Effects of β-arrestin 2 on cytokine production of CD4+ T lymphocytes of mice with allergic asthma

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β-arrestin 2 has been shown to participate in the pathogenesis of asthma by inducing Th2 cell migration to the lungs. Whether β-arrestin 2 regulates cytokine production of CD4+ T cells is still unknown. The aim of the present study was to investigate the effect of β-arrestin 2 on the cytokine production of CD4+ T lymphocytes and the mechanism involved in a mouse model for asthma. After silencing β-arrestin 2 expression in CD4+ T lymphocytes from asthmatic mice by RNA interference (RNAi), the interleukin-4 (IL-4) and interferon-γ (IFN-γ) levels in CD4+ T lymphocyte culture supernatants with or without terbutaline stimulation were determined. Cell-surface β2 adrenergic receptor (β2AR) as well as GATA3 expression of CD4+ T lymphocytes were also measured. CD4+ T lymphocytes of mice with allergic asthma expressed higher levels of β-arrestin 2 on both mRNA and protein levels. β-arrestin 2 RNAi decreased IL-4 (43.16%) and GATA3 (protein 77.21%, mRNA 62.98%) expression after terbutaline stimulation. Cell-surface β2AR of CD4+ T lymphocytes decreased (15.27%) after terbutaline treatment, but recovered after β-arrestin 2 RNAi down-modulation. These findings demonstrate that β-arrestin 2 regulates IL-4 production and GATA3 expression of CD4+ T lymphocytes partly through the β2AR signaling pathway in an allergic asthma model.

Keywords: Asthma, β-arrestin 2, β2 adrenergic receptors, CD4+ T lymphocytes, Interleukin-4

Asthma is a chronic inflammatory disease of the airways. CD4+ T cells play a crucial role in controlling inflammation in asthma by the production of several cytokines such as IL-4 and IFN-γ. Th2 cells play a key role in the pathogenesis of allergic asthma. The Th2 cytokine IL-4 is a major cytokine in the development of allergic inflammation.

β-arrestin 2, is an intracellular protein expressed ubiquitously, regulating G protein-coupled receptor (GPCR) function through multiple mechanisms. It was originally discovered to “arrest” G protein-mediated cell signaling events. In addition, β-arrestin 2 also acts as an adapter that couples GPCRs to a clathrin-coated pit endocytic mechanism and as a scaffold that links GPCRs to a second wave of cell signaling using several signaling pathways.

β-arrestin 2 deficiency augments polymorphonuclear leukocyte (PMN) chemotaxis and proinflammatory mediator production by PMNs, demonstrating that β-arrestin 2 is a negative regulator of inflammatory responses in leukocytes. Moreover, β-arrestin 2-deficient mice were considered to exhibit a defective CD4+ T cell migration to the lung, which provides evidence that β-arrestin 2 is involved in the manifestation of allergic asthma. Thus novel therapies focused on this protein may prove useful in the treatment of asthma. However, whether β-arrestin 2 plays a role in cytokine production of immune cells such as CD4+ T cells remains unclear.

The β2AR is a classical GPCR that regulates T lymphocyte activity. Impaired function of the β2AR system has been observed in peripheral blood lymphocytes during allergen-induced inflammatory reactions in asthma. The mechanism responsible for β2AR agonist-induced differential control of cytokines from Th1 and Th2 cells was shown to involve a differential level of β2AR expression in Th cells. Moreover, internalization and desensitization of β2ARs were considered to be closely related to β-arrestin 2. Since β-arrestin 2 regulates β2AR internalization which affects cytokine production of T lymphocytes, it is expected that β-arrestin 2 may regulate CD4+ T cell cytokine production via β2AR signaling pathway in allergic asthma.
The present study has been aimed to confirm the functional role of β-arrestin 2 on IFN-γ and IL-4 production, and GATA3 expression of CD4+ T cells by RNAi-mediated knock-down of β-arrestin 2. Moreover, whether the above-mentioned effects of β-arrestin 2 are caused by β-arrestin 2-mediated β2AR dysfunction of CD4+ T cells in allergic asthma was investigated by measuring cell-surface β2AR expression of CD4+ T cells and using β2AR agonist terbutaline stimulation.

Materials and Methods

Reagents and antibodies—Ovalbumin (OVA) Grade V, aluminum hydroxide gel and methacholine (Mch) were purchased from Sigma Chemical Co (USA). Mouse anti-CD4 microbeads and LS columns were purchased from Miltenyi Biotec (Germany) and terbutaline was obtained from AK Scientific (USA). Antibodies to β2AR, GATA3 and β-arrestin 2 as well as fluorescein-labeled goat anti-rabbit antibody were obtained from Santa Cruz Biotechnology (USA). IL-4 (sensitivity <2 pg/ml) and IFN-γ (sensitivity <2 pg/ml) ELISA kits were purchased from R&D systems Inc (USA). SiRNA targeting of the mouse β-arrestin 2 gene was designed and synthesized by Shanghai GenePharma Co.Ltd (China) and Lipofectamine 2000 was purchased from Invitrogen (USA).

Acute murine OVA-sensitization and challenge model of allergic asthma—Specific-pathogen-free grade female BALB/c mice 6-7 weeks old and weighing between 18–22 g were purchased from Slaikejingda Laboratory (Changsha, China). All mice were raised and kept on an OVA-free diet. The acute challenge were performed prior to tissue harvesting of the pulmonary function and methacholine challenge; airway responsiveness in mice was determined with a Buxco pulmonary function Apparatus. Briefly, after anesthesia with 5% chloral hydrate (500 mg/kg), mice received a tracheal intubation and were connected to a small animal respirator. First, the basis of airway resistance values was recorded for 1 min; subsequently airway resistance changes were determined after different concentrations of Mch aerosol challenge. Each atomization took 1 min and was recorded for 3 min. The concentrations of inspired Mch was from low to high as follows: 0.39, 0.78, 1.56 and 3.12 mg/ml after a saline challenge. Airway resistance (Rl) and dynamic lung compliance (Cdyn) were obtained by measuring mouse airway flow and pressure.

Magnetic activated cell sorting (MACS)—CD4+ T cells from splenic cell suspensions were purified by using a magnetic-activated cell sorter system according to the manufacturer’s instructions. Briefly, spleen cells were incubated with RBC lysate 3-5 times the volume of the cells (Beyotime Institute of Biotechnology, China) for 2 min at room temperature. After two washes, cells were incubated with streptavidin microbeads (100 µl/1 × 10^8 cells) at 1 × 10^6/ml for 15 min at 4°C in MACS buffer. After two additional washes, bead-bound cells were isolated using a separation column placed in a strong magnetic field and the positive fraction containing the CD4+ T cells was harvested.

RNA interference—SiRNAs targeting the mouse β-arrestin 2 gene were designed and synthesized by the Shanghai GenePharma Co.(China). The following siRNA preparations were used: siRNA-15 : 5-GGACCAGGGUCUUCAAGAATT-3, 5-UUCU UGAAGACCCUGGUCCCG-3; siRNA-1123 : 5- CGA AUUGCUAAACCUAUU-3, 5-AUAGUU GGUUACGAAUUCGAT-3; siRNA-585 : 5-GGA GGUCUCCACCUAGATT-3, 5-UCAUGGU GGAGGCCUCCGG-3. After transfection of CD4+ T cells as described below, silencing efficiency was examined by Western blot. The best siRNA was chosen for further experiments.
Cell culture and transfection—CD4+ T cells were transfected with siRNAs (see above) using the Lipofectamine 2000 according to modified manufacturer’s instructions. Briefly, siRNAs were diluted by DEPC water (1 OD/150 μl). siRNAs 7.5 μl and 250 μl Opti-MEM were mixed and incubated for 20 min at room temperature. Lipofectamine 2000 5 μl was diluted with 200 μl opti-MEM. Then the siRNA mixtures and lipofectamine 2000 were mixed by inversion and incubated for 5 min at room temperature. The entire transfection mixture was added to cell culture flasks containing 2.5×10^6 cells in 2.5 ml of RPMI-1640 medium. Cells were incubated for 24 h at 37°C and the medium was replaced by normal (serum-containing) growth medium. After an additional incubation for 24 h, silencing efficiency was examined by Western blot. Cells transfected with the best siRNA were used for further experiments.

Western blot—Splenic CD4+ T cells were harvested after treating with PMA (100 ng/ml) and ConA (5 μg/ml) for 24 h (1×10^6 cells/ml) with or without treatment with terbutaline (10^-5 M). Whole cell lysates were prepared from cells with RIPA lysis buffer containing protease inhibitors (Beyotime, Shanghai, China). Protein concentration was measured with a Bradford Protein Assay Kit (Beyotime, Shanghai, China). GAPDH was used as the loading control. Cells transfected with the best siRNA were used for further experiments.

Flow cytometry—Splenic CD4+ T cells were harvested after treatment with PMA (100 ng/ml) and ConA (5 μg/ml) for 24 h, with or without one last hour treatment with terbutaline (10^-5 M). After incubation in 5% fetal bovine serum (FBS) for 30 min, cells were incubated with a polyclonal rabbit anti-mouse β2AR Ab (1:100) at 4°C overnight, washed twice, incubated in fluorescein-labeled goat anti-rabbit antibody (1:100) at 4°C for 45 min in the dark. After surface labeling, cells were fixed with 2% paraformaldehyde in PBS. Data were analyzed on a FACS Calibur using CellQuest Pro (BD Biosciences, USA) software.

ELISA—Splenic CD4+T cells were treated with PMA and ConA for 24 h with or without treatment with terbutaline as described earlier. Supernatants were harvested from CD4+ T lymphocytes cultures. IL-4 and IFN-γ were quantified using the commercially available ELISA kits according to the manufacturer's recommendations. Samples were tested at least in duplicate.

Statistical analysis—Statistical analysis was performed using SPSS statistical software, version 17.0. All values were presented as mean ± SD. Rl, Cdyn and β-arrestin 2 mRNA expression of mice
as well as bronchoalveolar lavage cell counts were compared by using the Independent-Samples Student's t-test. The one-way analysis of variance (ANOVA) test was used for statistical analysis of differences in other tests between the groups. P<0.05 was considered statistically significant.

Results

Asthma model—After 24 h of last challenge, BALF was performed and the changes of lung functions of the mice from the PBS-treated group and the OVA-challenged group were measured. OVA-challenge greatly induced eosinophil, lymphocyte and neutrophil infiltration into the BALF and total cell numbers increased significantly in OVA-challenged mice (Table 1).

As the concentration of methacholine increased, lung resistance of the OVA-challenged group significantly increased as compared to the PBS-challenged group (Fig. 1A) and dynamic lung compliance of the OVA-challenged group was poorer than the control group (Fig. 1B) showing that the mice from the OVA-challenged group developed asthma in this model.

CD4+ T cells of mice from the asthma group express higher level of β-arrestin 2—β-arrestin 2 has been shown to be closely associated with allergic asthma. β-arrestin 2 expression of CD4+ T lymphocytes was first investigated. As shown in Fig. 2, β-arrestin 2 protein and mRNA of asthmatic mice were significantly higher than that of normal mice, suggesting that β-arrestin 2 plays a role in the pathogenesis of asthma. In order to uncover the precise role of β-arrestin 2, RNAi was used to knock-down β-arrestin 2 expression of CD4+ T lymphocytes. β-arrestin 2 protein of CD4+ T lymphocytes significantly decreased compared to the un-transfected lymphocytes in the asthmatic group after β-arrestin 2 RNAi (Fig. 2). Transfection of β-arrestins 2 siRNA reduced expression of the targeted β-arrestin 2 protein by 20.22 %.

β-arrestin 2 regulates IL-4 production of CD4+ T lymphocytes but not IFN-γ production—IL-4 and IFN-γ in the culture supernatant from CD4+ T lymphocytes was determined (Fig. 3). IFN-γ secretion of CD4+ T lymphocytes in the normal group was significantly higher than that in the asthmatic group while IL-4 levels in the normal group were significantly lower than those in the asthmatic group, which is consistent with previous studies.

Previous studies also showed that β-arrestin 2 is required for the manifestation of allergic asthma by sequestering T lymphocytes to the airways. However, whether β-arrestin 2 also influences the cytokine production of T lymphocytes is still unknown. As shown in Fig. 3, both IL-4 and IFN-γ levels of CD4+ T cells in asthmatic mice did not significantly change after β-arrestin 2 RNAi, which suggests that β-arrestin 2 does not directly affect cytokine production of CD4+ T lymphocytes. However, since β-arrestin 2 strongly influences β2AR, which is believed to regulate T lymphocyte activity, it is possible that β-arrestin 2 indirectly regulates cytokine secretion of CD4+ T lymphocytes in a β2AR-dependent manner.

Table 1—Bronchoalveolar lavage cell counts [Values are mean ± SD]

<table>
<thead>
<tr>
<th></th>
<th>Total cells</th>
<th>Mac</th>
<th>Lymph</th>
<th>Eos</th>
<th>Neu</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-treated</td>
<td>0.38 ± 0.28</td>
<td>0.35 ± 0.27</td>
<td>0.01 ± 0.01</td>
<td>0 ± 0</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>OVA-challenged</td>
<td>2.44 ± 0.90*</td>
<td>1.19 ± 0.50*</td>
<td>0.43 ± 0.16*</td>
<td>0.48 ± 0.18*</td>
<td>0.33 ± 0.11*</td>
</tr>
</tbody>
</table>

Mice were killed 24 h after the last challenge, and BALF was performed.

BALF: bronchoalveolar lavage fluid; OVA: ovalbumin; Mac: macrophage; Eos: eosinophil; Lymph: lymphocyte; Neu: neutrophil
Similar results were obtained in an earlier report which demonstrated that the exposure to β-agonists suppressed cytokine production of T lymphocytes, but may lose the control of Th2 lymphocytes after allergen challenge. As shown in Fig. 3A, IL-4 levels in the culture supernatant of CD4+ T lymphocytes are not significantly different between the asthma group and the terbutaline-treated asthma group, while IFN-γ levels of the asthma group decreased with terbutaline stimulation (Fig. 3B). Furthermore, the changes in the β-arrestin 2 RNAi group after terbutaline stimulation were studied. IL-4 levels of the terbutaline-treated asthma group decreased significantly after β-arrestin 2 RNAi, while IFN-γ did not change, suggesting that β-arrestin 2 could indirectly regulate IL-4 but not IFN-γ production of CD4+ T lymphocytes probably through β2AR. In addition, terbutaline inhibited IFN-γ production of CD4+ T lymphocytes in the asthma model.

β-arrestin 2 inhibits β2AR expression after terbutaline stimulation—One possible mechanism for the different effects on Th1 and Th2 cytokine expression is that the receptor responsible for mediating extracellular signals to the cell interior may be differentially expressed by CD4+ T cells of the different groups. To further test whether the regulatory role of β-arrestin 2 on the IL-4 production of CD4+ T cells was dependent on β2AR, cell-surface β2AR expression was determined. Cell-surface β2AR expression of cells from the asthma group was lower than from normal mice (Fig. 4). After terbutaline stimulation, β2AR expression decreased in the asthma group (Fig. 4). β2AR expression of the asthma group did not significantly change after β-arrestin 2 RNAi down-modulation (Fig. 4). However, after terbutaline stimulation, β2AR expression of the β-arrestin 2-transfected asthma group increased significantly compared to the non-transfected asthma group (Fig. 4). These data suggest that β-arrestin 2 RNAi knock-down may prevent β2AR internalization of CD4+ T lymphocytes of the asthmatic mice after terbutaline stimulation, which may play a regulatory role on cytokine production of CD4+ T lymphocytes in asthma.

β-arrestin 2 regulates GATA3 expression of CD4+T lymphocytes—GATA3 is a major Th2 regulatory transcription factor and promotes Th2 responses. GATA3 expression increased significantly in the asthma group and the terbutaline-treated asthma group compared to the control group on both protein and mRNA levels (Fig. 5). GATA3 protein levels did not show any differences between the asthma group and control group. After terbutaline stimulation, GATA3 protein levels significantly decreased in the asthma group (Fig. 5). However, after β-arrestin 2 RNAi down-modulation, GATA3 protein levels significantly increased in the asthma group (Fig. 5).}

Fig. 2—β-arrestin 2 expression of CD4+ T cells of murine asthmatic models and normal mice (ARRB2: β-arrestin 2). *Significant differences (p<0.05) compared to control; △Significant differences (P<0.05) compared to asthma group.

Fig. 3—IL-4 (A) and IFN-γ (B) production in CD4+ T cell supernatants (ARRB2: β-arrestin 2; Teb: terbutaline). *Significant differences (p<0.05) compared to control; △Significant differences (P<0.05) compared to asthma group; ▲Significant differences (P<0.05) compared to asthma+Terb group; ▲Significant differences (P<0.05) compared to asthma+ARRB2 RNAi group.
and the terbutaline-treated asthma group, but GATA3 mRNA decreased slightly after terbutaline treatment in asthma group (Fig. 5). More importantly, protein and mRNA expression of GATA3 decreased in the β-arrestin 2-transfected asthma group compared to the non-transfected asthma group. To our surprise, GATA3 expression was reduced to even a lower level when treated with terbutaline (Fig. 5).

Altogether, these results suggest that β-arrestin 2 may up-regulate GATA3 expression which promotes Th2 responses partly through β2AR.

**Discussion**

To our knowledge, this is the first study demonstrating that β-arrestin 2 regulates IL-4 and GATA3 expression of CD4⁺ T lymphocytes in a murine asthma model partly through mediating β2AR internalization, suggesting β-arrestin 2 plays an important role on CD4⁺ T lymphocyte functions not only through positive regulation of T lymphocyte chemotaxis to the lung but also through positive regulation of Th2-type cytokine production.

Walker et al.⁷ reported that allergen-sensitized mice having a targeted deletion of the β-arrestin 2 gene neither accumulate T lymphocytes in their airways nor show other inflammatory features characteristic of asthma partly because of defective CD4⁺ T cell migration to the lung. In addition, the expression of β-arrestin 2 in at least two different cell types contributed to the pathogenesis of the allergic airway disease. In the murine OVA model of allergic airway disease, eosinophilic and lymphocytic inflammation was shown to be dependent on the expression of β-arrestin 2 on hematopoietic cell types, while airway hyperresponsiveness was dependent on expression of β-arrestin 2 on stromal cells. Thus β-arrestin 2 was considered as a potential novel therapeutic target in asthma. Walker et al.⁷ also showed that IL-4 in the lavage fluid from wild type-OVA mice significantly increased relative to β-arrestin 2-deficient-OVA mice, which was attributed to defective chemotaxis of Th2 to the lung in β-arrestin 2-deficient mice. Nevertheless, in the present study it was shown that β-arrestin 2 also contributes to the pathogenesis of asthma through regulating cytokine production of CD4⁺ T lymphocytes, which further strengthens its potential importance as a therapeutic target in asthma.

β-arrestin 2 could regulate internalization, desensitization and signaling pathway of β2AR. Since β2AR-β-arrestin 2 interactions in intact cells depend on the presence of an agonist, terbutaline...
was used for β2AR stimulation in the present study. β-arrestin 2 induced β2AR internalization. Earlier studies also showed in the 12β6 cell line over-expressing β-arrestin 2 a marked reduction of β2AR on the plasma membrane after exposure to a β2AR agonist. A well-known mechanism involved in the β2AR internalization is that βARK phosphorylates β2AR and enables its binding to β-arrestins (especially β-arrestin 2), which prevents coupling of the receptor to the Gs protein. Th1 cells express a higher level of the β2AR cytoplasmic carboxyl terminus than Th2 cells. Another report showed that a detectable level of the β2AR was expressed on activated Th1 cells, but not on activated Th2 cells. Naïve CD4+ T lymphocytes in asthma mainly differentiate to Th1 and Th2 cells. Because Th2 cells express low to even undetectable levels of β2AR which has an inhibitory effect on cytokine production of CD4+ T lymphocytes, β2AR may have lost control of IL-4 levels in asthma. On the other hand, β2AR on Th1 cells shows a relatively smaller change, thus, β2AR could still inhibit IFN-γ production of CD4+ T lymphocytes in asthma.

Fig. 5—GATA3 protein (A, B) and mRNA (C) expression [ARRB2: β-arrestin 2; Teb: terbutaline. *Significant differences (P<0.05) compared to control; △Significant differences (P<0.05) compared to asthma group; #Significant differences (P<0.05) compared to asthma+Terb group; ▲Significant differences (P<0.05) compared to asthma+ARRB2 RNAi group]

Effects of several β2 agonists on T cells have been reported so far with seemingly conflicting results. Salmeterol suppressed IL-5 and IL-13 production by peripheral blood mononuclear cell (PBMC) stimulated with allergens and allergen-induced IL-5, IL-13 and IFN-γ production of PBMC were significantly suppressed by formoterol. While isoproterenol enhanced IL-5 expression by peripheral blood T cells activated by anti-CD3 and anti-CD28 antibodies, fenoterol, in contrast, inhibited IL-4, IL-5 and IL-13 mRNA expression of activated PBMC obtained from atopic asthmatics before allergen inhalation, but did no longer influence IL-4, IL-5 and IL-13 expressions after allergen challenge. The present results demonstrate that terbutaline...
inhibited IFN-γ production of CD4+ T lymphocytes of asthmatic mice, but did not influence IL-4 production unless β-arrestin 2 transfected, which is similar to several previous studies that β2 agonists may lose control of Th2 cells after allergen challenge. After β-arrestin 2 transfection, IL-4 of CD4+ T lymphocytes decreased in the terbutaline–treated asthmatic group, while IFN-γ decreased after terbutaline stimulation but did not get affected by β-arrestin 2. This indicates that β-arrestin 2 did not influence IFN-γ production but may increase IL-4 production of CD4+ T lymphocytes in asthma through β2AR agonistic stimulation.

Furthermore, β-arrestin 2 may up-regulate GATA3 partly through β2AR. Previous studies showed β-arrestin 2 acted as a positive regulator in β2AR-mediated ERK signaling and GATA3 was regulated by the ERK-MAPK pathway. It is possible that β-arrestin 2 may regulate GATA3 by influencing the β2AR-mediated ERK signaling pathway. However, GATA3 also decreased after β-arrestin 2 RNAi down-modulation without terbutaline stimulation, which indicates that β-arrestin 2 could also regulate GATA3 in a β2AR-independent manner. In addition, GATA3 expression decreased after β-arrestin 2 transfection while IL-4 did not significantly decline. This may be explained because cells used in this study were not CD4+ T cells of naïve mice but of asthmatic mice and GATA3 is less critical for maintaining the accessibility of IL-4 although it is important for initiating the Th2 polarization.

One of the limitations of this study is that the experiments were performed with mouse cells which don’t reflect the complicated situation of asthma patients. However, some experiments concerning β2AR of asthmatic patients were carried out. It was demonstrated previously that allergen exposure in asthma reduced the number of β2AR by 21% and function of β2AR by 40–50% in peripheral blood lymphocytes of allergic asthmatic patients, resulting in a loss of control over Th2-like cytokines. As shown in the present study, β2AR is likely to retain the control over CD4+ T cells in the asthma models when β-arrestin 2 was knocked-down which may be meaningful for the treatment of asthma.

In summary, the present study demonstrates that β-arrestin 2 regulates IL-4 production of CD4+ T lymphocytes in a murine allergic asthma model partly through mediating β2AR internalization and confirmed the effect of β2AR on cytokine production of CD4+ T lymphocytes in asthma. Further studies will aim to increase the knowledge on β-arrestin 2 for the differentiation of CD4+ T lymphocytes to further reveal its precise function in asthmatic patients.

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