Parasympathetic nerves play an important role in the pathogenesis of AR.[2,7,19,20] However, the mechanisms underlying the effects of parasympathetic nerves on immune cells in patients with AR are not clear. Although increasing evidence indicates the importance of parasympathetic nerves in the immune function of patients with other systematic diseases, studies of neuroimmune regulation in the field of AR have rarely been reported.[11,16,21-23] In the present study, by establishing an in-vitro neuroimmune coculture model of D-U87 neurons and CD4$^+$ T cells, we observed that cholinergic neuron-like D-U87 cells promoted the polarization of CD4$^+$ T cells from patients with AR toward Th2 cells, and inhibition of the cholinergic activity of D-U87 cells by IB decreased the preferential Th2 polarization by modulating Akt signaling.

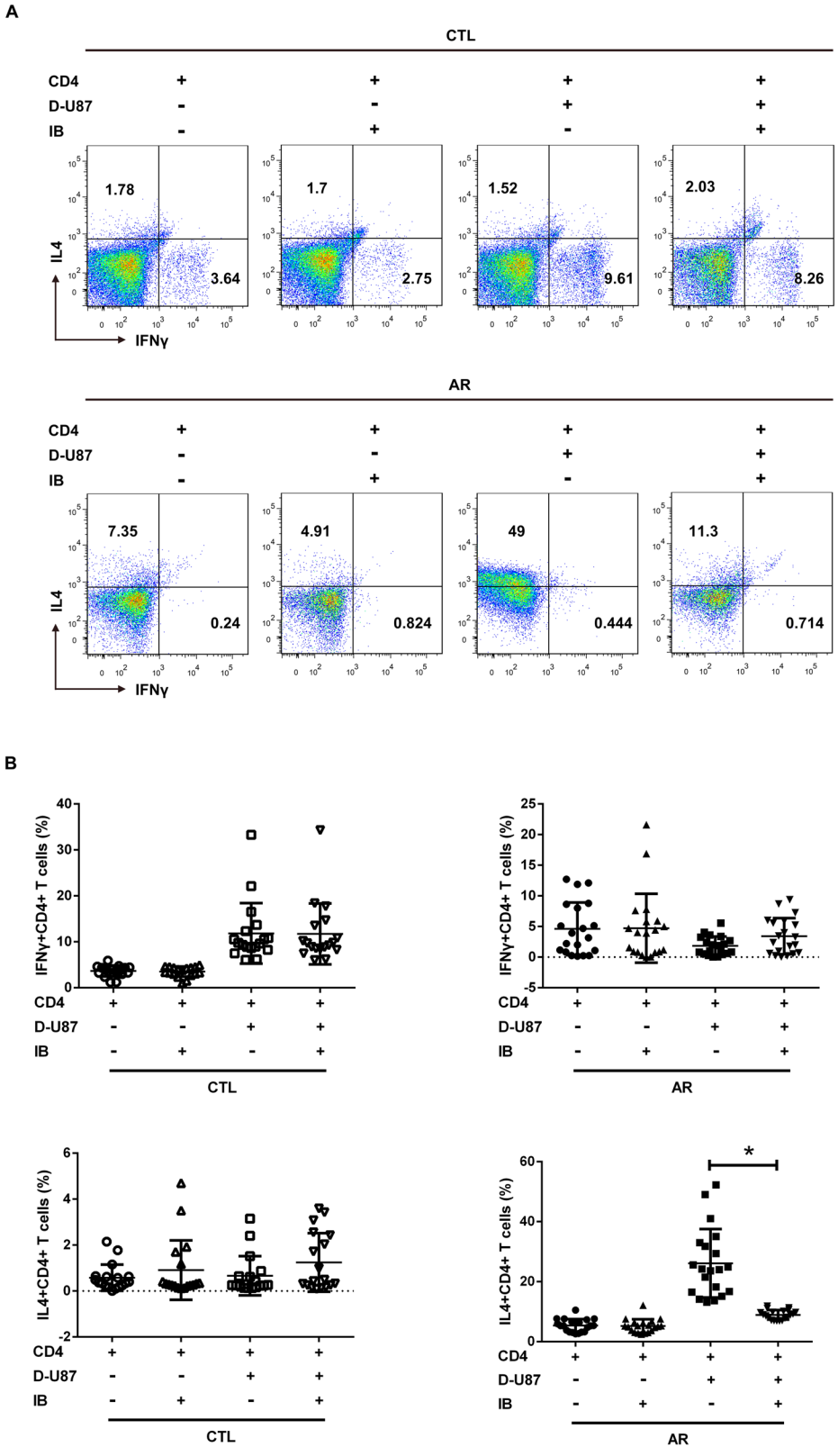

FIGURE 3. Effect of IB on the D-U87-cell-induced polarization of $CD4^+$ T cells from patients with AR. (A) Representative dot plots of Th1 and Th2 cells in $CD4^+$ T-cell-only cultures and cocultures treated with/without IB. (B) Percentages of Th1 and Th2 cells among $CD4^+$ T cells treated with IB (n = 18 for CTL, n = 20 for AR). Each symbol represents 1 donor. CD4 = $CD4^+$ T-cell-only culture; D-U87 = D-U87 cells; IB = ipratropium bromide; Th1 and Th2 = T-helper 1 and 2; +/− = cultured with/without the indicated cells/treatment. $^*p < 0.05$ vs coculture group.

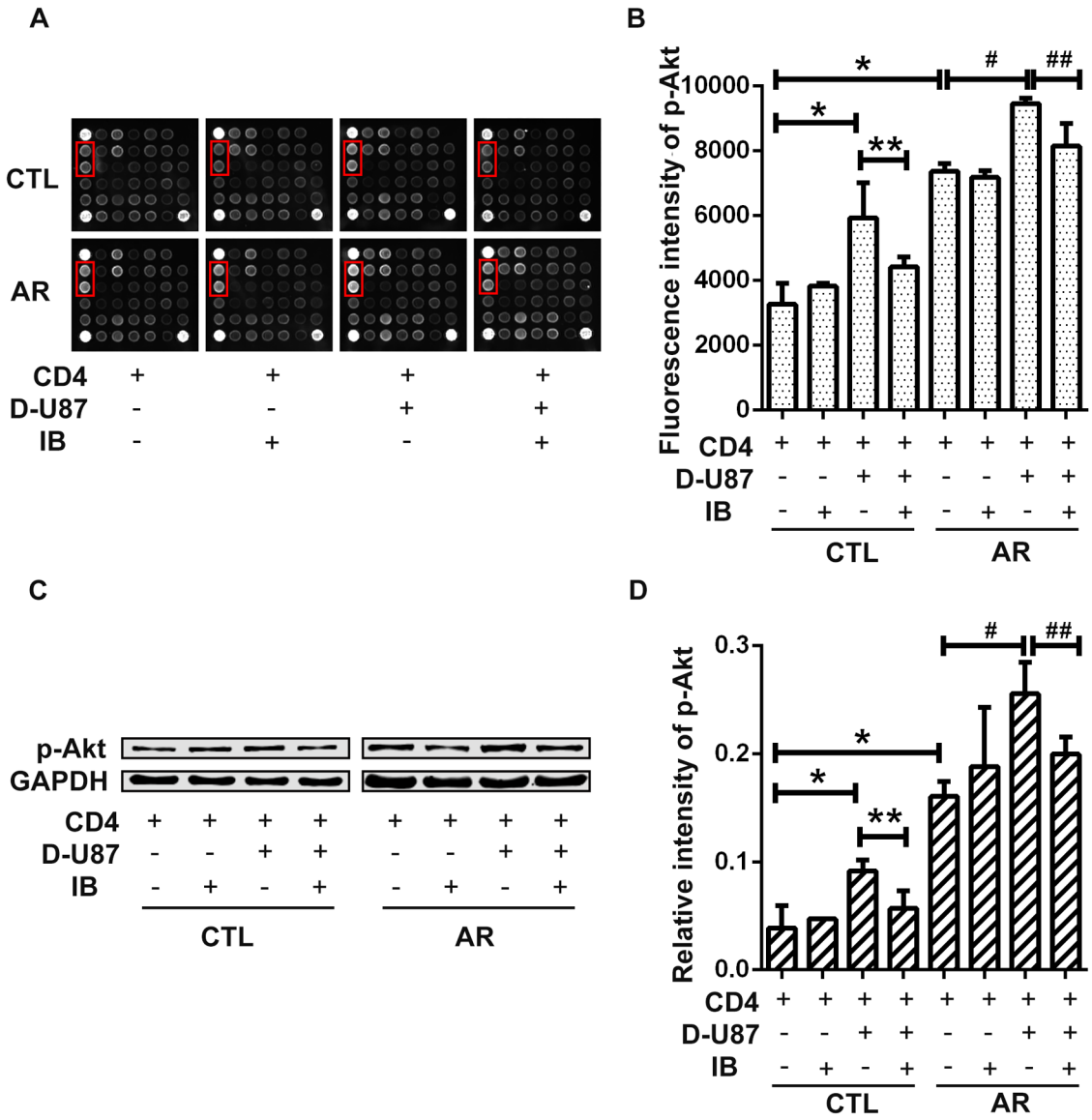

FIGURE 4. Levels of p-Akt protein in CD4$^+$ T cells after coculture with D-U87 cells and treatment with IB. (A) Representative fluorescence images acquired using the LI-COR® Odyssey imaging system. Each spot represents a specific antibody, and the red rectangular box shows the p-Akt (Ser473) spot. (B) Bar graph showing the fluorescence intensity of the p-Akt spot. (C) Representative Western blot bands for p-Akt. (D) Densitometry analysis of the Western blot bands. Data represent the results from at least 3 independent experiments (mean ± standard deviation). CD4 = CD4$^+$ T-cell-only culture; D-U87 = D-U87 cells; IB = ipratropium bromide; p-Akt = phosphorylated Akt; +/− = cultured with/without the indicated cells/drug. $^*p < 0.05$ vs control CD4$^+$ T-cell group; $^{**}p < 0.05$ vs control CD4$^+$ T cells cocultured with D-U87 neurons; $^{\#}p < 0.05$ vs AR CD4$^+$ T-cell group; $^{\#\#}p < 0.05$ vs AR CD4$^+$ T cells cocultured with D-U87 neurons.

To facilitate investigation of the role of cholinergic nerve function in patients with AR, an in-vitro cholinergic neuron model must be established. In this study, the human glioblastoma cell line U87 was used as our in-vitro model. Generally, cancer cells exhibit multilineage differentiation potential. Our previous study revealed that U87 cells differentiate into cells and show characteristics of cholinergic neurons.[17] In the present study, we further applied sodium butyrate, a common inhibitor of cell proliferation,[24-26] for 3 days to induce the differentiation of U87 cells. The levels of the cholinergic nerve–related ChAT, M1, and M3 proteins were significantly increased in D-U87 cells and decreased after IB treatment, indicating that D-U87 cells present characteristics of cholinergic neurons and are sensitive to IB. Therefore, we used the D-U87 neurons that had undergone differentiation for 3 days as a neuronal model in the subsequent experiments.

Most clinical investigations and animal studies have revealed an important role for nerves in AR[2,7]; however, controversy exists regarding whether parasympathetic nerves exert an effect on immune regulation. In our previous studies, nasal vidian neurectomy achieved a good therapeutic effect in patients with refractory AR, and IB has been shown to reverse the nasal mucosal Th2 advantage in BALB/c mice.[6,14,15] Similar to our previous studies, in the present in-vitro neuroimmune coculture

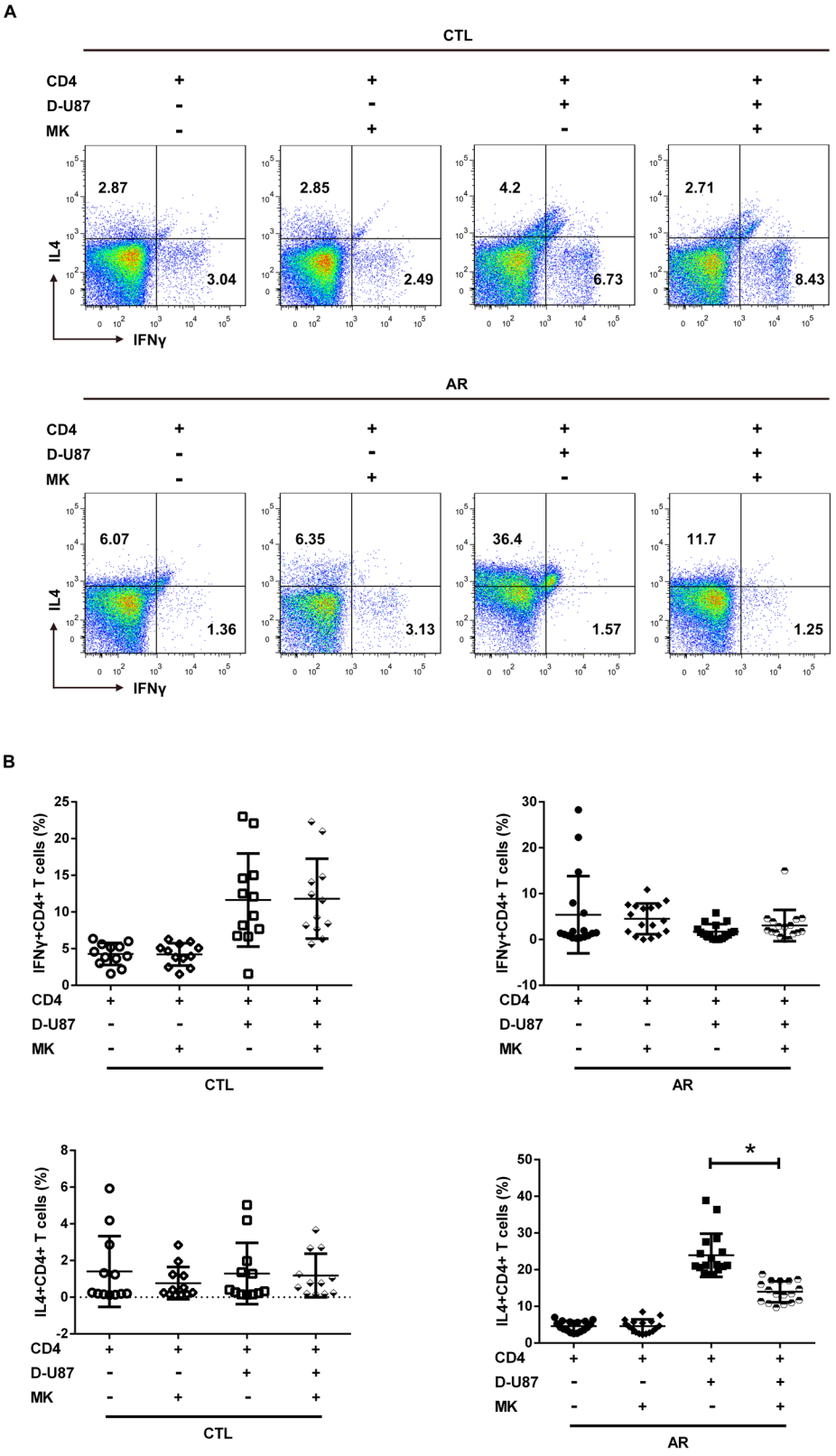

FIGURE 5. Effect of an Akt inhibitor on polarization of $CD4^+$ T cells from the AR group induced by D-U87 cells. (A) Representative dot plots of the percentages of Th1 and Th2 cells among $CD4^+$ T-cell-only cultures and cocultures from the healthy control and AR groups treated with/without MK-2206 (Akt inhibitor). (B) Percentages of Th1 and Th2 cells after $CD4^+$ T cells treated with and without MK-2206 (n = 12 for CTL, n = 17 for AR). $CD4^+$ Each symbol represents 1 donor. Cocultures were used as a control treatment group. AR = allergic rhinitis; CD4 = $CD4^+$ T-cell-only culture; CTL = control; D-U87 = D-U87 cells; MK = treated with MK-2206 Akt inhibitor; +/− = cultured with/without the indicated cells/drug Th1 and Th2 = T-helper 1 and 2. $^*p < 0.05$ vs coculture group.

study, D-U87 neurons promoted the polarization of $CD4^+$ T cells from patients with AR toward Th2 cells, and the increased percentage of Th2 cells was then reduced by IB treatment. Interestingly, in contrast to observations in cells from patients with AR, $CD4^+$ T cells from healthy controls polarized into Th1 cells after coculture with D-U87 neurons. However, IB treatment had no effect on the Th1 polarization induced by D-U87 cells. We propose several explanations for the completely opposite trends in Th2 and Th1 polarization observed between patients with AR and healthy individuals who were influenced by the D-U87 neurons and IB. First, Th2 polarization may be more closely related to muscarinic cholinergic receptors, whereas Th1 polarization may be strongly correlated with other neurotransmitter receptors, such as adrenergic receptors.[12,27] Second, the intrinsic differences between patients with AR and healthy subjects may also explain the differences. The $CD4^+$ T cells of patients with AR and healthy subjects are characterized by a Th2 advantage and a Th1 advantage, respectively, which provide completely different conditions for $CD4^+$ T-cell polarization. For example, increases in M1 and M5 levels are related to Th2 polarization, whereas a decrease in M3 levels is related to Th1 polarization.[12] In addition, stimulation of M1 and M3 increases the levels of secreted Th2-related cytokines, such as IL-2, IL-4, IL-10, and OX40, which promote Th2-related inflammation and inhibition of production of the Th1 cytokine IFN-γ.[11,23,28,29] Furthermore, these Th2-promoting cytokines, such as IL-4, not only contribute to Th2 polarization but also increase M1 and M3 activation.[12,27] Therefore, muscarinic receptors and IL-4 may participate in bidirectional communication and collaboratively contribute to Th2 polarization. Moreover, peripheral blood lymphocytes from patients with AR expressed M2 and M5 at higher levels than cells from healthy subjects,[30] suggesting that lymphocytes from patients with AR may be more sensitive to muscarinic receptor stimulation than lymphocytes from healthy individuals. These studies revealed a strong relationship between muscarinic cholinergic receptors and Th2 polarization. In our study, D-U87 cells exhibited decreased levels of M1 and M3 after IB treatment. We postulate that M1 and M3 may participate in the Th2 polarization process in cells from patients with AR. However, Th1 polarization appears to be more closely related to nonmuscarinic receptors based on the following reasons: (1) the stimulation of M1 and M3 inhibits secretion of the Th1 cytokine IFN-γ, and the stimulation of nicotinic receptor increases the level of IFN-γ[12]; and (2) the β_2-adrenergic receptor contributes to Th1 polarization, and activation of the calcitonin gene–related peptide (CGRP) receptor contributes to T-regulatory-cell polarization, which promotes Th1 polarization.[31] These findings indicate that D-U87 cells could induce T cells from healthy individuals to polarize into Th1 cells by activating CGRP or nicotinic receptors, but not muscarinic receptors, because the addition of IB had no effect on this process.

Akt regulates cell survival, proliferation, apoptosis, and differentiation. Although Akt is not related to Th2 polarization,[32] Akt activation was recently shown to positively regulate Th2 polarization. It was shown that: (1) activated Akt stimulates T-cell activation[33]; (2) Nox2-deficient T cells undergo Th2 polarization by increasing Akt activation[34]; (3) OX40L contributes to Th2 and Th17 polarization by activating PI3/Akt signaling[35]; (4) higher levels of the Akt protein were observed in splenic $CD4^+$ T cells from AR mice[36]; and (5) IL-4 also enhances Akt activation.[37] Consistent with these findings, higher levels of the Akt protein have been detected in cells from patients with AR than in cells from healthy controls in the present study, and an Akt inhibitor decreased Th2 polarization of cells from patients with AR but had no effect on Th1 polarization. Moreover, some studies revealed that muscarinic receptor activation promotes Akt activation,[38-42] indicating that muscarinic receptor activation may have a close relationship with Akt activation. In our study, IB not only reduced D-U87-cell-induced polarization of $CD4^+$ T cells from patients with AR into Th2 cells but also decreased the levels of the Akt protein. In addition, the Akt inhibitor also reduced the D-U87-cell-induced polarization of $CD4^+$ T cells from patients with AR into Th2 cells. Based on these results, the Akt pathway participates in D-U87-cell-induced polarization of $CD4^+$ T cells from patients with AR. In contrast, Akt inhibitor or IB treatment did not affect polarization of $CD4^+$ T cells from healthy controls, suggesting that D-U87 neurons may positively regulate the polarization of $CD4^+$ T cells from healthy individuals into Th1 cells through other neurotransmitter signaling pathways rather than through the M1 or M3/Akt pathway. Nevertheless, the exact mechanism by which cholinergic neurons promote $CD4^+$ T-cell polarization requires further study.

The in-vitro coculture model provides a convenient method for studies of the interactions between nerves and immune cells in patients with AR. However, additional studies are needed to confirm the roles of other neurotransmitter receptors and signaling molecules in immune-cell polarization.

Conclusion

Cholinergic neuron-like D-U87 cells contribute to the polarization of T cells from patients with AR, partially through Akt signaling. These findings expand our knowledge of the mechanism underlying cholinergic nerve–mediated inhibition of immune function in patients with AR. We believe that our findings provide insights for future studies investigating the crosstalk between cholinergic nerves and $CD4^+$ T cells in patients with AR.

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References

1. Brozek JL, Bousquet J, Agache I, et al. Allergic rhinitis and its impact on asthma (ARIA) guidelines—2016 revision. *J Allergy Clin Immunol*. 2017;140:950-958.
2. Druce HM, Wright RH, Kossolf D, Kaliner MA. Cholinergic nasal hyperreactivity in atopic subjects. *J Allergy Clin Immunol*. 1985;76:445-452.
3. Ishibe T, Yamashita T, Kumazawa T, Tanaka C. Adrenergic and cholinergic receptors in human nasal mucosa in cases of nasal allergy. *Arch Otorhinolaryngol*. 1983;238:167-173.
4. Konno A, Terada N, Okamoto Y. Changes of adrenergic and muscarinic cholinergic receptors in nasal mucosa in nasal allergy. *ORL J Otorhinolaryngol Relat Spec*. 1987;49:103-111.
5. Namimatsu A, Okazaki R, Go K, Hata T. Relationship between density of muscarinic receptors and nasal hypersecretion in a nasal allergic model. *Jpn J Pharmacol*. 1992;59:427-434.
6. Tan G, Ma Y, Li H, Li W, Wang J. Long-term results of bilateral endoscopic vidian neurectomy in the management of moderate to severe persistent allergic rhinitis. *Arch Otolaryngol Head Neck Surg*. 2012;138:492-497.
7. Kim D, Baraniuk JN. Neural aspects of allergic rhinitis. *Curr Opin Otolaryngol Head Neck Surg*. 2007;15:268273.
8. Long R, Zhou Y, Huang J, et al. Bencycloquidium bromide inhibits nasal hypersecretion in a rat model of allergic rhinitis. *Inflamm Res*. 2015;64:213-223.
9. Greiner AN, Meltzer EO. Overview of the treatment of allergic rhinitis and nonallergic rhinopathy. *Proc Am Thorac Soc*. 2011;8:121-131.
10. Kawashima K, Fujii T, Moriwaki Y, Misawa H. Critical roles of acetylcholine and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune function. *Life Sci*. 2012;91:1027-1032.
11. Gori S, Vermeulen M, Remes-Lenicov F, et al. Acetylcholine polarizes dendritic cells toward a Th2-promoting profile. *Allergy*. 2017;72:221-231.
12. Qian J, Galitovskiy V, Chernyavsky AI, Marchenko S, Grando SA. Plasticity of the murine spleen T-cell cholinergic receptors and their role in in vitro differentiation of naive CD4 T cells toward the Th1, Th2 and Th17 lineages. *Genes Immun*. 2011;12:222-230.
13. Ogawa T, Takeno S, Ishino T, Hirakawa K. Submucous turbinectomy combined with posterior nasal neurectomy in the management of severe allergic rhinitis: clinical outcomes and local cytokine changes. *Auris Nasus Larynx*. 2007;34:319-326.
14. Daoud A, Xie Z, MA Y, Wang T, Tan G. Changes of T-helper type 1/2 cell balance by anticholinergic treatment in allergic mice. *Ann Allergy Asthma Immunol*. 2014;112:249-255.
15. Hou M, Li W, Xie Z, et al. Effects of anticholinergic agent on miRNA profiles and transcriptomes in a murine model of allergic rhinitis. *Mol Med Rep*. 2017;16:6558-6569.
16. Nishijima H, Kondo K, Toma-Hirano M, et al. Denervation of nasal mucosa induced by posterior nasal neurectomy suppressed nasal secretion, not hypersensitivity, in an allergic rhinitis rat model. *Lab Invest*. 2016;96:981-993.
17. Liu H, Xia J, Wang T, et al. Differentiation of human glioblastoma U87 cells into cholinergic neuron. *Neurosci Lett*. 2019;704:1-7.
18. Cheng L, Chen JJ, Fu QL, et al. Chinese Society of Allergy guidelines for diagnosis and treatment of allergic rhinitis. *Allergy Asthma Immunol Res*. 2018;10:300-353.
19. Sarin S, Undem B, Sanico A, Togias A. The role of the nervous system in rhinitis. *J Allergy Clin Immunol*. 2006;118:999-1016.
20. Tai CF, Baraniuk JN. Upper airway neurogenic mechanisms. *Curr Opin Allergy Clin Immunol*. 2002;2:11-19.
21. Pongratz G, Straub RH. Role of peripheral nerve fibres in acute and chronic inflammation in arthritis. *Nat Rev Rheumatol*. 2013;9:117-126.
22. Olofsson PS, Steinberg BE, Sobbi R, et al. Blood pressure regulation by CD4+ lymphocytes expressing choline acetyltransferase. *Nat Biotechnol*. 2016;34:1066-1071.
23. Liu T, Xie C, Chen X, et al. Role of muscarinic receptor activation in regulating immune cell activity in nasal mucosa. *Allergy*. 2010;65:969-977.
24. Han R, Sun Q, Wu J, Zheng P, Zhao G. Sodium butyrate upregulates miR-203 expression to exert antiproliferation effect on colorectal cancer cells. *Cell Physiol Biochem*. 2016;39:1919-1929.
25. Mu D, Gao Z, Guo H, Zhou G, Sun B. Sodium butyrate induces growth inhibition and apoptosis in human prostate cancer DU145 cells by upregulation of the expression of Annexin A1. *PLoS One*. 2013;8:e74922.
26. Chang MC, Tsai YL, Liou EJ, et al. Effect of butyrate on collagen expression, cell viability, cell cycle progression and related proteins expression of MG-63 osteoblastic cells. *PLoS One*. 2016;11:e0165438.
27. Nakanishi M, Furuno T. Molecular basis of neuroimmune interaction in an in vitro coculture approach. *Cell Mol Immunol*. 2008;5:249-259.
28. Fujii T, Kawashima K. YM905, a novel M3 antagonist, inhibits Ca^{2+} signaling and c-fos gene expression mediated via muscarinic receptors in human T cells. *Gen Pharmacol*. 2000;35:71-75.
29. Nomura J, Hosoi T, Okuma Y, Nomura Y. The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system. *Life Sci*. 2003;72:2121-2126.
30. Ricci A, Mariotta S, Amenta F, Tayebati SK, Terzano C. Changes in muscarinic cholinergic receptor expression in human peripheral blood lymphocytes in allergic rhinitis patients. *Pulm Pharmacol Ther*. 2008;21:79-87.
31. Szklany K, Ruiter E, Mian F, et al. Superior cervical ganglia neurons induce Foxp3+ regulatory T cells via calcitonin gene-related peptide. *PLoS One*. 2016;11:e0152443.
32. Kane LP, Andres PG, Howland KC, Abbas AK, Weiss A. Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not Th2 cytokines. *Nat Immunol*. 2001;2:37-44.
33. Patra AK, Na SY, Bommhardt U. Active protein kinase B regulates TCR responsiveness by modulating cytoplasmic-nuclear localization of NFAT and NF-B proteins. *J Immunol*. 2004;172:4812-4820.
34. Kwon B, Kim TW, Shin K, et al. Enhanced Th2 cell differentiation and function in the absence of Nox2. *Allergy*. 2017;72:252-265.
35. Huang L, Wang M, Yan Y, et al. OX40L induces helper T cell differentiation during cell immunity of asthma through PI3K/AKT and P38 MAPK signaling pathway. *J Transl Med*. 2018;16:74.
36. Hu T, Fan X, Ma L, et al. TIM4-TIM1 interaction modulates Th2 pattern inflammation through enhancing SIRT1 expression. *Int J Mol Med*. 2017;40:1504-1510.
37. Reinhart R, Kaufmann T. IL-4 enhances survival of in vitro-differentiated mouse basophils through transcription-independent signaling downstream of PI3K. *Cell Death Dis*. 2018;9:713.
38. Damiano S, Sasso A, Accetta R, et al. Effect of mutated Cu, Zn superoxide dismutase (SOD1(G93A)) on modulation of transductional pathway mediated by M1 muscarinic receptor in SK-N-BE and NSC-34 cells. *Front Physiol*. 2018;9:611.
39. Luo L, Xi C, Xu T, et al. Muscarinic receptor mediated signaling pathways in hepatocytes from CCL4-induced liver fibrotic rat. *Eur J Pharmacol*. 2017;807:109-116.
40. Ahmed M, VanPatten S, Lakshminrusimha S, et al. Effects of novel muscarinic M3 receptor ligand C1213 in pulmonary arterial hypertension models. *Physiol Rep*. 2016;4:e13069.
41. Resende RR, Adhikari A. Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. *Cell Commun Signal*. 2009;7:20.
42. Krieg T, Qin Q, Philipp S, et al. Acetylcholine and bradykinin trigger preconditioning in the heart through a pathway that includes Akt and NOS. *Am J Physiol Heart Circ Physiol*. 2004;287:H2606-H2611.