

Altered β_2 -adrenergic regulation of T cell activity after allergen challenge in asthma

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Summary

Background Airway inflammation in asthma is orchestrated by recruitment of T helper (Th)2 lymphocytes to the lung and subsequent production of Th2-like cytokines upon allergen challenge.

Objective To examine whether allergen-induced dysfunction of the β_2 -adrenergic receptor (β_2 -AR) contributes to the enhanced T(h)2 cell activity in asthma.

Methods β_2 -adrenergic regulation of cytokine mRNA expression was studied in α -CD3/ α -CD28-activated peripheral blood lymphocytes from seven asthma patients before and 6 h after allergen challenge, in conjunction with the effects of β_2 -agonist fenoterol on T cell chemotaxis and signalling pathways.

Results A complete loss of β_2 -AR control over expression of the Th2 cytokines IL-4, IL-5 and IL-13, but not of the Th1 cytokine IFN- γ , was observed after allergen challenge. Furthermore, we found impaired β_2 -AR regulation of T cell migration as well as signal transduction pathways, i.e. the phosphorylation of cyclic adenosine monophosphate-responsive element binding protein and the inhibition of the mitogen-activated protein kinase pathway. The loss of β_2 -AR control was associated with increased β -adrenergic receptor kinase expression, which might be involved in β_2 -AR desensitization. In addition, we demonstrate for the first time that T cells exposed to the chemokine thymus and activation-regulated chemokine show hyporesponsiveness to fenoterol.

Conclusion Our results suggest that allergen-induced loss of β_2 -AR control, possibly mediated by chemokine release, plays an important role in enhanced Th2-like activity in asthma.

Keywords β_2 -adrenergic receptor, allergen challenge, asthma, chemotaxis, TARC, Th2 cytokines

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Introduction

Atopic asthma is a complex disorder characterized by allergen-induced airway inflammation with associated eosinophilia, bronchoconstriction, airway wall oedema and bronchial hyper-responsiveness [1–3]. Infiltrating T lymphocytes are thought to orchestrate the immune response associated with asthma by the production of T helper (Th)2-like cytokines, e.g. IL-4, IL-5 and IL-13. Enhanced expression of these cytokines has been observed in bronchoalveolar lavage and bronchial biopsies from asthma patients [4–6]. After allergen challenge, T cell activity increases, as reflected by further up-regulation of Th2 cytokines, enhanced expression of activation markers and migration of T cells into lung tissue [7–11]. Virtually all IL-4⁺ Th cells that enter the lung upon allergen challenge have been described to express the C–C chemokine receptor (CCR)4 [12, 13]. The production of one of the ligands for CCR4, i.e. thymus and activation-regulated chemokine (TARC), is enhanced in bronchial tissue

after allergen challenge and may thus be responsible for the attraction of T cells [12, 14]. CCR4 is preferentially expressed on Th2 cells [15], suggesting that primarily Th2-like T cells infiltrate lung tissue upon allergen challenge. Until now, the mechanisms underlying enhanced Th2-type activity in asthma are poorly understood.

We hypothesized that a defect in the β_2 -adrenergic system contributes to the increased Th2-like activity. The β_2 -adrenergic system can suppress inflammatory activity by activation of the G_s-coupled adenylyl cyclase (AC) system and a subsequent rise in intracellular cyclic adenosine monophosphate (cAMP) [16, 17]. T cell-mediated responses can be controlled by cAMP through inhibition of both Th1 and Th2 cytokines, although the ultimate effect of cAMP on Th2 cytokines is dependent on the activation status of the T cell and the presence of costimulatory signals [18–20]. A defect in the β_2 -adrenergic receptor (β_2 -AR)/AC system in asthma has been proposed a long time ago. The importance of the β_2 -adrenergic/AC system in asthma was first demonstrated by the observation that asthma-like symptoms develop in mice and rats when the β -adrenergic system is defective [21]. In addition, β_2 -adrenergic unresponsiveness can be induced in asthma patients by regular treatment with

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β_2 -agonists. This leads to an increased sensitivity to allergen exposure, suggesting that β_2 -adrenergic dysfunction may have important implications for allergic airway inflammation [22]. Allergen-induced dysfunction of the β_2 -AR/AC system has been observed in peripheral blood lymphocytes [23]. Because of the implications for allergic airway inflammation, we were interested in the effects of allergen-induced β_2 -AR dysfunction on T cell activity. Lung infiltration of activated T cells is thought to occur within 24 h after allergen challenge, whereas β_2 -AR desensitization in peripheral lymphocytes has already been observed after 3 h [24]. Therefore, we anticipated regulatory changes within 3–24 h after allergen challenge and studied β_2 -AR regulation of T cell activity 6 h after allergen challenge. We demonstrate that β_2 -AR control over Th2 cytokine production, T cell chemotaxis and signalling pathways is completely abolished after allergen challenge and we discuss the possible underlying mechanism.

Materials and methods

Subjects

Seven patients with a diagnosis of asthma, aged 18–45 years, were included on the basis of a late obstructive airway response after inhalation of house dust mite (HDM), cat or timothy grass (forced expiratory volume in 1 s (FEV₁)) during the late asthmatic response <15% from baseline, a provocation–concentration of histamine causing 20% fall in FEV₁ from the predicted FEV₁ value (PC₂₀ <8 mg/mL) and at least one positive skin prick test (for the allergen of interest). For clinical characteristics see Table 1. Patients did not receive oral corticosteroids on the preceding 2 months or any other medication on the preceding month, nor did they experience respiratory tract infections or acute asthmatic attacks within 4 weeks prior to the start of the study. None of the patients were smokers. The Medical Ethics Committee of the University Hospital of Groningen approved the study.

Study design

Blood samples (20 mL) were taken on two subsequent days at similar time-points, on the control day and the day of allergen challenge. The allergens used are described in Table 1. The extracts for allergen provocation were prepared from stock solutions of Der p (HDM), cat dander, or timothy grass, diluted in phosphate buffered saline containing 0.03% human

serum albumin and 0.5% phenol (ALK Benelux, Groningen, The Netherlands), producing fivefold increasing concentrations ranging from 1:4 to 1:128. Allergen provocation took place as described by Aalbers et al. [25]. In short, allergen concentrations were inhaled at 15 min intervals. The provocation was started with the lowest concentration and was finished at the concentration that evoked a 20% fall in FEV₁. Blood samples were taken 6 h after the induction of an early obstructive reaction, which was determined by at least a 20% fall in FEV₁ within the first hour after allergen challenge.

Cell cultures, cytokines and reagentia

Peripheral blood mononuclear cells (PBMCs) from asthmatic subjects or healthy donors were isolated by Ficoll–Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. T cells were isolated by rosetting with 2-aminoethylisothionium bromide (AET)-treated sheep red blood cells (SRBCs), as described before [18]. After isolation, the PBMCs or T cells were incubated for 30 min at 37 °C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 0.5%, 1% or 5% fetal calf serum (FCS; Hyclone, Logan, UT, USA), supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. T cell-specific stimulation with α -CD3 and α -CD28 (50 µL/mL) was used to induce cytokine expression, as described before [18]. Fenoterol (Sigma, St Louis, MO, USA), prostaglandin E₂ (PGE₂) (Sigma) and sodium fluoride (Sigma) were used in a concentration of 10 µM, 100 nM and 10 mM, respectively, as previously described [26]. TARC (R&D Systems, ITK Diagnostics, Uithoorn, the Netherlands) was used in a concentration of 100 ng/mL.

Measurement of cytokine mRNA expression by LightCycler PCR

3×10^6 PBMCs/mL in RPMI 1640 containing 5% FCS were stimulated with α -CD3/ α -CD28 during 5 h, in the presence and absence of fenoterol and PGE₂. RNA was isolated using the TRIzol method (GIBCOBRL, Burlington, ON, Canada) and cDNA was synthesized as described before [18]. Expression of cytokine mRNA was analysed by quantitative real-time-PCR, using the LightCycler system (Roche, Basel, Switzerland). Two microlitres FastStart DNA SybrGreen I mix (Roche), 2 mM MgCl₂, 0.5 µM of forward and reverse primer and 2 µL cDNA were used. Gene expression was measured by relative quantification, which compares the threshold cycle (Ct) of the sample of interest to the Ct

Table 1. Clinical characteristics of the asthmatic subjects

Patient	Gender	Age (years)	FEV ₁ (%pred)	FEV ₁ (L) <i>t</i> = 0	FEV ₁ (L) <i>t</i> = 6	PC ₂₀ hist mg/mL	Allergen
1	m	54	104	3.57	2.62	4.84	Grass
2	m	38	73	3.34	2.04	0.22	HDM
3	m	19	105	3.90	2.17	1.84	HDM
4	m	42	83	3.32	2.48	0.37	Cat
5	m	21	81	3.80	2.42	0.57	HDM
6	m	27	100	4.85	2.41	1.67	HDM
7	m	18	115	4.02	2.88	1.81	HDM

FEV₁ (%pred), percent of predicted value of FEV₁; *t* = 0, baseline; *t* = 6, 6 h after allergen challenge; PC₂₀ hist, PC₂₀ histamine as described in the methods; HDM; house dust mite.

generated by a reference sample (in our case the α -CD3/ α -CD28-stimulated PBMCs). Cytokine gene expression was normalized to expression of the housekeeping gene β -2-microglobulin ($\beta_2\mu$ G). The Δ Ct was calculated as the difference between Ct values for stimulated PBMCs in the absence and presence of fenoterol or PGE₂. The relative difference in cytokine expression between cells was determined using the equation $2^{-\Delta\text{Ct}}$. This equation is valid only if the amplification efficiencies of $\beta_2\mu$ G and the cytokines to be measured are approximately equal. To confirm this, standard curves were generated by serial dilution of samples and the efficiency for each primer set was determined. To exclude inter-assay variations, all cDNA samples from one patient were run together in one PCR. Cytokine gene expression was normalized to expression of the housekeeping gene $\beta_2\mu$ G, with approximately equal amplification efficiency. The Δ Ct was calculated as the difference between the Ct values, determined using the equation $2^{-\Delta\text{Ct}}$. The following specific primer pairs for $\beta_2\mu$ G, IL-4, IL-5, IL-13 and IFN- γ were obtained from Biolegio BV (Malden, the Netherlands): $\beta_2\mu$ G: 5'CCAGCAGAGAATGGAAAGTC3' sense and 5'GATGCTGCTTACATGTCTCG3' antisense, IL-4: 5'TGCCTCCAAGAACAACACTG3' sense and 5'AACGTACTCTGGTTGGCTTC3' antisense, IL-5: 5'GCTTCTGCATTTGAGTTTGCTAGCT3' sense and 5'TGGCCGTCAATGTATTCTTTATTAAG3' antisense, IL-13: 5'CGGTCATTGCTCTCACTTGCCCTT3' sense and 5'TTACCCCTACCTAACCCTCCTT3' antisense, IFN- γ : GCAGAGCCAAATTGTCTCCT3' sense and 5'ATGCTCTTCGACCTCGAAAC3' antisense. PCR conditions were a denaturation step at 94 °C for 10 min followed by 40 cycles of 94 °C, 10 s; 58 °C, 10 s; 72 °C, 20 s.

Migration assay

The migration assay was performed using a microchamber transwell system with 5 μ m pores (Corning Costar Incorporated, New York, MA, USA). 2×10^5 T cells/100 μ L RPMI 1640 containing 1% FCS were applied to the upper compartment of the transwell system. Migration was induced by TARC present in the lower compartment. T cells were allowed to migrate to the lower well for 2 h, in the presence and absence of fenoterol added to the upper well (10 μ M). The upper well was removed and the T cells in the lower well were counted and expressed as percentage of the total amount of T cells added to the upper well. Experiments were carried out in duplicate.

Immunodetection by Western blotting

Phosphorylation of cAMP-responsive element binding (CREB) protein and extracellular signal-regulated kinase (ERK) and the expression of β ARK2 were analysed by Western blotting. 3×10^6 PBMCs/mL in RPMI 1640 containing 0.5% FCS were stimulated with or without fenoterol, PGE₂ or NaF for 60 min. In healthy donors, cells were incubated with or without TARC (100 ng/mL) for 6–12 h prior to stimulation. Total cell lysates were prepared, separated by SDS-10% PAGE and transferred to a nitrocellulose membrane. The immunodetection of phospho-CREB, phospho-ERK (New England Biolabs, Hitchin, Herts, UK), β ARK2, pan-ERK and actin (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) was performed according the manufacturer's guidelines (ECL, Amersham, Buckinghamshire, UK). Relative protein levels were quantified using the gelscan program ImageMaster (Pharmacia, Uppsala, Sweden).

Measurement of IL-5 protein

3×10^6 T cells isolated from healthy controls were pre-incubated with or without TARC for 12 h in 1 mL RPMI containing 5% FCS and stimulated with α -CD3/ α -CD28 during 8 h. Fenoterol was added 5 min prior to stimulation. IL-5 protein was measured in cell-free supernatants, using ELISA, performed as previously described by Heijink et al. [27].

Statistical analysis

Analysis was performed by using the Wilcoxon's signed rank test for paired observations in case of mRNA measurements or by using the Student's *t*-test for paired observations in case of protein level measurements or T cell migration. Data are expressed as median (range) or mean $\pm$ SEM. Statistical significance of the secretion data was set at $P < 0.05$.

Results

β_2 -adrenergic receptor control over T helper 2, but not T helper 1, cytokine mRNA expression is abolished after allergen challenge

The α -CD3/ α -CD28-induced mRNA expression of IFN- γ on the control day was not significantly different from the expression after allergen challenge (data not shown). On the control day, fenoterol exerted a strong inhibitory effect on α -CD3/ α -CD28-induced IFN- γ mRNA expression (Fig. 1), the median (range) expression being reduced from 100% to 45% (21–66%). Six hours after inhalation of the allergen, the expression of IFN- γ was still inhibited by fenoterol, median 42% (31–59%), the inhibitory effect being not significantly altered.

Similar to IFN- γ , no significant change could be observed in the α -CD3/ α -CD28-induced expression of Th2 cytokines after allergen challenge, although the expression of IL-4 (but not IL-5 or IL-13) tended to be up-regulated (data not shown). In contrast to IFN- γ , β_2 -AR regulation of Th2-type cytokines was significantly altered after challenge. Before allergen challenge, fenoterol modestly but significantly inhibited IL-4, IL-5 and IL-13 mRNA expression (median 82% (68–100%), 75% (58–100%) and 70% (30–90%), respectively, Fig. 1). Allergen inhalation induced a complete loss of β_2 -adrenergic control over Th2 cytokine expression. As shown in Fig. 1, IL-4, IL-5 and IL-13 expressions were no longer significantly inhibited by fenoterol and in some patients even an up-regulatory effect could be observed (median 104% (72–268%), 121% (92–200%) and 120% (60–150%), respectively). In contrast to fenoterol, the effect of PGE₂ on IL-4, IL-5, IL-13 and IFN- γ expression was not significantly altered upon allergen challenge. Median expressions of IL-4, IL-5, IL-13 and IFN- γ were 63% (32–100%), 56% (10–100%), 30% (18–84%) and 23% (9–56%),

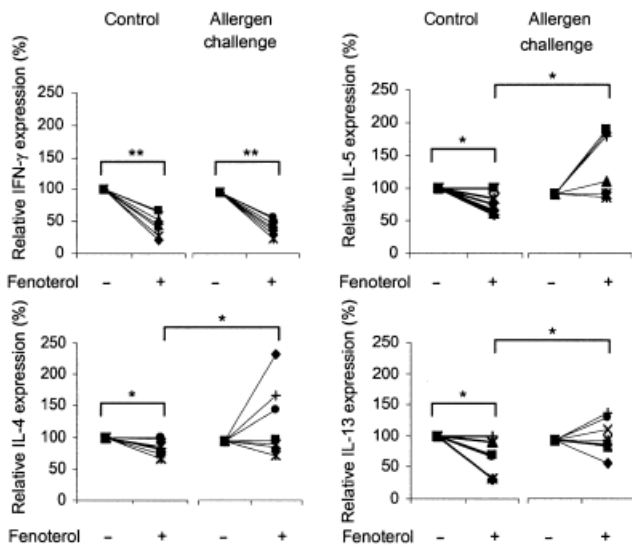


Fig. 1. mRNA expression of the T helper (Th)1 cytokine IFN- γ and the Th2 cytokines IL-4, IL-5 and IL-13 in peripheral blood mononuclear cells isolated from seven asthmatic subjects before (control day) and 6 h after allergen challenge, stimulated with α -CD3/ α -CD28 for 5 h in the absence and presence of fenoterol. mRNA levels were normalized for β -2-microglobulin and expressed as percentage of the secretion after stimulation with α -CD3/ α -CD28 in the absence of fenoterol, both before and after allergen challenge. * P < 0.05 and ** P < 0.01 between the indicated values.

respectively, on the control day and 62% (46–100%), 72% (10–100%), 58% (15–84%) and 19% (10–29%) respectively, after allergen challenge in the presence of PGE₂, P = 0.73, 0.07, 0.08 and 0.25, respectively. This indicates that the allergen-induced changes in Th2 cytokine regulation are specific for β_2 -AR function.

The β_2 -adrenergic system controls T cell chemotaxis in healthy controls and stable asthma

To study if the infiltration of T cells is also controlled by the β_2 -adrenergic system, we examined the migratory response of T cells *in vitro*. Little information is available about β_2 -AR of T cell migration, therefore we first studied this in freshly isolated T cells from healthy controls. Because of its implications for asthma, TARC was used to induce T cell migration. As demonstrated in Fig. 2a, some spontaneous migration was found (mean $\pm$ SEM: $2.4 \pm 0.7\%$ of the total number of T cells, n = 8). The number of T cells migrating to the lower well was significantly enhanced by the addition of TARC, with a maximal response at 100 ng/mL (Fig. 2a). The addition of 10^{-8} M (data not shown) and 10^{-5} M (Fig. 2a) fenoterol significantly inhibited the TARC-induced T cell migration, by approximately 40% (P < 0.01, n = 8).

The effect of fenoterol on T cell migration was examined in three asthmatic subjects, before and 6 h after allergen challenge. Before the challenge, similar results were obtained compared with healthy donors, i.e. TARC induced an approximately twofold increase in T cell migration, which was inhibited by fenoterol (Fig. 2b). After the allergen challenge, β_2 -adrenergic control over T cell migration was significantly reduced (P < 0.05, n = 3), indicating that the loss of β_2 -AR control is not specific for Th2-like cytokine production and may serve to facilitate T cell migration.

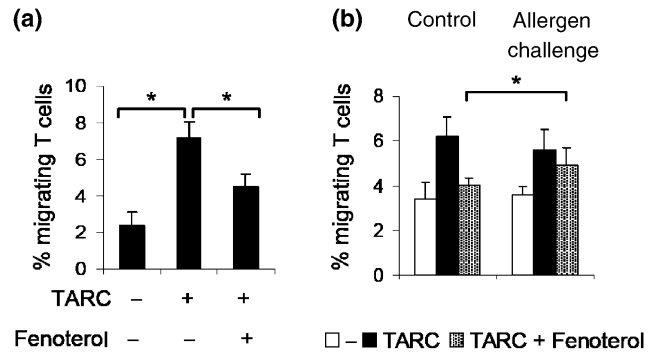


Fig. 2. (a) Thymus and activation-regulated chemokine (TARC)-induced migration of T cells isolated from healthy donors, in the absence and presence of fenoterol. Migrating T cells are expressed as percentage of the total amount of T cells added to the upper compartment (mean $\pm$ SEM, n = 8). * P < 0.001 between the indicated values. (b) TARC-induced migration of T cells isolated from three asthmatic subjects before (control day) and 6 h after allergen challenge, in the absence and presence of fenoterol. T cell migration is expressed as percentage of the total amount of T cells added to the upper compartment (mean $\pm$ SEM, n = 3). * P < 0.05 between the indicated values.

Impaired CREB phosphorylation by β_2 -adrenergic stimulation after allergen challenge

To investigate whether the reduced sensitivity to fenoterol was caused by the inability to activate the AC/cAMP system, we studied the activation of cAMP downstream effector CREB. In PBMCs derived on the control day, fenoterol induced an increase in the phosphorylation of CREB, which was observed for each patient (Fig. 3a). The ability of fenoterol to induce CREB phosphorylation was impaired in PBMCs isolated 6 h after the allergen challenge. In some patients, the induction of CREB phosphorylation was reduced, whereas in others no induction of CREB phosphorylation could be observed at all. In contrast to fenoterol, both PGE₂ and direct activation the G protein by NaF induced a strong, significant increase in CREB phosphorylation after the challenge (Fig. 3a), suggesting a defect at β_2 -AR level. The magnitude of reduction in fenoterol-induced CREB phosphorylation could not directly be related to the magnitude of changes in the regulation of cytokine expression.

Impaired regulation of extracellular signal-regulated kinase activity by β_2 -agonist fenoterol after allergen challenge

Th2-like cytokines are unlikely regulated by CREB, yet may be regulated by the interference of cAMP with other pathways. An important pathway in T cell activation that is controlled by cAMP is the Ras/Raf/ERK pathway [28]. cAMP has been described to inhibit this pathway, whereas the desensitized β_2 -AR may activate this pathway [29]. We studied the effect of fenoterol on ERK activation before and after allergen challenge. Fig. 3b demonstrates an inhibitory effect of fenoterol on ERK phosphorylation in PBMCs isolated on the control day. After allergen challenge, the inhibitory effect of fenoterol on ERK phosphorylation was abolished in two out of four patients, while ERK phosphorylation was even slightly up-regulated by fenoterol in two others. In the latter two, we also observed up-regulation of Th2 cytokine expression by fenoterol. This further illustrates the altered function of the β_2 -AR upon allergen challenge.

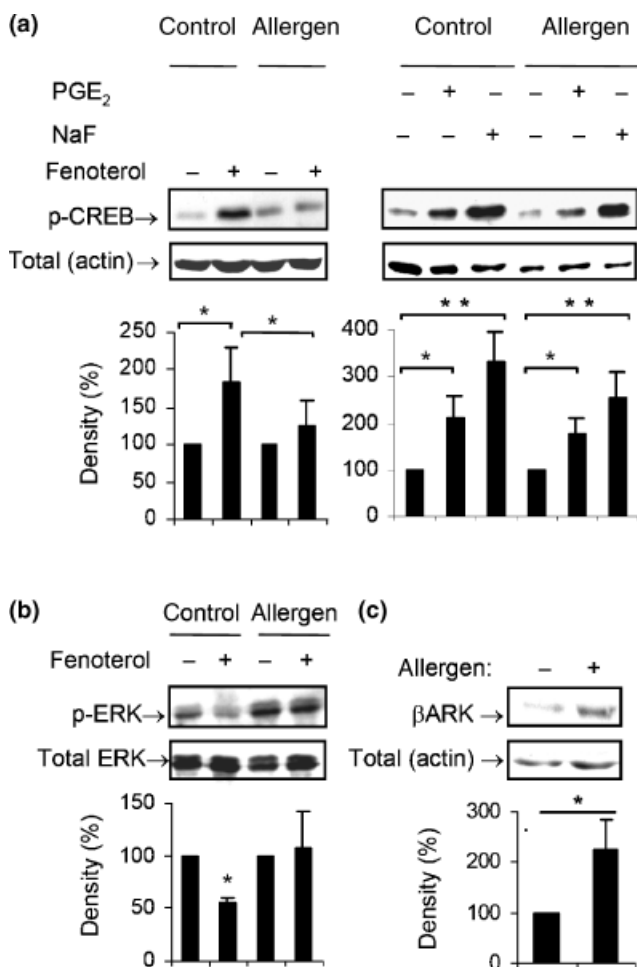


Fig. 3. Immunodetection of phospho-CREB, phospho-ERK and β -adrenergic receptor kinase 2 (β ARK2) in peripheral blood mononuclear cells (PBMCs) isolated from asthma patients before (control day) and 6 h after the allergen challenge. (a) PBMCs were stimulated with fenoterol, prostaglandin E₂ (PGE₂) or NaF for 60 min. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). The phospho-CREB levels were normalized for actin. The levels in the absence of fenoterol, PGE₂ or NaF were set to 100% and mean relative phospho-CREB values $\pm$ SEM of seven asthmatic subjects are depicted in the corresponding diagram. * $P < 0.05$ and ** $P < 0.01$ between the indicated values. (b) PBMCs were incubated with fenoterol for 60 min. Phospho-ERK is depicted in the upper panel and total protein levels (ERK) are shown in the lower panel (marked by arrows). The phospho-ERK levels were normalized for actin. The levels in the absence of fenoterol were set to 100% and mean relative phospho-ERK values $\pm$ SEM of four asthmatic subjects are depicted in the corresponding diagram. * $P < 0.01$ between the indicated values. (c) β ARK2 is depicted in the upper panel and actin in the lower panel. The β ARK2 levels were normalized for actin. The levels on the control day were set to 100% and mean relative β ARK2 values $\pm$ SEM of seven asthmatic subjects are depicted in the corresponding diagram. * $P < 0.05$ between the indicated values. Representative blots are shown.

Up-regulation of β -adrenergic receptor kinase 2 expression after allergen challenge

G protein-coupled receptor kinases (GRKs) induce phosphorylation and desensitization of G protein-coupled receptors. GRK2 and GRK3, also called β -adrenergic receptor kinase (β ARK)1 and β ARK2, are specific for the β_2 -AR. Agonist-induced β_2 -AR desensitization can be enhanced by β ARK overexpression [29–33]. To study whether or not

β ARK up-regulation may play a role in allergen-induced β_2 -AR desensitization, the protein levels of β ARK were analysed before and 6 h after an allergen challenge. The levels of β ARK1 were not clearly altered upon allergen challenge (data not shown). Interestingly, the expression of β ARK2 significantly increased about a twofold after the allergen challenge (Fig. 3c). Possibly, this enhanced expression of β ARK2 contributes to the β_2 -AR hyporesponsiveness.

Activation of C-C chemokine receptor 4 by TARC induces β_2 -adrenergic hyporesponsiveness

Because activation of the G_i protein is known to counteract the effects of G_s signalling, we hypothesized that the activation of G_i-coupled chemokine receptors might be involved in the reduced β_2 -adrenergic responsiveness. We used TARC to induce CCR4 activation and studied the effect on β_2 -adrenergic function and β ARK expression in T cells obtained from healthy donors. T cells incubated with TARC for 12 h show stronger β ARK2 expression compared with T cells incubated without TARC (Fig. 4a). In addition, incubation with TARC reduced the capacity of β_2 -agonist fenoterol to induce CREB phosphorylation (Fig. 4b). This effect was already observed after 6 h, but more pronounced after 12 h of pre-incubation. Likewise, pre-incubation with TARC (12 h) reduced the capacity of fenoterol to control the secretion of Th2 cytokine IL-5 (Fig. 4c), while Th1 cytokine

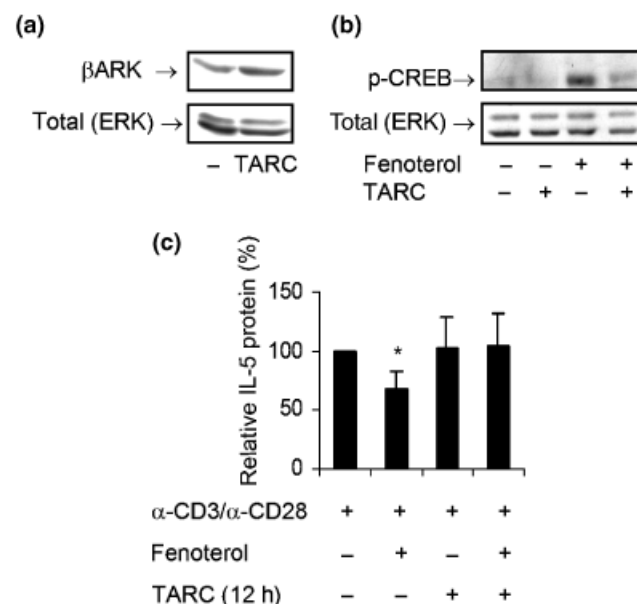


Fig. 4. (a) Immunodetection of β -adrenergic receptor kinase 2 (β ARK2) in T cells isolated from healthy donors and incubated with and without thymus and activation-regulated chemokine (TARC) for 1 h. β ARK2 is depicted in the upper panel and total ERK is depicted in the lower panel. A representative blot of three independent experiments is shown. (b) Immunodetection of phospho-CREB in T cells from healthy donors, after 12 h of pre-incubation with and without TARC and 60 min of stimulation with fenoterol. Phospho-CREB is depicted in the upper panel and total ERK levels are shown in the lower panel (marked by arrows). A representative blot of five independent experiments is shown. (c) Secretion of IL-5 protein in T cells isolated from healthy donors that were pre-incubated with TARC for 12 h and subsequently stimulated with α -CD3/ α -CD28 for 8 h in the absence and presence of fenoterol. IL-5 protein levels are expressed as percentage ($x \pm$ SEM, $n = 8$) of the secretion after stimulation with α -CD3/ α -CD28 in the absence of fenoterol. * $P < 0.05$ for the cytokine secretion levels after pre-incubation with fenoterol compared with the level without.

IFN- γ was still strongly inhibited (by approximately 70%, $P = 0.01$, data not shown). Thus, TARC appears to induce β_2 -adrenergic hyporesponsiveness and β ARK2 up-regulation, similar to allergen exposure in asthma.

Discussion

Allergen-induced bronchoconstriction in asthma is known to be associated with enhanced Th2 activity [7–11]. In this study, we show that a defect in the β_2 -adrenergic system may contribute to Th2-mediated inflammation in asthma. Allergen inhalation induced a loss of β_2 -AR control in peripheral lymphocytes, leading to an enhanced cellular activation status. While the β_2 -agonist fenoterol exerted inhibitory effects on both Th1 and Th2-like cytokines in stable asthma, Th2 cytokines were no longer inhibited after allergen challenge, whereas the control over Th1 cytokine IFN- γ remained. This may thus favour Th2 activity at the sites of allergen-induced inflammation, where T cell cytokine production is induced. The reduced sensitivity of peripheral lymphocytes to fenoterol appears to be a consequence of impaired activation of the cAMP-dependent pathway. The associated up-regulation in β ARK2 expression may be one of the mechanisms involved in allergen-induced β_2 -adrenergic desensitization. Our findings may explain why airway inflammation in asthma cannot be suppressed by the treatment with β_2 -agonists and why β_2 -AR desensitization *in vivo* upon regular use of β_2 -agonists renders asthmatic airways more sensitive to allergen [22].

In addition to the inhibition of T cell cytokine production, we observed that fenoterol inhibited T cell migration, suggesting that the β_2 -adrenergic system functions to retain T cells in the circulation. This inhibitory effect of fenoterol on T cell chemotaxis was abolished upon allergen challenge. Thus, particularly T cells that lose their β_2 -adrenergic control may infiltrate the lung upon allergen challenge. A part of the T(h2) cells may already have migrated to the lung 6 h after challenge. However, based on a previous study where priming for IL-5 production was found in peripheral blood lymphocytes after 24 h [34], we expect that a substantial number of Th2 cells is still present in the circulation 6 h after challenge.

Previously, it has been demonstrated that allergen challenge induces impaired AC activity in response to β_2 -agonists. The membrane expression of the β_2 -AR was only slightly reduced, suggesting uncoupling of the receptor from the AC complex. This was observed in lymphocytes from asthma patients, but not in healthy controls [23]. Furthermore, obstructive reactions caused by histamine inhalation did not impair β_2 -AR function [35], indicating that the release of inflammatory mediators during the allergic reaction rather than the stress induced by airway obstruction is responsible for the β_2 -AR dysfunction. This is further supported by the observation that epinephrine levels are not increased upon allergen exposure in asthma [36], suggesting the involvement of heterologous factors.

Our findings concerning CREB indicate that the reduced sensitivity of T cells to β_2 -agonist fenoterol is a consequence of impaired activation of the cAMP-dependent pathway,

specific for the β_2 -AR. Activation of the G_s protein/AC-coupled PGE₂ receptor still clearly induced activation of the cAMP-dependent pathway after allergen challenge. Moreover, direct activation of the G protein by NaF strongly induced phosphorylation of CREB after allergen challenge. This suggests reduced coupling of the β_2 -AR to the G_s protein and AC system instead of impaired G_s or AC function. A well-known mechanism involved in the desensitization of the β_2 -AR is the activation of β ARK [30–32]. β ARK phosphorylates the β_2 -AR and enables its binding to β -arrestin, which prevents coupling of the receptor to the G_s protein [29, 31, 33]. Overexpression of β ARK promotes agonist-induced phosphorylation of the β_2 -AR [31]. A β ARK2-mediated mechanism may be involved in the allergen-induced β_2 -adrenergic dysregulation, because we observed that the β ARK2 expression was increased upon allergen challenge. Instead of activation of the AC/cAMP pathway, after its coupling to β -arrestin the β_2 -AR has been described to induce a new set of signalling events, including activation of the MAPK pathway [29]. Indeed, we found that the inhibitory effect of fenoterol on ERK activation was abrogated after the allergen challenge and in some patients we even observed induction of the MAPK/ERK pathway. This might lead to a more activated status of lymphocytes and contribute to the up-regulation of Th2 cytokines as observed in some patients.

In a previous study, loss of β_2 -adrenergic control over both IFN- γ and IL-5 has been observed 24 h after allergen challenge [34]. This is in contrast to our findings and possibly the result of further down-regulation of β_2 -AR function or down-regulation in another T cell subset. In contrast to Th2 cytokines, IFN- γ expression remained inhibited by fenoterol after the allergen challenge in our setting. As production of IFN- γ appears to be more sensitive to cAMP than production of Th2 cytokines, the resting activity of the desensitized β_2 -adrenergic AC/cAMP system might still be able to efficiently inhibit IFN- γ . Another explanation for the differential effect on Th1 and Th2 cytokines might be that β_2 -adrenergic desensitization predominantly occurs in Th2-like cells. As virtually all T cells that enter lung tissue after allergen challenge have been demonstrated to express the Th2-specific chemokine receptor CCR4, it is tempting to speculate that particularly these cells lose their negative feedback. The limited quantity of sampled blood did not allow us to study the composition of T cells both before and after the allergen challenge. Because the migratory response of T cells was in general inhibited by fenoterol, we do not render it likely that the reduced β_2 -AR function in peripheral blood is caused by a selective efflux and lung recruitment of T cells expressing a functional β_2 -AR. Instead, our observation suggests that impaired β_2 -adrenergic function may be a prerequisite for T cells to enter the lung. TARC is one of the ligands for CCR4 and the release of TARC into the circulation may be responsible for the attraction of peripheral T cells to the lung after the allergen challenge [12, 14, 37]. Most chemokines induce migration through the activation of the receptor-associated G_i protein. The activation of G_i -coupled receptors is known to counteract the effects of the G_s protein and may induce β ARK-mediated desensitization of G protein-coupled receptors [38–40]. We found that *in vitro* pre-incubation with TARC induced β_2 -adrenergic hyporesponsiveness in T cells from healthy donors, with similar alterations as observed in

asthmatic subjects. Because T cell migration was inhibited by fenoterol, the TARC-induced β_2 -adrenergic desensitization might facilitate the migration of CCR4⁺ cells. Thus, upon the allergen-induced release of TARC into the circulation, peripheral CCR4⁺ T cells may change their β_2 -AR function. As a consequence, the impaired negative feedback control may lead to enhanced activity of the T cells that enter the inflamed tissue. Recently, it has been described that β -arrestin-2 is required for the accumulation of T cells in the airways and the development of asthma in allergen-sensitized mice [41]. As β -arrestin is involved in β_2 -AR desensitization [29], this further supports a role for impaired β_2 -AR/G_s signalling in the pathophysiology of asthma.

In summary, our data demonstrate a loss of β_2 -adrenergic control in peripheral blood lymphocytes 6 h after allergen challenge. This impaired β_2 -AR function is reflected by a loss of control over TARC-induced T cell migration and Th2 cytokine expression, but not Th1 cytokine expression. The loss of β_2 -adrenergic control may promote T cell-mediated inflammation and contribute to enhanced Th2-like activity after allergen exposure in asthma.

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