

Role of muscarinic receptor activation in regulating immune cell activity in nasal mucosa

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Abstract

Background: The prevalence of airway inflammatory disorders keeps rising; its pathogenic mechanism is still not fully understood.

Objective: The present study aimed to investigate the role of muscarinic receptor (M receptor) in regulating the immune cell activity in nasal mucosa by using surgical removed nasal mucosa from patients with nasal polyposis (NP) as a study platform.

Methods: Human nasal mucosal sample was collected from inferior turbinectomy of 86 patients with NP or/and allergic rhinitis. Expression of tumor necrosis factor alpha (TNF-alpha), M receptor, OX40 ligand was measured in nasal mucosa by enzyme-linked immunosorbent assay, flow cytometry, and Western blotting assay.

Results: When compared with non-NP (nNP) nasal mucosa, contents of TNF-alpha and TNF-alpha(+) cells markedly increased in NP nasal mucosa; immune staining colocalized M3 receptor(+) and TNF-alpha(+) cells in NP nasal mucosa; exposure of isolated CD4(+) T cells to methacholine induced the release of TNF-alpha. We also found CD11c(+)/M3 receptor(+) cells in NP nasal mucosa. Methacholine increased the expression of OX40L in dendritic cells. Staphylococcal (S) aureus and S. enterotoxin B (SEB) were detected in NP nasal mucosa. Exposure of dendritic cells or naïve CD4(+) T cells to SEB initiated the expression of M3 receptor at mRNA and protein levels.

Conclusions: The present data demonstrate that parasympathetic activity has the capacity to activate dendritic cells to release OX40 ligand, the latter induces CD4(+) T cells to produce IL-4 and TNF-alpha that may further contribute to the pathogenesis of NP.

Nasal polyposis (NP) features as a chronic immune inflammation in nasal mucosa that is usually arise from inflammatory nasal mucosa complicating with chronic rhinosinusitis; the prevalence of chronic rhinosinusitis is >10% of the general population in the world (1). The pathogenesis is not fully elucidated. The treatment of NP is not satisfactory currently. Recurrence of NP after surgical remove or other treatments is very often (2, 3) because the causative factors of NP are not clear.

Not only causing the nasal stuffiness, NP is also associated with inflammatory disorders in the lower airways, such as hypersensitivity reaction to normal antigens. Over 84% of sinonasal CT scans shows abnormal results in severe asthmatic patients (4). Asthmatic patients usually benefit from removing the pathogenic tissue from nasal cavity and sinuses (5). Because T helper 2 (Th2) cytokines and tumor necrosis factor α (TNF- α) were found in NP nasal mucosa (6), these mediators may be released and

absorbed into the blood stream to affect the immune homeostasis in other organs, such as in the lower airways, of the body, but the direct evidence is still scarcely available.

TNF- α is a proinflammatory cytokine that is involved in an array of inflammatory process such as asthma (7), NP (6, 8), inflammatory bowel disease (9), and rheumatic arthritis (10). Yet apart from the finding that levels of TNF- α were increased in NP nasal mucosa, from which cell type the TNF- α derived from is not clear.

Nasal mucosal DCs from chronic nonallergic rhinitis (nAR) patients or healthy subjects are positive for CD1a, HLA-DR, CD11c, CD116, CD1c, TLR4, CD40, CD80, CD86, and CCR6 (11). High levels of CD86 were detected in dendritic cells (DC) of AR nasal mucosa (12). Among the cell markers, CD11c is generally selected as DC markers (13) although it is overlapped with macrophages. It is proposed that OX40/OX40 ligand (OX40L) interaction in DCs induces CD4⁺ T cells to produce TNF- α and IL-4 (14) that plays an important role in the pathogenesis of immune inflammation such as airway allergy (15). Activated DCs produce OX40L upon proper stimulation such as exposure to thymic stromal lymphopoietin (16). However, little information is published yet on whether nasal mucosal DCs produce OX40L.

It is accepted that cholinergic activity plays a role in nasal mucosal inflammation. Five types of muscarinic receptors (M receptor) were identified in the blood vessels and glands in nasal mucosa (16, 17); whether immune cells in nasal mucosa express M receptors is not clear. On the other hand, microbial products have a close relation with the regulation of immune responses (18, 19); but whether microbial products modulate the parasympathetic activity, and immune reaction in the nasal mucosa is not fully understood. Therefore, based on the previous information, we hypothesized that microbial product can be one of the important factors that modulate the immune responses in the nasal mucosa to play an important role in the pathogenesis of NP. The present study used surgical removed nasal mucosa as a study platform and aimed to analyze (i) TNF- α contents and TNF- α ⁺ cells in NP nasal mucosa; (ii) M receptor expression/activation influencing the function of nasal mucosal immune cells; and (iii) microbial products modulating the expression of M receptor in immune cells of nasal mucosa. The results showed that microbial products up regulate the expression of M3 receptor in nasal mucosal immune cells that further increases the production of OX40L in nasal DCs and drives the production of TNF- α in nasal mucosa.

Materials and methods

Reagents

IgE antibody detection system (CAP system, Pharmacia Diagnosis, Sweden). Magnetic cell sorting reagents (Miltenyi Biotec, Auburn, CA, USA). ELISA kits of TNF- α and OX40L (R&D Systems, Burlington, ON, Canada). Methacholine, collagenase D, DNase I, dispase II, and DTT (Sigma Aldrich, Oakville, ON, Canada). All the fluorescently labeled antibodies for flow cytometry assay, permeable reagent kit (BD Bioscience, Mississauga, ON, Canada). Anti-TNF- α , OX40L, beta-actin, and M3 receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). iScriptTM cDNA Synthesis Kit; nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). SuperScript III Platinum SYBR Green Two-Step qPCR Kit; precast NuPAGE gel system (Invitrogen, Mississauga, ON, Canada). LPS levels in all reagents were detected using the limulus assay (Limulus amoebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). LPS contaminated reagents with no alternative sources available were treated with polymyxin B-agarose to eliminate the LPS. The reagents used in this study contained <0.2 U LPS/10 μ g reagents.

Patients

Eighty-six patients were recruited into this study that included 30 NP/AR, 30 NP/nAR (not AR), 18 not NP (nNP)/AR, and 8 nNP/nAR (Table 1). The demographic data were listed in Table 2. The diagnosis of AR was followed the established criteria at our department that was also published elsewhere (20). All patients had severe symptoms of nasal obstruction because of the hypertrophic inferior turbinate. All AR patients had positive allergy responses for mite nasal allergy, determined by a clinical history, a nasal smear positive for eosinophils, positive intradermal testing, serum mite-specific IgE antibodies (all the patients had >2.0 kU_A/l serum mite (Der p 1)-specific IgE that

Table 1 Definition of study groups

Group	Definition
NP/AR	Patients with both NP and AR
NP/nAR	Patients with NP but not AR
nNP/AR	Patients did not have NP, but have AR
nNP/nAR Control	Patients with no NP, no AR All patients had inferior turbinate hypertrophy Patients with nasal cancer

NP: Nasal polyposis. AR: Allergic rhinitis.

Table 2 Demographic data

Group	Number	Male	Female	Age (year)	Case history (year)
NP/AR	30	14	16	18–56, 38.3 $\pm$ 6.9	8–15, 12.1 $\pm$ 7.5
NP/nAR	30	17	13	21–49, 31.5 $\pm$ 7.8	6–10, 8.5 $\pm$ 4.1
nNP/AR	18	8	10	25–57, 34.6 $\pm$ 10.3	5–30, 8.7 $\pm$ 7.1
nNP/nAR	8	5	3	24–42, 31.8 $\pm$ 8.6	5–11, 7.5 $\pm$ 5.2
Control	5	3	2	55–72, 61.8 $\pm$ 5.6	2–3, 1.8 $\pm$ 1.1

ranged as 0.21–18.8 kU_A/l), positive nasal provocation test responses to mite allergen and negative test responses for other common aeroallergens. NP diagnosis was based on nasal cavity examination and pathoanatomic examination on NP-like tissue biopsies. Five patients with nasal cancer were also recruited. The marginal nasal mucosa was excised from surgically removed tissue using as control. All the patients were repeatedly treated with medical intervention (including nasal spray of decongestion and steroids) that did not get satisfactory results and asked for surgical treatment with inferior turbinectomy, remove NP and some with endoscopic sinus surgery. Written informed consent was obtained from each patient. The study protocol was approved by the Human Research Ethic Committee at Shanxi Medical University. All subjects did not use any medicines in past 2 weeks. The patients recruited in the present study did not have nasal surgery history.

Enzyme-linked immunosorbent assay (ELISA)

Serum mite (Der p 1)-specific IgE, contents of TNF- α and OX40L were determined by ELISA with commercial reagent kits following the manufacturer's instruction.

Flow cytometry

Cells were collected and incubated with fluorescently labeled primary antibodies (or isotype IgG) on ice for 30 min (For the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permeabilization reagents for 30 min on ice). The stained cells were analyzed using a FACS-array (BD Bioscience, San Jose, CA, USA). Data were analyzed with software FLOW JO.

Immunohistochemistry

Nasal mucosal cryosections were fixed with acetone for 20 min. After blocking with 2% bovine serum albumin for 30 min, the sections were incubated with primary antibodies (anti-M3 receptor, 1 : 1000; anti-

TNF- α , 1 : 200) or isotype IgG at 4°C overnight. Sections were incubated with fluorescence-labeled second antibodies for 1 h at room temperature. Propidium iodide (10 μ g/ml, 15 min) was used as contrast staining. Washing with PBS was performed after each incubation. Sections were observed with a confocal microscope (LSM510).

Quantitative real time RT-PCR (qPCR)

Total RNA was extracted from collected cells using an RNeasy Mini kit. cDNA was synthesized using iScriptTMcDNA Synthesis Kit. The resulting cDNA was subjected to qPCR that was performed with a LightCycler using a SuperScript III Platinum SYBR Green Two-Step qPCR Kit. The amplified product was detected by the presence of an SYBR green fluorescent signal. β -actin cDNA was used as the internal control. The primers of human OX40L were forward: 5'-gtgaatggcggagaactgat-3'; reverse: 5'-gccaggatctgcttctgttc-3' (NCBI: X79929).

Western blotting

Total proteins were extracted from surgical removed nasal mucosa or cultured cells with established procedures (18). Equal amounts protein extracts of each sample were separated on a precast NuPAGE gel system and blotted onto nitrocellulose membrane. The membranes were then blotted with primary antibodies against target proteins. The proteins were detected using horseradish peroxidase-conjugated second antibodies. Horseradish peroxidase enzymatic reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

Immune cell isolation

Surgical removed nasal tissue (1~2.5 g tissue/patient was obtained in the present study) obtained from all patients that were cut into small pieces and treated with predigestion solution (1 $\times$ HBSS containing 5 mM EDTA and 1 mM DTT) at 37°C for 30 min

under slow rotation. The tissue was collected by centrifugation ($126 \times g$, 10 min) and incubated in digestion solution (Dissolve 0.05 g of collagenase D, 0.05 g of DNase I and 0.3 g of dispase II in 100 ml of 1 times PBS) at 37°C for 60 min under slow rotation. Cells were filtered with a cell strainer. Isolation of CD11c⁺ DCs and CD4⁺ T cells was performed with commercial magnetic cell sorting kits. The purity of all the isolated cells was over 90% as checked by flow cytometry. One gram nasal tissue could harvest $0.3\sim 1.2 \times 10^5$ DCs and $0.5\sim 3.8 \times 10^6$ CD4⁺ T cells in the present study.

Generation of DCs from peripheral blood mononuclear cells (PBMC)

Immature DCs were generated from PBMC with our established protocol (21) that was also described elsewhere (22). Briefly, PBMCs were prepared from healthy volunteers by gravity density centrifugation. The isolated PBMCs were then transferred into 12-well plates at a density of 0.5×10^6 cells/ml and placed in a humidified incubator at 37°C . The cells were grown in RPMI1640 media supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$), IL-4 (10 ng/ml) and GM-CSF (20 ng/ml). The cytokine-supplemented medium was replaced weekly, and the nonadherent cells were collected and split to fresh medium. CD11c, CD80 and CD86 expression were used as an indicator of DCs. As shown by flow cytometry, over 95% cells were CD11c positive in day 6 with mild expression of CD80 and CD86 (compared with LPS-stimulated DCs; data not shown); these cells were used for further experiments.

Statistics

The data were expressed as means $\pm$ SD of at least three independent experiments. The values were analyzed using the two-tailed unpaired Student's *t*-test when data consisted of two groups or by ANOVA when three or more groups were compared. $P < 0.05$ was set as the statistically significant.

Results

TNF- α level is increased in nasal mucosa of patients with NP

Published data indicate that the proinflammatory cytokine TNF- α is associated with an array of immune diseases such as allergic rhinitis (AR) (23), low airway hypersensitivity (24), rheumatoid arthritis (25), and

intestinal inflammation (26). Because NP is also an immune disorder (27), we speculated that the TNF- α might play a role in the pathogenesis of NP. To test the hypothesis, we evaluated the levels of TNF- α in surgically removed nasal mucosal extracts from patients with nasal mucosal hyperplasia with or without NP. As shown by ELISA data, levels of TNF- α were significantly higher in patients with NP than that from nNP. Further analyses showed that the levels of TNF- α in nasal mucosal specimen from patients with both NP and AR were not significantly higher than those with NP/nAR (Fig. 1).

TNF- α ⁺ cells increase in NP nasal mucosa

A subtype of immune cells produces TNF- α in inflammatory tissue. We had detected the increase in TNF- α in nasal mucosa (Fig. 1). Thus, it was conceivable that TNF- α ⁺ cells might be increased in NP nasal mucosa. To prove the speculation, we isolated CD4⁺ T cells from surgically removed nasal mucosa with or without NP and stained with anti-TNF- α antibody. As shown by flow cytometry data, significantly more TNF- α ⁺ cells were detected in those from NP nasal mucosa than cells from nNP nasal mucosa (Fig. 2).

Increase in muscarinic receptor 3 (M3) in immune cells in nasal mucosa

It is not clear what factors cause TNF- α ⁺ cell increase in NP nasal mucosa. Published data indicate that human nasal mucosa expresses M receptor 1–5 (24). M3 is the subtype that most abundantly expresses in nasal mucosa (24). Using M receptor blockers suppresses the clinical symptoms of nasal

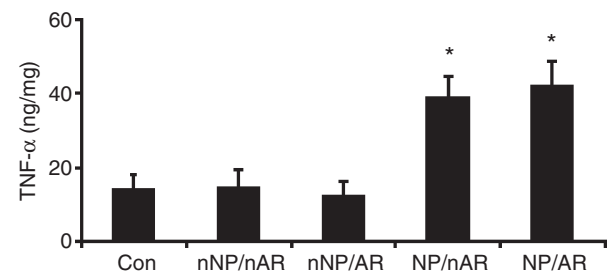


Figure 1 Levels of TNF- α in nasal mucosa. Total protein was extracted from surgically removed nasal mucosa. Bars indicate the levels of TNF- α in extracts that were determined by ELISA. * $P < 0.05$, compared with control group (con; from the margin tissue of nasal cancer removed in surgery). NP, nasal polyposis; nNP, non-NP; AR, allergic rhinitis; nAR, non-AR.

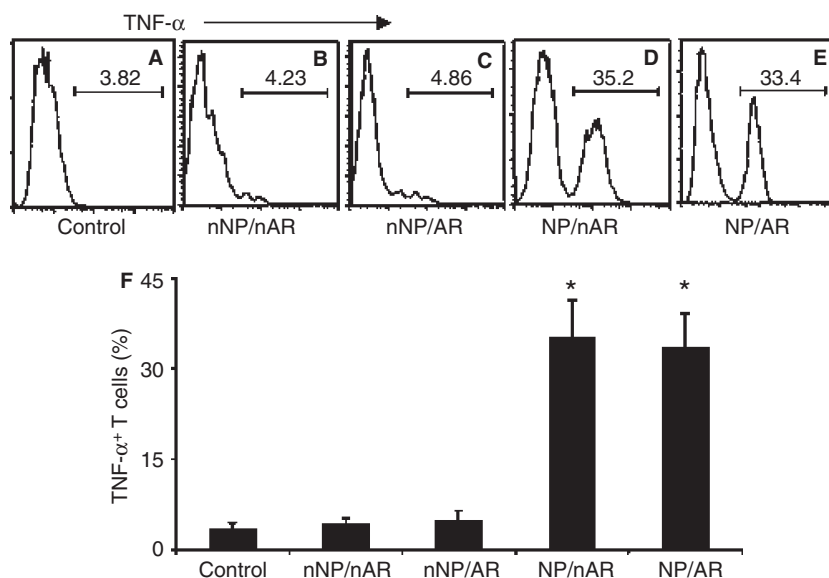


Figure 2 TNF- α ⁺ T cells in nasal mucosa. CD4⁺ T cells were isolated from surgically removed nasal mucosa and stained with anti-TNF- α antibody. A–E, flow cytometry histograms indicate the rate of TNF- α ⁺ T cell population (the gated area) in iso-

lated CD4⁺ T cells. F, bars indicate the TNF- α ⁺ T cell population in isolated CD4⁺ T cells. Data were presented as mean $\pm$ SD from all patients. * P < 0.05, compared with nNP/nAR patients.

immune disorders (28). The present data show that TNF- α ⁺ cells are increased in NP nasal mucosa (Fig. 2) that implies M3 may be increased in NP nasal mucosa. To this end, we employed the double immune staining technique with antibodies against TNF- α and M receptor 3 to stain cryosections of nasal mucosa. The results showed that number of TNF- α ⁺ M3⁺ cells was significantly more in NP nasal mucosa than those of nNP (Fig. 3). It is noteworthy that apart from co-stained with TNF- α , many other cells were also stained positively with anti-M3 antibody. To identify the cell types of the TNF- α ⁺ M3⁺ cells in nasal mucosa, M3⁺ cells were isolated from surgically removed nasal mucosa by magnetic cell sorting (MACS) and stained with antibodies against CD11c, IL-4, INF- γ , Foxp3, IL-17 and M3, and analyzed by flow cytometry. The results showed that apart from TNF- α ⁺ M3⁺ cells, CD11c⁺ M3⁺ cells and IL-4⁺ M3⁺ cells were also identified. The results indicate that DCs in nasal mucosa also express M3. In addition, we detected a small number of M3 cells was IL-4⁺, or INF- γ ⁺, or Foxp3⁺ (Fig. 3).

Interaction of methacholine (Mch)/M3 triggers TNF- α release from CD4⁺ T cells in NP nasal mucosa

Because we found that nasal mucosal immune cells co-expressed M3 and TNF- α , we wondered whether activation of M3 could be a factor to trigger the

release of TNF- α . To this end, CD4⁺ T cells were isolated from surgically removed NP nasal mucosa. As prechecked by flow cytometry with sample cells, these CD4⁺ T cells expressed high levels of TNF- α (data not shown). The cells were exposed to Mch at graded concentrations for 30 min. As expected, TNF- α was detected in culture media that was in a Mch dose-dependent manner (Fig. 4).

Acetylcholine/M3 interaction increases expression of OX40L in DCs of NP nasal mucosa

Based on the fact that high levels of TNF- α were detected in NP nasal mucosa (Fig. 1), NP nasal mucosal DCs expressed M3 (Fig. 3), we hypothesized that parasympathetic nervous ending-derived acetylcholine might activate the M3 on DCs; which might initiate the gene transcription machinery in DCs to promote the expression of OX40L. Exposure to OX40L has the potential to induce naïve CD4⁺ T cells to become TNF- α -producing cells. To test the hypothesis, DCs were isolated from surgically removed nasal mucosa by MACS. The expression of M3 on isolated DCs was checked with flow cytometry; about 30% DCs expressed M3 (Fig. 3F). The isolated DCs were exposed to Mch in culture at graded concentrations for 48 h. As shown by ELISA data, the expression of OX40L was markedly increased in culture media of DCs from NP patients that was higher than that from nNP patients. The results were further confirmed by

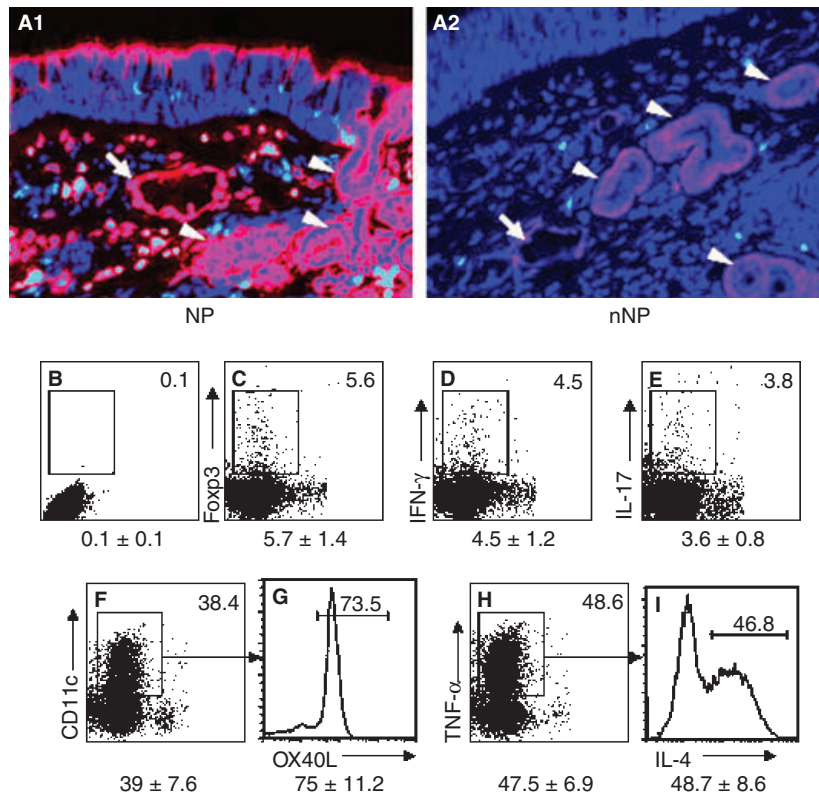


Figure 3 Nasal mucosal immune cells express M3. Surgically removed nasal mucosa was collected and subjected to assessment of the expression of M3. A1 and A2, confocal images show M3 expression (in red) on the surface of mucosa, blood vessels (arrow), glands (arrow heads) and TNF- α cells (in green) in nasal mucosa of NP (A1) and nNP (A2). B–F and H, M3⁺ cells were

isolated from nasal mucosa and analyzed by flow cytometry. Panel B is an isotype IgG control. Histograms indicate positive stained cell population (the gated cells). G, OX40L⁺ cells in gated CD11c⁺ DCs in panel F. I, IL-4⁺ cells in gated TNF- α ⁺ cells in panel H. The means $\pm$ SD of gated cell populations were presented below each flow cytometry graph.

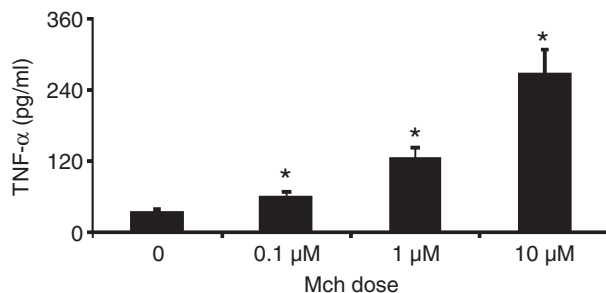


Figure 4 Interaction of M3/Mch triggers TNF- α release. CD4⁺ T cells were isolated from nasal mucosa by MACS and exposed to Mch in culture for 30 min. Bars indicate TNF- α level in culture media that were determined by ELISA. * P < 0.05, compared with group "0".

Western blotting assay (Fig. 5). To clarify whether DCs constitutively express M3, we isolated DCs from the peripheral blood. As detected by flow cytometry, the expression of M3 was very low in peripheral DCs (data not shown). The results demonstrate that M3 is

not constitutively expressed in DCs; but can be induced somehow.

Staphylococcal enterotoxin B (SEB) promotes M3 expression in DCs

Because DCs from NP nasal mucosa expressed M3, while peripheral DCs did not, other factors within nasal mucosa must be triggers for the expression of M3 in nasal mucosal DCs. We previously found that SEB drove DCs to express T-cell immunoglobulin mucin domain 4 (TIM4) in DCs that plays a critical role in the initiation of skewed Th2 polarization (18, 19); we therefore wondered whether the stimulation of SEB could initiate the expression of M3 in nasal mucosal DCs. To prove this point, our first attempt was to check whether these patients with NP also had *Staphylococcus aureus* (*S. aureus*) colonization in nasal mucosa. With published approaches (29), we isolated *S. aureus* from surgical removed nasal mucosa of 13/30 NP patients. Statistical assay resulted in positive

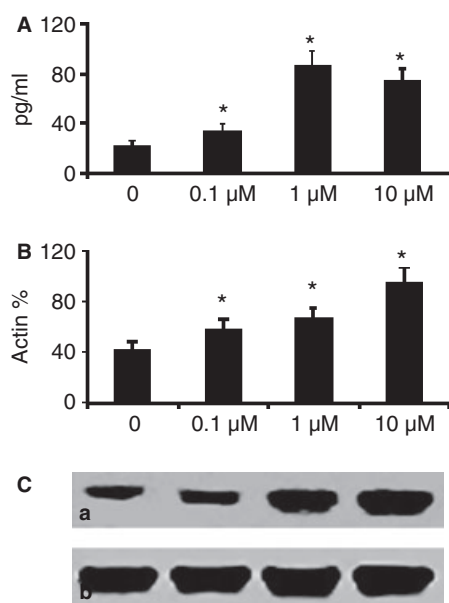


Figure 5 Mch induces DCs to produce OX40L. CD11c⁺ DCs were isolated from nasal mucosa and exposed to Mch in culture for 48 h. Levels of OX40L in culture media (or cellular extracts) were measured by ELISA (or Western blotting). A and B, bars indicate the levels of OX40L protein in culture media (A) and mRNA in cellular extracts (B). C, immune blots show proteins of OX40L in DC cellular extracts. Gel a show OX40L; gel b show beta-actin blots. *P < 0.05, compared with '0' group.

correlation to M3 expression ($r = 0.65$, $P < 0.05$) in isolated DCs (Fig. 6G). In nNP patients, *S. aureus* was detected in 3/30; the correlation assay resulted $r = 0.105$, $P > 0.05$. In an alternative approach, we assessed the levels of SEB in protein extracts from surgically removed nasal mucosa. As shown by ELISA, 27/30 NP patients had high levels of SEB in their nasal mucosa (mean = 45.8 ± 12.6 ng/mg protein); positive relationship ($r = 0.93$, $P < 0.001$) was also determined between SEB levels and M3 expression in isolated DCs. On the other hand, SEB was only detected in 5/30 patients with nNP (2.6 ± 2.1 ng/mg protein) ($r = 0.26$ between SEB and M3 expression, $P > 0.05$).

The results in the observation of *S. aureus* and SEB in nasal mucosa imply a positive connection between SEB and M3 expression existing in DCs in NP nasal mucosa. To confirm the speculation, we generated DCs from peripheral mononuclear cells with our established protocol (21). The DCs were exposed to SEB in culture at graded concentrations for 48 h. As shown by flow cytometry assay, the expression of M3 in DCs was increased in SEB dose-dependent manner. The results were further confirmed by qPCR and Western blotting assay (Fig. 6).

Discussion

With the advantage of obtaining relatively large volume of human tissue from inferior nasal turbinectomy of patients with chronic hyperplasia rhinitis and NP, we isolated sufficient immune cells that gave us opportunity to study the roles of these cells in the pathogenesis of immune inflammation in nasal mucosa. The study had several novel findings that include (i) nasal mucosal DCs express muscarinic receptor 3 (M3); (ii) activation of M3 by Mch induced the expression of OX40L in DCs; (iii) IL-4⁺ TNF- α ⁺ T cells were abundant in nasal mucosa of patients with NP; (iv) M3 expression was also identified in IL-4⁺ TNF- α ⁺ T cells of NP nasal mucosa; (v) exposure to Mch drove NP nasal mucosa-derived IL-4⁺ TNF- α ⁺ T cells to release IL-4 and TNF- α .

It is proposed that NP is resulted from chronic inflammation. Allergy is one of the major factors in the induction of NP in which IgE and mast cell degranulation are regarded as the major effector factors (30). TNF- α is one of the chemical mediators of mast cell that is released upon mast cell activation. Previous studies also found that increases in TNF- α in NP nasal mucosa (31). Our data are in line with these studies by revealing increases in TNF- α in protein extracts of nasal mucosa as well as found increases in TNF- α ⁺ cell number in NP nasal mucosa. TNF- α has the capacity to induce cell death via apoptotic pathway and induces inflammation in local tissue. The present data imply an important role of TNF- α in the pathogenesis of NP and chronic rhinitis.

It seems most inflammatory cells produce TNF- α upon activation, which includes mast cells, Th1 cells and Th2 cells. Thus, the mechanism of activation of inflammatory cells is important in studying the pathogenic mechanism of NP and chronic inflammation in the airway. Hyperactivity of parasympathetic nerve in nasal mucosa is associated with both allergic and nonallergic nasal inflammation (32). Previous studies indicate that M3 receptors are extensively distributed on nasal mucosa, especially in the inferior turbinate (16, 17). The M3 receptor is probably important in the physiology of the human inferior turbinate. The cholinergic activities stimulate M3 muscarinic receptors on the submucosal glands to cause the exocytosis and thick mucous discharge (16, 17). In accordance with these previous reports, the present data show that hyper expression of M3 receptor in nasal mucosa of NP patients with or without AR. Furthermore, we found that the expression of M3 receptors not only localized in blood vessels and glands as reported previously, we also unraveled that most immune cells also

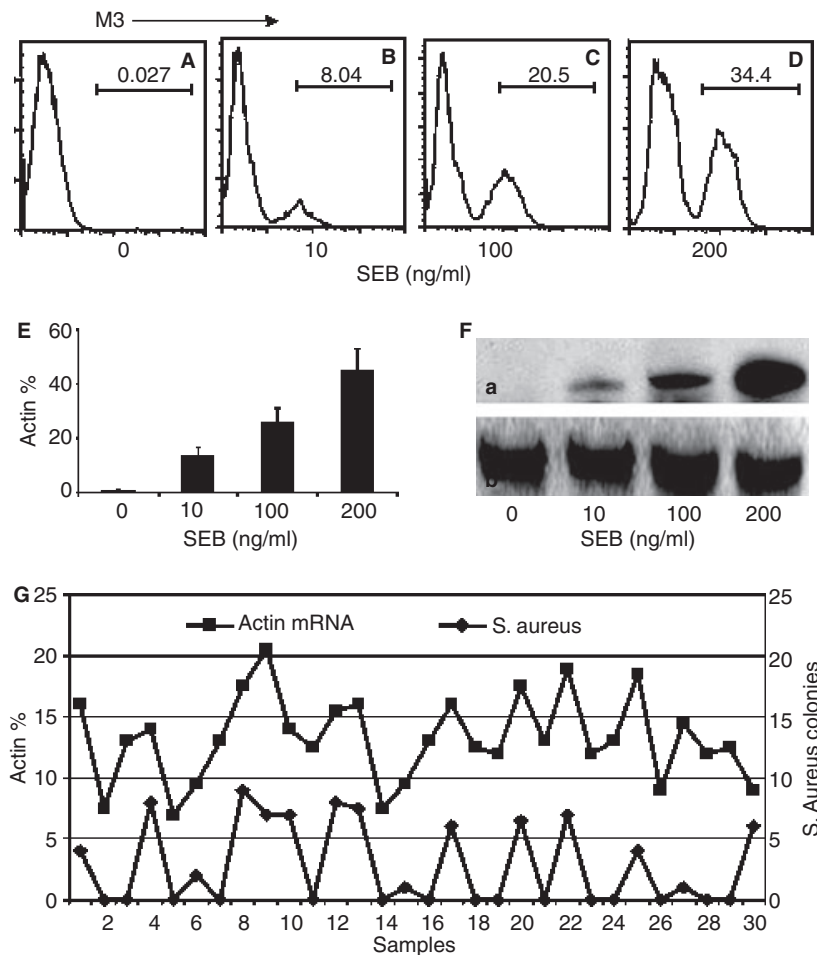


Figure 6 SEB promotes expression of M3 in DCs. Peripheral DCs were prepared and exposed to SEB in culture at graded concentrations. M3 expression in DCs was analyzed by flow cytometry, qPCR and Western blotting. A–D, histograms indicate the M3⁺ DC population. E, bars indicate M3 mRNA expression in DCs that was

presented as percentage of β -actin. F, immune blots show M3 protein in DC cellular extracts. Gel a: M3 protein; gel b: β -actin. Each experiment was repeated 3 times. G, scatter plots show the relation between the expression of β -actin mRNA in isolated DCs and the *S. aureus* colony numbers cultured from nasal mucosal specimens.

expressed M3 receptors, especially in DCs and CD4⁺ T cells.

Parasympathetic nervous transmitters, such as acetylcholine, can bind M receptors to activate mitogen-activated protein kinase and subsequently initiate the signal transduction pathway and activates the M receptor-bearing cells (33). In this way, parasympathetic nervous system is involved in regulating the immune function in the body, such as modulating the antibody production, inflammatory cell infiltration, and lymphocyte proliferation (34–36). The present data add a novel aspect to the immune regulatory aspects of parasympathetic nervous system that can drive immune cells to release inflammatory mediators, e.g. exposure to Mch induced the release of TNF- α from CD4⁺ T cells isolated from NP, but not those from

nNP, nasal mucosa as observed in the present study. Collectively, the data also suggest deregulation of parasympathetic activities in the pathogenesis of NP. To regulate the parasympathetic function in nasal mucosa may be a potential therapeutic remedy for NP that is worth further exploring.

OX40 and OX40L belong to the tumor necrosis factor receptor/tumor necrosis factor superfamily; they are expressed on activated lymphocytes and on a number of other lymphoid and nonlymphoid cells (37). Interaction of OX40/OX40L modulates cytokine synthesis in immune cells and is involved in the process of immune inflammation (38). Epithelial cell-derived TSLP is a factor driving DCs to express OX40L that further binds to OX40 on CD4⁺ T cells to promote the production of inflammatory cytokine, such as

TNF- α (14). The present data indicate that, similar to TSLP, cholinergic activity can also up regulate the expression of OX40L in DCs. Because the innervations of parasympathetic nerve are ubiquitously distributed in the body, apart from in the subepithelial region where epithelial cell-derived TSLP can directly influence DC's function, the cholinergic activity may influence DC's function, such as inducing the expression of OX40L, in most part of the body.

The regulation of M receptor expression in immune cells is unclear. Our data provide a connection between *S. aureus* colonization on nasal mucosa and M3 receptor expression in DCs and CD4⁺ T cells in NP nasal mucosa. Additional evidence obtained in the present study is SEB was detected in over 90% NP nasal mucosa. SEB is a superantigen and has high immune regulatory activity. Our previous studies indicate that SEB is involved in the initiation of skewed Th2 response (18, 19). The present data add another novel point to SEB's capacity on immune regulation by showing that SEB increases the expression of M3 receptor in DCs and CD4⁺ T cells at mRNA and protein levels.

Based on the present data, we may envisage a scenario that exposure to microbial products (such as SEB) up regulates the expression of M3 receptor on nasal mucosal DCs and CD4⁺ T cells. Cholinergic agents activate M3 receptor to induce DCs to release OX40L; the latter drives Th2 cells to express TNF- α (14). Activation of M3 receptors on these TNF- α -producing Th2 cells induce the release of TNF- α to induce inflammation to occur in local tissue such as inducing NP formation (8).

In summary, the present study revealed that high levels of TNF- α and TNF- α ⁺ IL-4⁺ T cells were detected in NP nasal mucosa. Both DCs and IL-4⁺ T cells expressed high levels of M3 receptor. Interaction of Mch/M3 increased the expression of OX40L in DCs and the expression of TNF- α and IL-4 in CD4⁺ T cells. The present study demonstrates that dysregulation of parasympathetic function affects the immune system in nasal mucosa and induces inappropriate immune responses and eventually induces immune inflammation in the nasal mucosa such as NP.

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Conflict of interest

The authors do not have any conflict of interest on this paper.

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