

MECHANISM OF ACTION

Th2 cytokine-primed airway smooth muscle cells induce mast cell chemotaxis via secretion of ATP

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Abstract

Objective: Mast cell infiltration into airway smooth muscle (ASM) bundle is an important feature of asthma. Extracellular adenosine triphosphate (eATP) contributes to the initiation of airway inflammation. eATP induces mast cells migration by acting through purinergic receptors. CD39 is an ectonucleotidase that degrades ATP to ADP and AMP. Whether eATP participates in the migration of mast cell towards ASM cells is still unknown. **Methods:** Airway smooth muscle cells (ASMCs) were isolated from C57/BL6J mice sensitized and challenged with OVA. ASMCs were *in vitro* cultured and stimulated with IL-4 + IL-13 in the presence or absence of exogenous CD39 or CD39 inhibitor ARL67156. ATP level in the supernatants was measured with ATP content determination kit. CXCL10 concentration in the ASMCs supernatants was measured by ELISA, the mRNA expression of CXCL10 in ASMCs was determined with real-time PCR. Human mast cell line HMC-1 was cultured in Iscove's-Modified Dubecco's Medium. The expression of CXCR3 in HMC-1 cells was determined with flow cytometry and real-time PCR, respectively. HMC-1 migration rates were determined with transwell system. **Results:** In the supernatants of Th2 cytokine-stimulated ASMCs, ATP level was higher than that without stimulation. CD39 decreased, whereas ARL67156 increased ATP level in the supernatants. Both ATP and the supernatants of Th2 cytokine-stimulated ASMCs induced migration of HMC-1 cells. The surface and mRNA expression of CXCR3 in HMC-1 cells, and the mRNA expression and secretion of CXCL10 in ASMCs were increased after stimulation with ATP or Th2 cytokines. All these effects were partially inhibited by CD39. **Conclusion:** Our data suggested ASMCs in the asthma microenvironment promoted the migration of mast cells via secretion of ATP and the expression of CXCL10/CXCR3 axis. CD39 could reverse this effect and may be a new target for the treatment of asthma.

Keywords

Airway smooth muscle cells, ATP, CD39, chemotaxis, mast cells

History

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Introduction

Allergic asthma is a common airway inflammatory disease which is characterized by airway remodeling and bronchial hyperresponsiveness (BHR) [1]. Different cell types within the lungs are implicated in the initiation and development of airway inflammation and airway hyperresponsiveness, among which mast cells are the major players of allergic responses [2].

Studies on asthma patients have highlighted the importance of both mast cells and airway smooth muscle cells (ASMCs) in the pathogenesis of asthma [3,4]. Infiltration of mast cells into airway smooth muscle (ASM) bundle is found in asthmatics but not in patients with eosinophilic bronchitis or healthy controls [5], suggesting that this is a major feature of asthma [6,7]. Besides induction of airway constriction, ASMCs also play a role in airway inflammation in that they

are capable of releasing cytokines after stimulation. The underlying mechanisms of the infiltration of mast cells into ASM bundle are still not completely understood.

Extracellular adenosine triphosphate (eATP) serves as a danger signal to alert the immune system upon tissue damage. Allergen challenge causes acute accumulation of ATP in the airways of asthmatic subjects and mice model of asthma [8]. Of relevance to asthma, ATP can modify the parasympathetic limb of the autonomous nervous system, which has been proved to contribute to the generation of BHR [9]. ATP has also been shown to modify the recruitment and/or function of inflammatory cells involved in asthma pathogenesis, including eosinophils, dendritic cells, lymphocytes and mast cells [9,10]. In asthma, ATP and adenosine 5'-diphosphate (ADP) act through the P2 purinoceptors of macrophages and dendritic cells, and induce the production of inflammatory cytokines [11]. The steady-state cytosolic ATP concentration is 3–10 mM, whereas eATP concentration is ~10 nM. This minimal concentration of eATP is maintained by extracellular ectoapyrases and ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, CD39), which metabolize ATP into ADP, adenosine 5'-monophosphate (AMP) and adenosine [12].

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Previous studies showed that ATP levels were increased in BALF of both asthma patients and murine models of asthma [8]. In addition, conjugation of ATP with purinoceptors on the surface results in degranulation, cytokine secretion, chemotaxis and apoptosis of mast cells [13,14]. eATP is degraded by CD39 and CD73. CD39 expressed on regulatory T cells is an important mechanism of their immune suppressing effect [15,16]. A recent study showed that CD39-deficient mice had attenuated airway inflammation, which may be caused by P2Y receptor desensitization due to the accumulation of eATP [17]. These data suggest that eATP is involved in the initiation and/or maintenance of allergic airway inflammation. Whether increased eATP level causes the infiltration of mast cells into ASM bundle under asthma condition has not been reported.

In this study, we tested the migration of HMC-1 cells toward ATP and OVA-sensitized and -challenged ASMCs under Th2 cytokine stimulation. Meanwhile, the effect of exogenous CD39 on the migration of mast cells induced by ATP and the supernatants of Th2 cytokine-stimulated ASMCs was also examined.

Materials and methods

Mice model of allergic asthma

C57/BL6J mice were obtained from Experimental Animal Center of Medical colleges of Wuhan University. Mice were sensitized via intraperitoneal injection of OVA/Alum (20 µg chicken OVA, grade V; Sigma, St Louis, MO; adsorbed to 2 mg Imject Alum, Thermo Scientific Pierce Biotechnology, Rockford, IL) in total volume of 200 µl PBS on day 0 and 14, and challenged with OVA solution (100 µg OVA in 50 µl PBS) via intranasal administration on days 25–27.

Isolation and culture of mice ASMCs

Airway smooth muscle cells were isolated from OVA-sensitized and -challenged C57/BL6J mice and cultured as previously described [13]. Briefly, the bronchi were dissected from mice under sterile condition. After removing adherent connective tissues and epithelia, the smooth muscles were cut into small pieces. The muscle pieces were then put into 2 ml of PBS containing 1 mg/ml collagenase I and 1 mg/ml papain. After enzymatic digestion at 37 °C for 30 min, the suspension was centrifuged and then washed with Dulbecco's-Modified Eagle's medium F12 (DMEM; Gibco, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA). Cells were cultured in DMEM medium supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere of 5% CO₂–95% air. After confluent, cells were passaged with 0.25% trypsin–0.02% EDTA solution (Gibco, Invitrogen, Carlsbad, CA). Cells between three and eight passages were used for experiments. Typical ASMCs grew in a hill-valley form and were confirmed with immunohistochemistry identification of smooth muscle-specific α-actin (purity >98%).

Airway smooth muscle cells were dissolved and placed into 6-well plates with a density of 1×10^5 cells per well. After grown in 10% FBS-containing medium for 3 days,

ASMCs were growth-arrested for 24 h with serum deprivation. Then cells were divided into different groups and stimulated for 24 h with Th2 cytokines IL-4 and IL-13 (10 ng/ml of each, Peprotech, Rocky Hill, NJ) combined with CD39 (10 ng/ml, R&D system, Abingdon, UK) or CD39 inhibitor ARL67156 (10 µg/ml, Sigma-Aldrich, Germany). The concentrations of these cytokines were chosen since they were optimal or had the maximal responses as described in previous studies [18]. Supernatants were collected and stored at –80 °C for later use.

Culture of human mast cells

Human mast cell line (HMC-1) was provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). The cells were cultured in Iscove's-Modified Dubecco's Medium (IMDM, Sigma-Aldrich, Gillingham, Dorset, UK) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (pH 7.4) [13].

Extracellular ATP measurements

Airway smooth muscle cells from OVA-sensitized and -challenged C57B/L6 mice were placed into 6-well plates (1.0×10^5 cells in 2 ml DMEM supplemented with 10% FBS). After cultured for 72 h, the cells were growth-arrested for 24 h in serum-deprived medium. The cells were then divided into four groups and stimulated with Th2 cytokines IL-4 + IL-13, IL-4 + IL-13 + D39, IL-4 + IL-13 + ARL 67156 or PBS as control for 30 min. The cells were collected and centrifuged at 1500 rpm for 10 min, the supernatants were collected and stored at –80 °C for later use. The eATP levels were measured using ATP content determination kit (from Tondcy, Beijing, China) according to the manipulator's instruction.

Detection of HMC-1 cell surface antigens by flow cytometry

The expression of specific chemokine receptors on HMC-1 cells was determined by flow cytometry. Briefly, HMC-1 cells were placed into 6-well plates (1.0×10^6 cells per well). After reaching 70% confluence, the cells were stimulated with ATP (10 µM) in the absence or presence of exogenous CD39 or ARL67156 (10 µg/ml). After incubation for 24 h, cells were collected and washed with PBS for two times. The cells were then resuspended in PBS and incubated with PE-conjugated anti-human-CXCR3 (CD183) (Ebioscience, San Diego, CA) at 4 °C for 30 min in the dark. Then, cells were washed with PBS for two times, resuspended in 500 µl PBS and subjected to analysis with a flow cytometer (Epic Altra; Beckman, CA) immediately. The levels of chemokine receptors on the cell surface were expressed by percentages of positive cells.

Quantitative real-time PCR analysis of mRNA expression

Total RNA from ASMCs and HMC-1 cells was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA (1 µg) was transcribed into cDNA using Revertra Ace RT kit (TOYOBO, Japan).

Sequence-specific PCR primers used for the mouse or human target genes and housekeeping genes were selected after searching with the publicly available databases (GenBank). The specific primer sequences were as follows: for mouse CXCL10 (product size 178bp) forward: 5'-TCC ACAGCGCTTCTATTCT-3', reverse: 5'-CTATGGCTTTC AGGGTGCAT-3'; for mouse CXCL11 (product size 107bp) forward: 5'-GGATGGCTGCTAGCTCTG-3', reverse: 5'-TGAGCTAGGGAGGACAAGGA-3'; for mouse β -actin (product size 89bp) forward: 5'-ACCAGCGCAGCGATA TCGTC-3', reverse: 5'-TCCTTCGTTGCCGGTCCAC-3'; for human CXCR3 (product size 151bp) forward: 5'-GGTGG ACATCCTCATGGACC-3', reverse: 5'-CCGGAAGTTGAC CCCTACAA-3'; for human GAPDH (product size 95bp) forward: 5'-GGTGTGAACCATGAGAAGTATGACA-3', reverse: 5'-GGTGTGAACCATGAGAAGTATGACA-3'. To half-quantify the mRNA level, real-time PCR was performed using a ROTOR Gene 3000 system, total reaction volume of 20 μ l. We used SYBR Green Supermix (TaKaRa, Japan) to indicate the PCR products. Amplification was conducted under the following condition, initial denaturation at 94 °C for 1 min, then 40 cycles at 94 °C for 15 s, 60 °C for 10 s and 72 °C for 10 s. Measurements were all performed in duplicate. The expression levels of target genes were calculated using the comparative $2^{-\Delta\Delta C_t}$ method, where C_t indicates the cycle number of threshold at which the amplification is exponential.

HMC-1 migration assay

Chemotaxis assays were performed using transwell system with 8.0 μ m pore polycarbonate membrane insert (Corning Costar, Oxford, UK) as previously described [19]. About 2.0×10^5 HMC-1 cells were suspended in 50 μ l culture medium supplemented with 2% FBS and placed into the upper chamber, and 450 μ l of medium containing different concentrations of ATP (0.1 μ M–1 mM), CD39 (10 ng/ml), ARL67156 (10 μ g/ml), 10 μ M ATP + CD39, 10 μ M ATP + ARL67156 or the supernatants of ASMCs stimulated with IL-4 + IL-13 were included in the lower chambers. PBS was used as normal control and included in the lower chamber. All measurements were performed in triplicate. After incubation for 5 h at 37 °C, the amounts of HMC-1 cells in the lower chamber were counted using a haemocytometer by Kimura stain. The migration rates of HMC-1 cells were calculated as the fold increase of migrated cells compared with that of PBS-treated control cells [19].

ELISA

Airway smooth muscle cells were stimulated with IL-4 + IL-13, IL-4 + IL-13 + CD39 or IL-4 + IL-13 + ARL67156. After incubation for 24 h, the supernatants were collected and stored at –80 °C for later use. The concentrations of CXCL10 in the supernatants were measured by ELISA kit (eBioscience Inc., San Diego, CA) according to the manufacturer's instruction. The minimal detectable concentration for mouse CXCL10 was 6.5 pg/ml. All samples were measured in duplicate.

Statistics

All data were expressed as means $\pm$ SEM. Parametric data were analyzed using *t*-tests or ANOVA. All analyses were performed with SPSS 17.0 software (IBM, Armonk, NY). $p < 0.05$ was taken as being statistically significant.

Results

Th2 cytokines stimulation increases eATP concentration in ASMCs supernatants

Adenosine triphosphate concentration was higher in the supernatants of Th2 cytokine-stimulated ASMCs than that in those of PBS-stimulated control cells ($p < 0.01$, Figure 1). CD39 (10 ng/ml) decreased the concentrations of eATP in the supernatants of both control and Th2 cytokine-stimulated ASMCs ($p < 0.05$, Figure 1). Consistently, the ATPase inhibitor ARL67156 increased ATP concentration in the supernatants of Th2 cytokine-stimulated ASMCs ($p < 0.05$, Figure 1). These data suggest that Th2 cytokines were capable of promoting the production of ATP from *in vitro* cultured ASMCs.

Th2 cytokine-stimulated ASMCs promote HMC-1 cells migration via secretion of ATP

We firstly determined the migration rates of HMC-1 cells towards different concentrations of ATP (1×10^{-8} M– 1×10^{-4} M) using transwell system. As shown in Figure 2(A), the migration rate of HMC-1 cells towards 10 μ M ATP was 2.3-fold of control ($n = 12$; $p < 0.05$, Figure 2A); ATP at 1 μ M also slightly increased the migration rate of HMC-1 cells ($p < 0.05$). Other concentrations of ATP had no significant effect on the migration rates of HMC-1 cells. The following experiments of ATP were then performed at the concentration of 10 μ M. We then tested the effect of CD39 and ARL67156 on the migration rates of HMC-1 cells promoted by ATP. In the presence of CD39 (10 ng/ml), ATP-induced HMC-1 cell migration decreased to 1.2-fold of

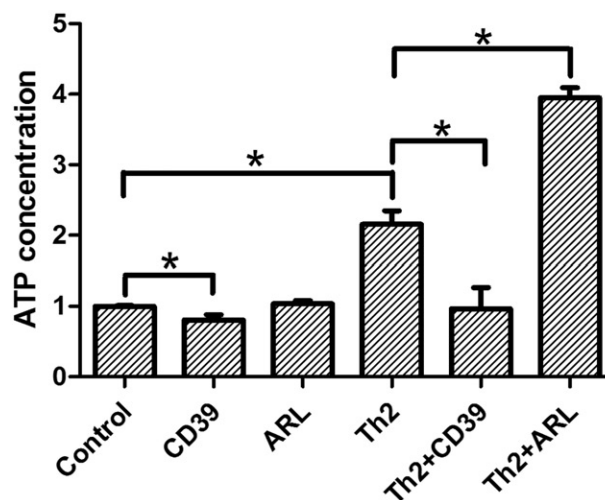


Figure 1. Concentrations of extracellular ATP in the supernatants of cultured ASMCs. Appropriate passages of ASMCs were differently stimulated as indicated in the figure for 30 min, the extracellular ATP concentrations were then determined. Results were set as percentages of control group. Data are averaged of three independent tests. * $p < 0.05$.

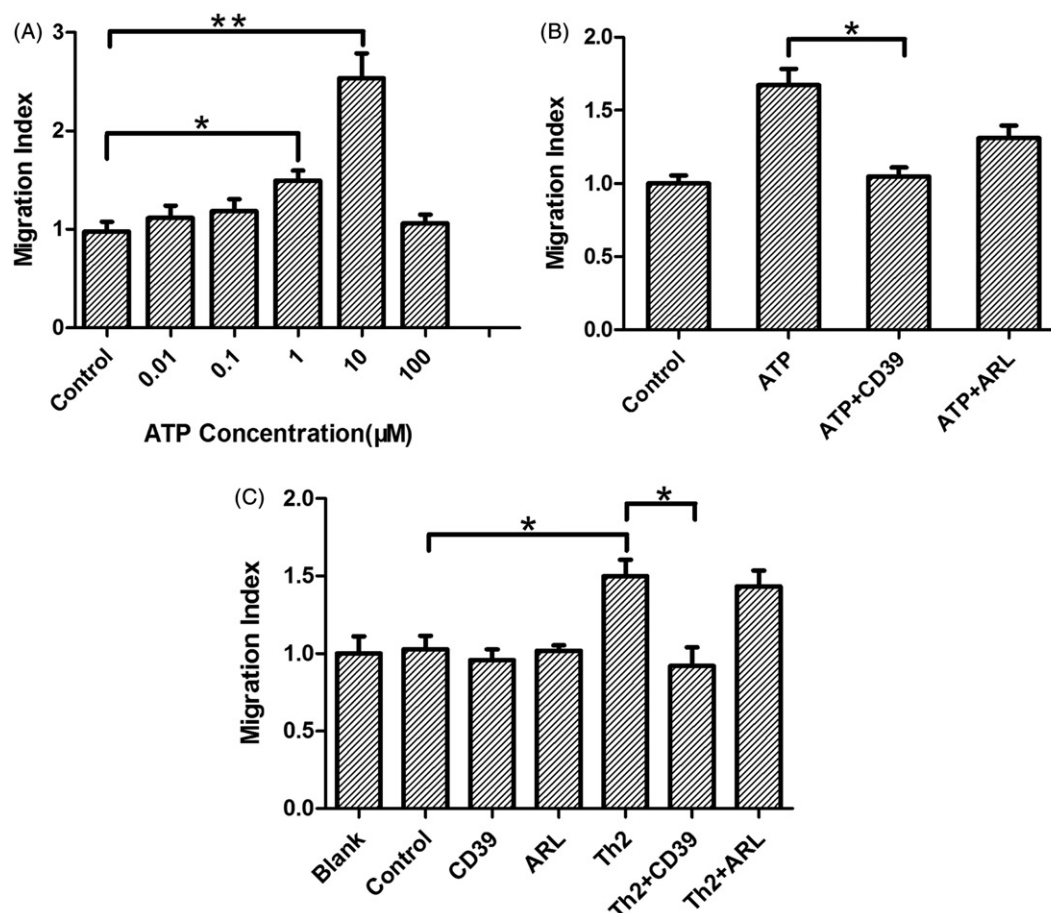


Figure 2. HMC-1 cell migration assay. By using the two-chamber transwell system, the HMC-1 cells were stimulated with ATP of different concentrations (A), ATP (10 μM) + CD39 or ARL67156 (B) and the ASMCS supernatants stimulated with PBS (control), CD39, ARL67156, Th2 cytokines (Th2), Th2 cytokines in the presence of CD 39 (10 ng/ml) or ARL67156 (ARL, 10 μg/ml) (C). The migration rate was set as the fold of control. Data are averaged of four experiments done in triplicate each. * $p < 0.05$ and ** $p < 0.01$.

control, which is significantly lower than that of ATP alone (Figure 2B). However, presence of ARL67156 (10 μg/ml) had no significant effect on the migration rate of HMC-1 cells when compared to that of ATP alone ($p > 0.05$). These results confirmed that ATP could promote mast cell migration at proper concentration.

As shown above, IL-4 + IL-13 stimulation increased the concentration of ATP in the supernatants of ASMCS, we then determined whether ASMCS could promote HMC-1 migration by secreting ATP. As shown in Figure 2(C), the supernatants of control ASMCS had no effect on HMC-1 migration when compared to that of PBS-stimulated HMC-1 cells. Both CD39 and ARL-67156 had no effects on the migration rates of HMC-1 cells stimulated by supernatants of control ASMCS. Interestingly, when ASMCS were cultured in the presence of Th2 cytokines IL-4 + IL-13, the supernatants were able to increase the migration rates of HMC-1 cells (1.4-fold of control) ($p < 0.05$). Presence of exogenous CD39 diminished the promoting effect of supernatants of Th2 cytokine-stimulated ASMCS on the migration of HMC-1. Instead, ARL67156 had no similar effect on the migration of HMC-1 cells. These data suggest that ATP mediates the promoting effect of supernatants of ASMCS stimulated by Th2 cytokines on the migration of mast cells.

Expression of CXCR3 on HMC-1 cells

The migration of mast cell toward chemokines is mediated by different receptors on their surface. Previous studies demonstrated that CXCL10/CXCR3 axis mediates human lung mast cells migration to asthmatic ASM bundles [19]. We then examined the expression of CXCR3 on HMC-1 cells by flow cytometry. The surface expression of CXCR3 on HMC-1 cells increased significantly after treatment with ATP (10 μM) for 24 h when compared to PBS-treated control cells (2.5% versus 1.7%, $p < 0.05$, Figure 3A). CD39 decreased the surface expression of CXCR3 on both control and ATP-treated HMC-1 cells. Interestingly, ARL67156 (10 μM) increased the surface expression of CXCR3 on ATP-treated HMC-1 cells (Figure 3B).

We then determined the surface expression of CXCR3 on HMC-1 cells treated with supernatants of ASMCS. As shown in Figure 3(C), the surface expression of CXCR3 was higher on HMC-1 cells treated with the supernatants of Th2 cytokine-stimulated ASMCS in comparison with that on HMC-1 cells treated with supernatants from PBS-treated control ASMCS. CD39 decreased, whereas ARL67156 had no effect on the surface expression of CXCR3 in HMC-1 cells treated with supernatants of Th2 cytokine-stimulated ASMCS (Figure 3C). We also determined the expression the

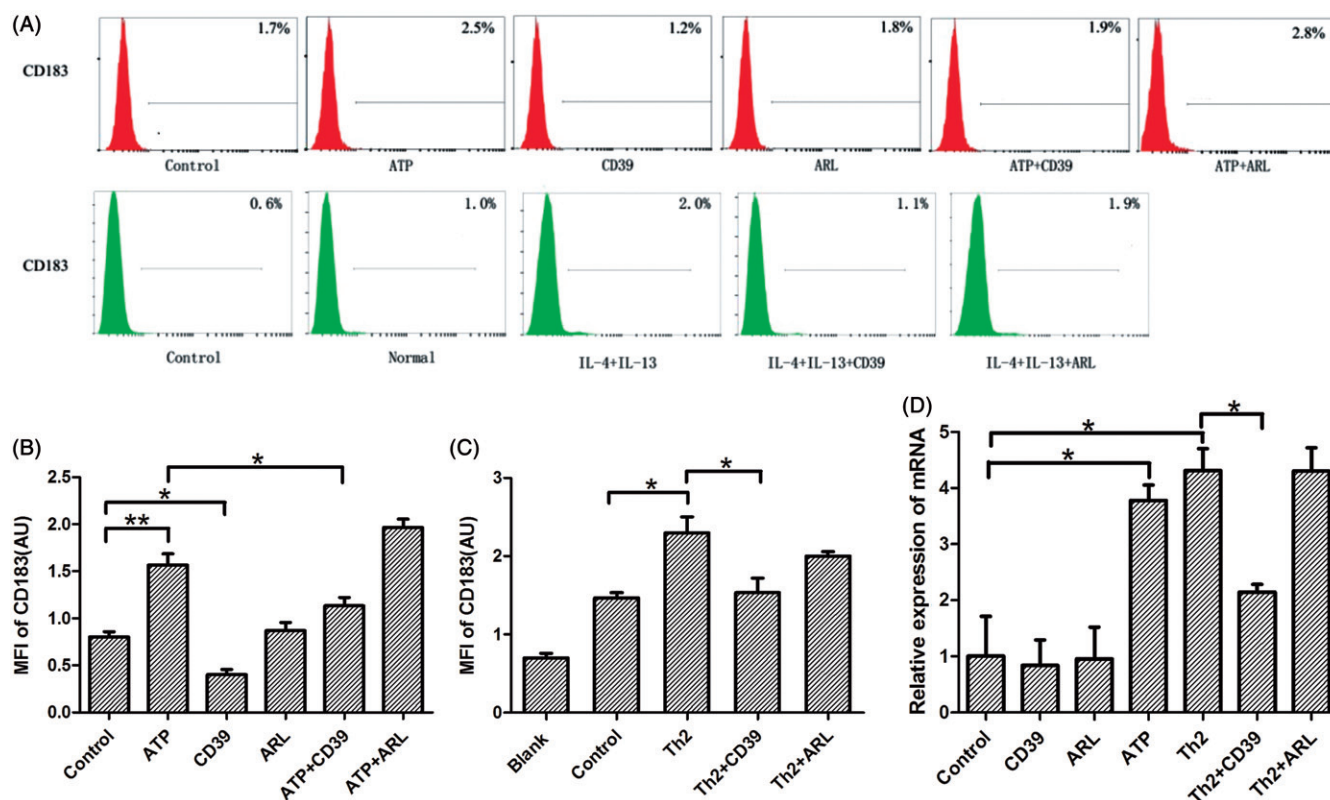


Figure 3. Expression of chemokine receptor CXCR3 on HMC-1 cells. (A) Representative flow cytometry assays show the surface expression of CXCR3 (CD183) in HMC-1 cells. Upper rows: HMC-1 cells treated with PBS (control), ATP, ARL, CD39 and ATP in the presence of CD39 or ARL. Lower row: HMC-1 cells treated with PBS (control), ASMCM supernatants without Th2 cytokine stimulation (normal) or Th2 cytokines IL-4 + IL-13-stimulated ASMCM supernatants, IL-4 + IL-13 together with ARL or CD39. (B) MFI of CD183 on HMC-1 after stimulation with ATP, CD39, ARL, ATP + CD39 or ATP + ARL; (C) MFI of CD183 on HMC-1 after treatment with supernatants of ASMCMs stimulated by IL-4 + IL-13 (10 ng/ml of each) alone, in the presence of CD 39 (10 ng/ml) or ARL67156 (ARL, 10 µg/ml). (D) Expression of CXCR3 mRNA in HMC-1 cells. Data are averaged of three individual tests. * $p < 0.05$; ** $p < 0.01$.

chemokine receptors CCR3 and CXCR1, which are expressed on human mast cells. However, they were not detected on HMC-1 cells (data not shown).

We also checked the mRNA expression of CXCR3 on HMC-1 cells. As shown in Figure 3(D), the HMC-1 cells were treated differently for 24 h and then collected for PCR assay. CD39 or ARL67156 alone had no effect on mRNA expression of CXCR3. ATP and the supernatants of Th2 cytokine-stimulated ASMCMs increased mRNA expression of CXCR3 in HMC-1 cells, respectively ($p < 0.05$). The promoting effect of Th2 cytokines on CXCR3 expression was inhibited by CD39 ($p < 0.05$) but not by ARL67156. Taken together, our ELISA and PCR experiments clearly demonstrated that Th2 cytokine-stimulated ASMCMs promoted CXCR3 expression on HMC-1 cells by secretion of ATP.

Expression of CXCL10 in ASMCMs

Next, we checked the concentration of CXCL10 in the supernatants of ASMCMs stimulated with 10 µM ATP, CD39, ARL67156, IL-4 + IL-13, IL-4 + IL-13 + CD39 or ARL67156. Our results showed that stimulation with ATP and Th2 cytokines for 24 h increased CXCL10 secretion in OVA-sensitized and -challenged ASMCMs. This effect was inhibited by CD39 ($p < 0.05$, Figure 4A). Stimulation with CD39 or ARL67156 alone did not alter CXCL10 release from ASMCMs.

The mRNA expression of CXCL10 in ASMCMs was elevated after incubation with IL-4 + IL-13 or ATP (10 µM) for 24 h ($p < 0.05$, Figure 4B). Presence of CD39 (10 ng/ml) inhibited CXCL10 mRNA expression increased by IL-4 + IL-13 or ATP. By contrast, ARL67156 had no effect on Th2 cytokine-promoted CXCL10 mRNA expression. It is interesting that the mRNA expression of another important chemokine receptor ligand for CXCR3, CCL11, on ASMCMs was not influenced by IL-4 + IL-13 or ATP (10 µM). These data indicate that increased production of CXCL10 from Th2 cytokine-stimulated ASMCMs also contributes to increased migration of HMC-1 cells.

Discussion

Our results suggest that ATP concentration was higher in the supernatants of Th2 cytokine-stimulated ASMCMs than that in control cells. We found that eATP could increase HMC-1 cell migration. Our findings also indicate that the supernatants of Th2 cytokine-stimulated ASMCMs-induced HMC-1 cell migration. The effect of ATP and the supernatants of Th2 cytokine-stimulated ASMCMs on HMC-1 migration were partly inhibited by ATP hydrolase CD39. Meanwhile, the level of CXCR3 on HMC-1 cells was elevated after treatment with ATP and the supernatants of Th2 cytokine-stimulated ASMCMs, and was inhibited by CD39. The production of CXCL10 by ASMCMs was elevated by ATP or Th2 cytokines and inhibited by

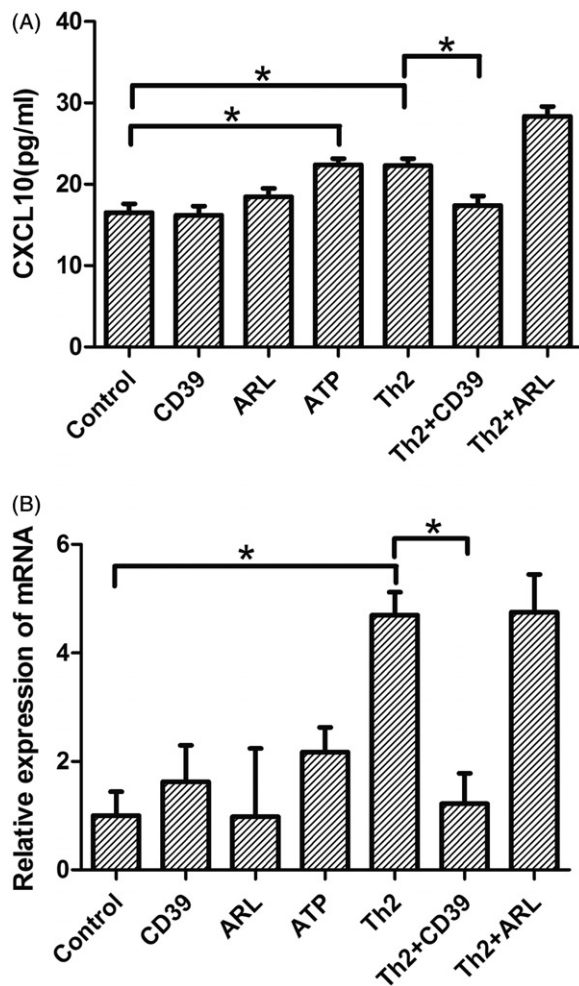


Figure 4. Expression of CXCL10 by ASMCs. (A) CXCL10 concentrations in the supernatants of ASMCs stimulated with ATP (10 μ M), IL-4 + IL-13 and CD39 (10 ng/ml) or ARL67156 (10 μ g/ml). (B) Representative mRNA expression of CXCL10 of ASMCs while treated with ATP (10 μ M), IL-4 + IL-13, IL-4 + IL-13 + CD39 or +ARL67156. Data are averaged of four independent tests; all samples were measured in duplicate. * p < 0.05.

CD39, suggesting that ATP and Th2 cytokine-stimulated ASMCs could induce HMC-1 migration by activating CXCR3/CXCL10 axis. Our results discovered a new mechanism of mast cell-ASM interaction that ASMCs induce mast cell migration under Th2 cytokine-mediated inflammation via secretion of ATP, which then promotes the expression CXCL10/CXCR3 axis.

Infiltration of mast cells into ASM bundle was found in asthmatics but not eosinophilic bronchitis or healthy controls, suggesting that this is a major determinant feature of asthma [20]. Increased expression of Th2 cytokines has been shown in the bronchoalveolar lavage fluid (BALF) [21] and bronchial biopsy specimens from asthma patients [22–24]. ASMCs stimulated with Th2 cytokines are capable of secreting proinflammatory cytokines and thus facilitate airway inflammation [12]. The eATP concentration in the supernatants of ASMCs was increased after stimulation with Th2 cytokines and decreased by CD39, consistent with previous studies [8,12]. The ATPase inhibitor ARL67156 slightly increased eATP concentration in the supernatants but without statistical difference. It was shown that increased

eATP contributes to the bronchial hyperresponsiveness of asthma [25]. eATP binds to P2 purinoceptors and induces cytoplasmic calcium oscillation, which promotes ASM contraction [25]. We demonstrated here that eATP could induce HMC-1 cell migration. Similar results were found in dendritic cells (DCs) that ATP induces DCs migration via activation of P2Y receptors [6]. It was noticeable that ATP-induced HMC-1 cell migration was inhibited by CD39. Accordingly, HMC-1 cell chemotaxis was also induced by Th2 cytokines-treated ASMCs supernatants and inhibited by CD39. These data confirmed the hypothesis that ASMCs induce mast cell migration by secretion of ATP under the asthmatic airway inflammation microenvironment.

However, the mechanisms underlying the effect of eATP on HMC-1 cell migration towards ASMCs remain elusive. Previous studies showed that different chemokine receptors are expressed on human lung mast cells (HLMCs), such as CCR3, CXCR1, CXCR3 and CXCR4 [26,27]. The respective ligands for these receptors are CCL11, CXCL8, CXCL10 and CXCL12, which mediated HLMCs chemotaxis [19,27]. Experiments also showed that CXCR1 and CXCR4 contribute to HLMC chemotaxis induced by asthmatic ASM-conditioned media, but CCR3 had no effect on HLMC chemotaxis even in high concentration [19]. In addition, CXCL10 production is markedly increased following segmentally challenged with allergen in asthma [19,27]. Therefore, CXCL10 might be an important chemokine ligand for mast cells recruited to the airway in response to a variety of stimuli [28–30]. Similarly, mast cell migration toward BALF is correlated with the CXCL10 concentration [31]. Interestingly, ASM of asthmatics preferentially release the CXCR3 ligand CXCL10, and blockade of CXCR3 inhibited the chemotactic activity of mast cell [32]. The CXCL10/CXCR3 axis is therefore important to the migration of mast cell towards ASM bundle in asthma [19]. Furthermore, ASMCs supernatants from asthmatics stimulated with Th2 cytokines *in vitro* had greater mast cell chemotactic activity than normal controls [32]. Similarly, we found that the supernatants of ASMCs stimulated with IL-4 + IL-13 were chemotactic for HMC-1 cells. Stimulation with IL-4 + IL-13 increased the CXCL10 concentration in ASMCs supernatants, this effect of Th2 cytokines was decreased by CD39. The expression profile of CXCR3 in HMC-1 was similar to that of CXCL10 in ASMCs. IL-4 + IL-13 stimulation increased the CXCR3 mRNA expression, while CD39 reversed the CXCR3 expression increased by this treatment. ATP treatment also increased mRNA expression of CXCL10 in ASMCs. Stimulation with Th2 cytokine upregulated the mRNA expression of CXCL10 in ASMCs and the mRNA expression of CXCR3 in HMC-1 cells. Similarly, a recent study showed phenotype alteration of lung mast cells after infiltration into ASM bundles [33]. We also tested the expression of another CXCR3 ligand CCL11 in ASMCs but no significant difference was found after stimulation with IL-4 + IL-13 (data not shown). However, treatment with ATP or Th2 cytokine-stimulated ASMCs supernatants had no significant effect on CXCR4 expression in HMC-1 cells. These data showed that the expression of CXCL10/CXCR3 axis is regulated by eATP and CXCL10/CXCR3 axis plays an important role in the migration of HMC-1 cells towards ASMCs. Since CD39 could reduce the

eATP concentration in the supernatants of ASMCs stimulated with IL-4 + IL-13, inhibit the migration of HMC-1 induced by these supernatants and suppress the expression of CXCL10/CXCR3 axis, it might be a useful target to control the airway hyperresponsiveness in asthma.

In conclusion, our results demonstrated that in the Th2 cytokine microenvironment, ASMCs induce the mast cell migration via secretion of ATP and upregulation of CXCL10/CXCR3 axis. CD39 could be used to block the interactions between mast cells and ASMCs in asthma by degradation of ATP.

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Declaration of interest

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