

Effect of Ciclesonide Treatment on Allergen-Induced Changes in T Cell Regulation in Asthma

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Key Words

Asthma allergic · Corticosteroid · Chemokine · Th2 cells · Adrenergic receptor

Abstract

Background: The allergen-induced release of CCL17/thymus and activation-regulated chemokine (TARC) may be crucial in asthmatic airway inflammation by recruitment of Th2 cells. In addition, it might lead to aberrant Th2 cell activity through impairment of β_2 -adrenergic receptor (β_2 -AR) control. We questioned how chemokine patterns change upon allergen challenge and whether treatment with the inhaled steroid ciclesonide can reduce chemokine release and subsequently prevent allergen-induced changes in Th2 cell regulation and migration. **Methods:** Asthma patients were double-blindly treated with placebo or 80 μ g ciclesonide for 7 days. We studied allergen-induced changes in sputum chemokines, migration of peripheral blood T cells and control of β_2 -agonist fenoterol over T cell migration and α -CD3/ α -CD28-induced cytokine production. **Results:** Treatment with 80 μ g ciclesonide significantly diminished the late asthmatic response. The late asthmatic response was associated with increased sputum levels of CCL17 and CCL4 (but none of the other chemokines measured) and loss of β_2 -AR control over T cell migration and Th2-type cytokine production. Although ciclesonide treatment did not prevent chemokine

release nor altered β_2 -AR function in circulating T cells, it exerted an inhibitory effect on TARC-induced T cell migration and α -CD3/ α -CD28-induced cytokine production. **Conclusion:** Our data support the hypothesis that CCL17 is involved in allergen-induced dysregulation of Th2 cell migration and cytokine production. Ciclesonide treatment inhibits T cell migration and cytokine production upon allergen inhalation, which is regulated independently from reducing CCL17 release, but may contribute to beneficial effects of ciclesonide on Th2-mediated airway inflammation.

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Introduction

Atopic asthma is a complex disorder, with airway inflammation underlying its pathophysiology. T cells are thought to orchestrate this immune response by production of Th2-type cytokines interleukin (IL)-4, IL-5 and IL-13. Enhanced expression of these cytokines has been observed in bronchoalveolar lavage and bronchial biopsies from asthmatic subjects [1, 2]. Allergen exposure induces a further increase in T cell activity, as reflected by the upregulation of activation markers in lung and peripheral blood lymphocytes and the recruitment of allergen-specific T cells to the lung tissue [3–7]. The allergen-specific T cells of asthmatics appear to be primed for the

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production of Th2-type cytokines IL-5 and IL-13, and the capacity of bronchoalveolar lavage and peripheral blood T cells to produce IL-5 further increases after allergen challenge [7–10]. Thus, allergen challenge might induce the migration of a primed and allergen-specific T cell subset from peripheral blood to the lung. CD4⁺ T cells that infiltrate the asthmatic airways upon allergen challenge have been described to express the C-C chemokine receptor CCR4, which is predominantly expressed on Th2 cells [11–14]. Although the expression of CCR4 in lung T cells does not differ significantly in asthma versus normal subjects [15, 16], a marked increase in the percentage of CCR4⁺ alveolar T cells has been observed following specific allergen challenge [14]. CCL17/thymus and activation-regulated chemokine (TARC) is one of the ligands of CCR4. There is emerging evidence that CCL17 plays a critical role in the attraction of Th2 cells to the asthmatic airways [11, 14, 17–21]. In addition to its role in Th2 cell recruitment, we have observed that CCL17 may contribute to uncontrolled Th2 cell activity by reducing β_2 -adrenergic control over T cells [22].

The β_2 -adrenergic system normally suppresses T cell activity by activation of the G_s -coupled adenylyl cyclase (AC) system and cAMP accumulation [23, 24], which controls both cytokine production and migration. An allergen-induced dysfunction of the β_2 -adrenergic receptor (β_2 -AR)/AC system has been observed in peripheral blood Th2 lymphocytes in asthma [22, 25]. Although the allergen-induced release of proinflammatory mediators is held responsible for this β_2 -AR desensitization [26], the exact cause has remained unresolved. Interestingly, we recently observed that CCL17 exposure can impair β_2 -AR function in T cells [22], most likely by the activation of β_2 -adrenergic receptor kinase (β ARK) and β -arrestin [27].

Inhaled corticosteroids are widely used for treatment of persistent asthma due to their potent effects on inflammatory cell migration and secretion of multiple proinflammatory mediators. Corticosteroids can have serious side effects; therefore, newly developed corticosteroids are designed to have high local anti-inflammatory efficacy and fast metabolic inactivation in the circulation. Amongst these is ciclesonide, an ester prodrug that requires cleavage by esterases in the lung in order to obtain sufficient receptor affinity [28, 29]. In the current study, we aimed to investigate the effect of low-dose ciclesonide on Th2 cell-mediated airway inflammation in allergen-induced asthma. We questioned whether ciclesonide is able to reduce the release of proallergic chemokines and thereby not only prevent Th2 cell infiltration in the air-

ways, but also improve β_2 -AR regulation of peripheral blood T cells. We investigated the allergen-induced changes in chemokine levels and regulation of CCL17-induced T cell migration and cytokine production, and studied the effect of ciclesonide treatment on these parameters. In addition to CCL17, we studied CCL11/eotaxin, CCL5/regulated on activation normal T cell expressed and secreted (RANTES), CCL2/monocyte chemoattractant protein (MCP)-1, CCL8/MCP-2, CCL7/MCP-3 and CXCL1/growth-related oncogene- α , since these chemokines or their receptors have been implicated in asthma [30–33], as well as the levels of CCL3/macrophage inflammatory protein (MIP)-1 α , CCL4/MIP-1 β , CXCL9/monokine-induced by interferon (IFN)- γ and CXCL10/IFN- γ -inducible protein 10, since these are known to attract Th1 cells instead of Th2 cells.

Material and Methods

Subjects

Patients (18–60 years) were included on basis of a diagnosis of mild atopic asthma. Inclusion criteria required patients to be non-smokers, free of other lung diseases and without lower respiratory tract infection for 6 weeks prior to entering the study. For randomization, subjects were required to have baseline methacholine PC₂₀ (the provocative concentration of methacholine causing a 20% fall in FEV₁) <16 mg/ml and to have stable asthma with FEV₁ >70% of predicted with no regular use of asthma medication during the study other than infrequent inhaled β_2 -agonists, which was withheld for 8 h before each visit. Patients did not receive systemic corticosteroids on the preceding 6 weeks or inhaled steroids for at least 4 weeks and had no asthma exacerbations for 6 weeks or exposure to sensitizing allergens apart from house dust mite (HDM). Before morning visits to the lab, subjects were to refrain from tea or coffee. The Medical Ethics Committee of the University Hospital of Groningen approved the study, and signed informed consent was given to participate.

Study Design

The patients in this study participated in a multi-center study, which was designed as a double-blind, randomized, placebo-controlled, three-period change-over study. Subjects who developed an early asthmatic response (at least 20% fall in FEV₁ within 2 h after allergen inhalation) and late asthmatic response (LAR, at least 15% fall in FEV₁ between 3 and 8 h after allergen inhalation) during the screening allergen inhalation challenge were enrolled in the study. All patients had a positive skin prick test for HDM. Each patient was treated with 80 μ g ciclesonide, 40 μ g ciclesonide and placebo once daily in the morning for 7 days each, separated by a washout phase of at least 3 weeks. In the present study, we have evaluated data from the highest dose of ciclesonide. Medication was administered by the use of a metered-dose inhaler. At day 5 of treatment, sputum was induced. Allergen challenge took place at day 6 of treatment as described by O'Byrne et al. [34]. Subjects inhaled the same dose of allergen for the three treatment

periods. In our center, 5 patients were included (for clinical characteristics, see table 1). Only from these patients, blood samples (30 ml) were taken before and 6 h after allergen challenge, and T cells were isolated. Sputum was again induced 24 h after allergen challenge.

Sputum

Sputum was induced and processed using the method described by Pizzichini et al. [35] and treated with dithiothreitol. Sputum supernatants were stored in a final dilution of 1:9 at -20°C until use.

Cell Cultures, Cytokines and Reagents

Peripheral blood mononuclear cells from asthmatic subjects were isolated by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density-gradient centrifugation. T cells were isolated by rosetting with 2-aminoethylisothionium bromide-treated sheep red blood cells, as described before [36]. After isolation, T cells were incubated for 60 min at 37°C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 0.5, 1 or 5% fetal calf serum (FCS; Hyclone, Logan, Utah, USA), supplemented with 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. T-cell-specific stimulation with $\alpha\text{-CD3}$ and $\alpha\text{-CD28}$ (50 $\mu\text{l}/\text{ml}$) was used to induce cytokine expression, as described before [36]. Fenoterol (Sigma, St. Louis, Mo., USA) was used in a concentration of 10 μM . TARC (R&D Systems, ITK Diagnostics, Uithoorn, The Netherlands) was used in a concentration of 100 ng/ml.

Migration Assay

The migration assay was performed using a microchamber transwell system with 5- μm pores (Corning Costar Incorporated, New York, NY, USA). Migration was induced by 100 ng/ml CCL17 present in the lower compartment, based on our previous findings [27]. T cells were allowed to migrate to the lower well for 2 h, in presence and absence of fenoterol (10 μM) added to the upper well, as described previously [22]. The upper well was removed and transmigrated cells were counted under the microscope by the use of a counting chamber. Migration of T cells was expressed as percentage of the total amount of T cells added to the upper well. Experiments were carried out in duplicate.

Measurement of Cytokine Protein

T cells, 2×10^6 , were stimulated with 50 μl $\alpha\text{-CD3}/\alpha\text{-CD28}$ in 1 ml RPMI containing 5% FCS as described previously [37] during 12 h. Fenoterol (10 μM) was added just prior to stimulation, as described before [38]. Secreted IL-4, IL-13, IL-5 and IFN- γ proteins were measured in cell-free supernatants, using enzyme-linked immunosorbent assay (ELISA) kits for IL-4, IL-13 and IFN- γ . The IL-5 ELISA was performed as previously described by Hoekstra et al. [39]. Cytokine production was expressed as percentage of the level induced by $\alpha\text{-CD3}/\alpha\text{-CD28}$ stimulation in absence of fenoterol before allergen challenge.

Measurement of Chemokine Levels

For detection of CCL17 sputum levels, an ELISA kit was used (R&D Systems). Detection of CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11 and CXCL1, CXCL9 and CXCL10 was performed on the Luminex-100 system and the XY Platform (Luminex Corporation, Austin, Tex., USA). Calibration microspheres for classification and reporter readings as well as sheath fluid were also

Table 1. Subject characteristics at study screening visit

Subject	Age years	Gender	Predicted FEV ₁ , %	Methacholine PC ₂₀ , mg/ml	Allergen
1 ^a	19	M	103	0.70	HDM
2 ^a	58	M	107	8.80	HDM
3 ^a	40	M	84	0.44	HDM
4 ^a	49	F	100	0.21	HDM
5 ^a	33	F	85	0.33	HDM
6	26	F	83	1.07	HDM
7	28	F	121	1.11	cat
8	25	F	96	1.42	cat
9	23	M	99	1.47	cat
10	26	M	93	2.21	HDM
11	38	M	112	1.56	HDM

^a Blood samples were taken from these patients.

purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the MasterPlex™ QT software (version 1.2; MiraiBio, Inc., San Francisco, Calif., USA). Sputum concentrations were measured by a Biosource Chemokine 10-Plex Panel (Biosource International, Inc., Camarillo, Calif., USA) according to the manufacturers' protocols.

Cytosolic and Membrane Fractionation

For subcellular localization of βARK and $\beta\text{-arrestin}$, 5×10^6 T cells were rested in 1 ml RPMI containing 0.5% FCS for 1 h. Nuclear and cytosol fractions were prepared as described before [27] and resuspended in 1 $\times$ sample buffer. Samples were subjected to SDS 10% PAGE and blotted to a polyvinylidene fluoride membrane. Immunodetection was performed with antibodies against $\beta\text{ARK}/\text{G-protein-coupled kinase 2}$ (GRK2), $\beta\text{-arrestin 2}$, IL-1R and tubulin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) according to the manufacturer's guidelines (ECL, Amersham, UK). Protein levels were quantified using the gelscan program ImageMaster (Pharmacia, Uppsala, Sweden) and related to IL-1R or tubulin levels, as described before [27].

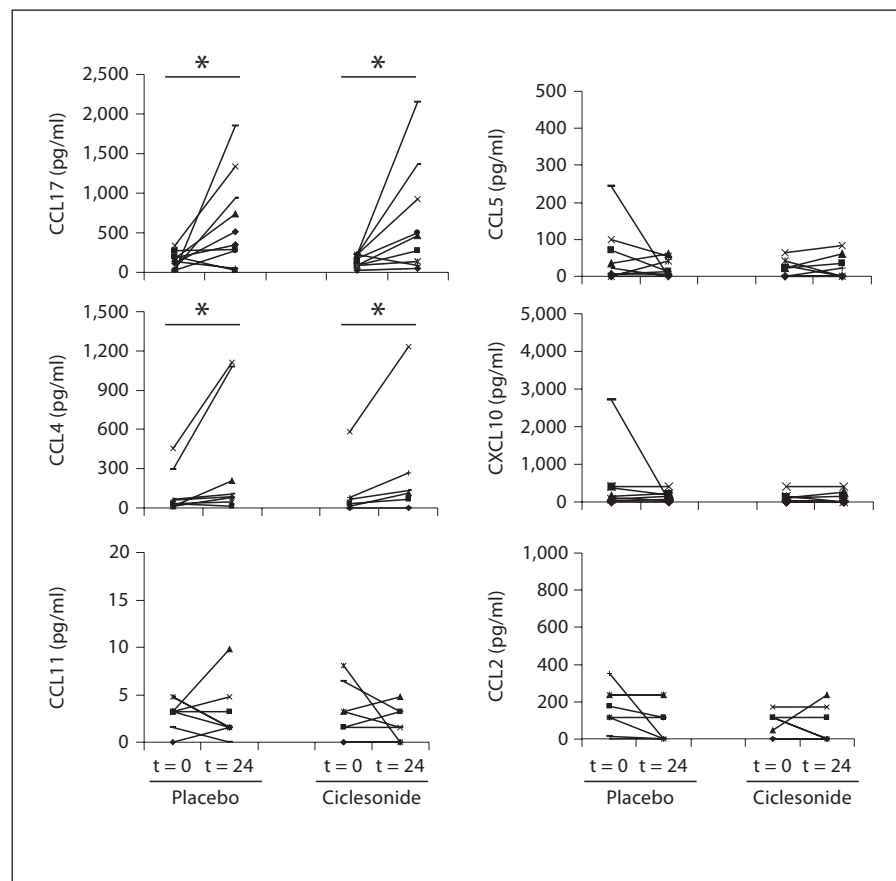
Immunodetection by Western Blotting

Phosphorylation of cAMP-responsive element-binding protein (CREB) was analyzed by Western blotting. T cells, $2 \times 10^6/\text{ml}$, in RPMI 1640 containing 0.5% FCS were stimulated with or without fenoterol for 10 and 60 min. Total cell lysates were prepared. Immunodetection of phospho-CREB (New England Biolabs, Hitchin, UK) and pan-ERK (Santa Cruz Biotechnology) was performed as described before [27].

Statistical Analysis

Analysis was performed by using the Wilcoxon signed rank test for the chemokine levels, transwell assays and densitometry on the immunoblots. Based on previous results, we assumed normal distribution for cytokine secretion levels and we used Student's t test for paired observations in these experiments. Data are expressed as mean $\pm$ SEM. Statistical significance of the secretion data was set at $p < 0.05$.

Fig. 1. Effect of allergen challenge on chemokine levels in the placebo and 80- μ g ciclesonide treatment period. Sputum chemokine levels before ($t = 0$) and 24 h after allergen challenge ($t = 24$) are shown. * $p < 0.05$ between the indicated values.



Results

Effect of Ciclesonide on the Early and Late Asthmatic Response

During placebo treatment, all subjects enrolled in the multicenter trial demonstrated early and late airway responses after allergen inhalation challenge. In the 5 patients included in our center, the mean maximum ($\pm$ SEM) percent fall in FEV₁ was $20.5 \pm 5.8\%$ during the early response and $24.9 \pm 9.9\%$ during the late response. Eighty micrograms ciclesonide significantly reduced the maximum percent fall during the LAR by $47.6 \pm 15.6\%$ (from 24.9 ± 9.9 to $12.9 \pm 7.4\%$; $p = 0.032$). In one of the subjects, ciclesonide treatment had no effect on maximum percent fall in FEV₁ during the LAR nor on any of the parameters measured in the present study; therefore, we did not include this subject in analyses on the effect of ciclesonide.

Effect of Ciclesonide on Chemokine Sputum Levels before and after Allergen Challenge

To test our hypothesis on the role of CCL17 release in allergen-induced changes of T cell regulation and activation, we studied CCL17 levels in sputum before and after allergen challenge. One of the patients included in our center was not able to give up sputum. In addition to our center, sputum samples from 6 additional patients who participated in the multicenter trial were provided by the group of Prof. L.P. Boulet. These additional patients all developed an early and late asthmatic response after the inhalation of cat or HDM extracts. We observed a clear and significant allergen-induced increase in CCL17 sputum levels (from 150 ± 85 to 637 ± 465 pg/ml; $p < 0.05$, $n = 10$) (fig. 1). Since other chemokines may affect T cell regulation as well, we subjected sputum samples to a chemokine 10-plex panel containing CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11 and CXCL1, CXCL9 and CXCL10. Of these, only CCL2, CCL4, CCL5, CCL11 and CXCL10 could be measured in sputum samples. Concen-

trations of these chemokines in sputum were above detection level of the assay. In addition to CCL17, we observed a significant increase in sputum levels of CCL4 after allergen challenge, albeit to a somewhat smaller extent (from 122 ± 127 to 342 ± 377 pg/ml; $p < 0.05$, $n = 10$) (fig. 1). No change could be observed in sputum levels of CCL2 and CCL11, while CCL5 and CXCL10 levels even tended to decrease upon allergen challenge.

Ciclesonide treatment did not prevent the allergen-induced release of CCL17 and CCL4 in sputum. Similar to placebo, an increase in CCL17 and CCL4 sputum levels was observed after allergen inhalation (from 157 ± 66 to 663 ± 544 and from 137 ± 148 to 363 ± 348 pg/ml, respectively; $n = 9$, $p < 0.05$) (fig. 1). Furthermore, sputum levels of none of the other chemokines measured were significantly changed by ciclesonide treatment (fig. 1).

Effects of Ciclesonide Treatment on the T Cell Migration Response and β_2 -AR Control before and after Allergen Challenge

To examine if ciclesonide treatment exerts an effect on CCL17-induced migration of T cells or on β_2 -AR control over T cell migration, we studied the capacity of peripheral blood T cells isolated before and after challenge to migrate towards CCL17. The presence of CCL17 enhanced spontaneous T cell migration by approximately 2.5 fold ($p < 0.01$, $n = 5$). Although the CCL17-induced T cell migration response tended to decrease upon allergen challenge, still a significant and 1.8 ± 0.4 fold increase in migration could be induced by CCL17 ($p < 0.05$, $n = 5$). Ciclesonide treatment had no significant effect on spontaneous T cell migration before allergen challenge. However, interestingly, a significant decline in CCL17-induced T cell migration was observed after ciclesonide treatment, both before and after allergen challenge ($p < 0.05$, $n = 4$) (fig. 2a). In addition, the CCL17-induced T cell migration was significantly inhibited upon allergen challenge when compared with prechallenge values in the ciclesonide treatment group.

During the placebo treatment period, the β_2 -agonist fenoterol was able to significantly reduce CCL17-induced T cell migration ($p < 0.05$, $n = 5$). Upon allergen challenge, this inhibitory effect of fenoterol was completely abolished, while the migration response towards CCL17 was not significantly altered (fig. 2b). This supports our previous data on allergen-induced loss of β_2 -AR control over T cell migration [22]. We observed no significant differences in the effect of fenoterol before or after allergen challenge when compared with placebo. Thus, although treatment with ciclesonide could not prevent the

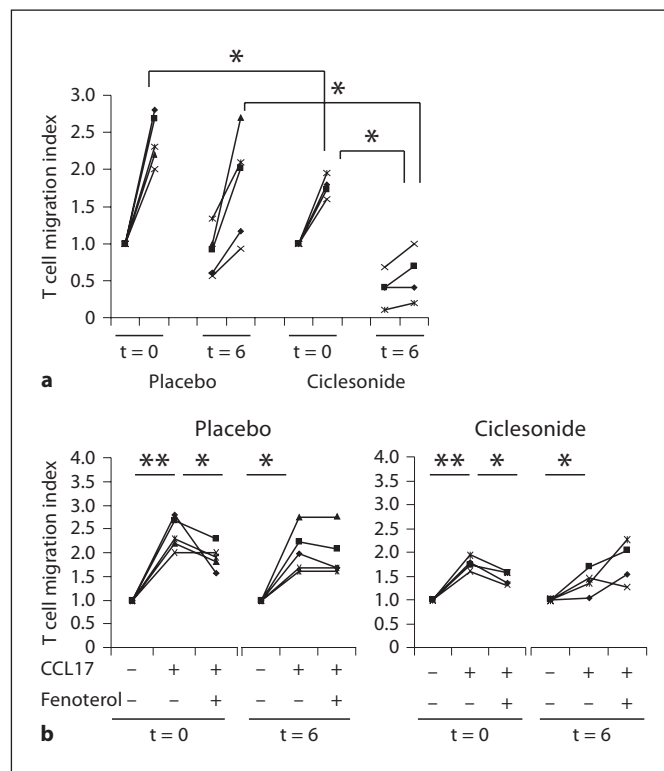


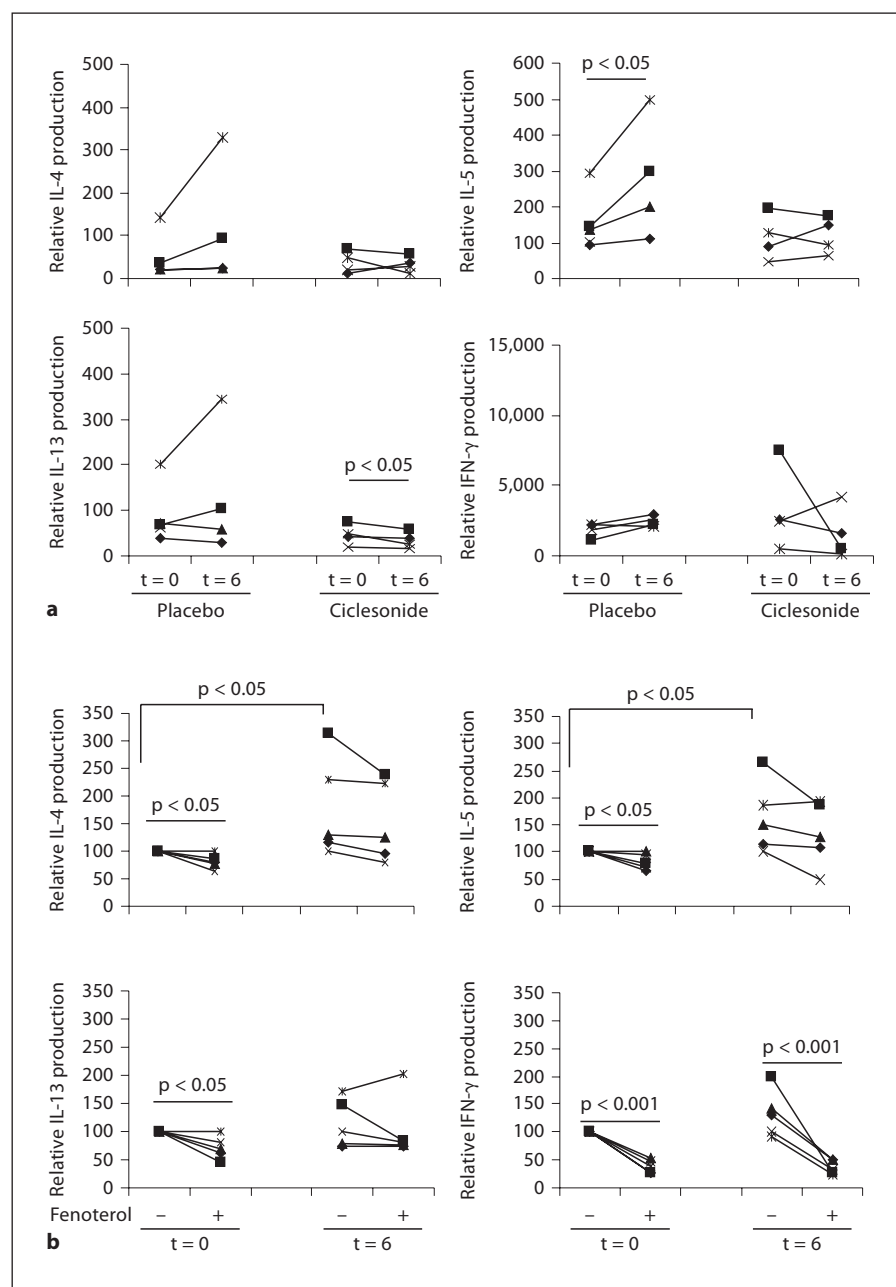
Fig. 2. The CCL17-induced migration response of T cells isolated from asthma patients before (t = 0) and 6 h after allergen challenge (t = 6) in the placebo (n = 5) and ciclesonide (n = 4) treatment periods. **a** Effect of ciclesonide (80 μ g) treatment. T cells were allowed to migrate through 5- μ m pores of a transwell system for 2 h. T cell migration was induced by CCL17 (100 ng/ml). Migrating T cells were counted, expressed as percentage of the total amount of T cells added to the upper compartment and related to the spontaneous migration. * $p < 0.05$ between indicated values. **b** Effect of β_2 -agonist fenoterol. T cells were allowed to migrate through 5- μ m pores of a transwell system for 2 h. T cell migration was induced by CCL17 (100 ng/ml), in the presence and absence of fenoterol (10 μ M). Migrating T cells were counted, expressed as percentage of the total amount of T cells added to the upper compartment and related to the spontaneous migration. * $p < 0.05$ and ** $p < 0.01$ between indicated values.

allergen-induced loss of β_2 -AR control over T cell migration, it did reduce the migration response of T cells towards CCL17, most profoundly after allergen challenge.

Effects of Ciclesonide Treatment on Cytokine Production and β_2 -AR Control before and after Allergen Challenge

In addition to the effect of ciclesonide on the migration response, we were interested in the effect of ciclesonide treatment on Th2-type cytokine secretion and

Fig. 3. Allergen-induced changes in cytokine production in T cells isolated from asthma patients before ($t = 0$) and 6 h after allergen challenge ($t = 6$). **a** Effect of ciclesonide treatment on α -CD3/ α -CD28-induced production of Th2-type cytokines IL-4, IL-5 and IL-13 and Th1-type cytokine IFN- γ . After isolation, T cells were stimulated with α -CD3/ α -CD28 for 8 h. $p < 0.05$ between indicated values ($n = 4$). α -CD3/ α -CD28-induced production of Th2-type cytokines IL-4, IL-5 and IL-13 and Th1-type cytokine IFN- γ in T cells isolated from asthma patients before ($t = 0$) and 6 h after allergen challenge ($t = 6$). **b** Effect of β_2 -agonist fenoterol on cytokine production before and after allergen challenge in the placebo treatment period. After isolation, T cells were stimulated with α -CD3/ α -CD28 for 8 h, in the presence and absence of fenoterol (10 μ M). The α -CD3/ α -CD28 secretion level before allergen challenge in the absence of fenoterol was set to 100%. Significance between the values is indicated ($n = 5$).



their β_2 -AR regulation. We found a significant increase in absolute levels of α -CD3/ α -CD28-induced IL-5 by peripheral blood T cells (fig. 3a) and a significant fold increase in the ability to produce both IL-4 and IL-5 (fold induction 1.97 ± 0.8 and $1.80 \pm 0.5\%$, respectively; $p < 0.05$, $n = 5$) 6 h after allergen challenge. No significant change could be observed in the absolute levels of IL-13 and IFN- γ or in the fold increase in IL-13 and IFN- γ production (fig. 3a). Upon treatment with 80 μ g of inhaled

ciclesonide, the α -CD3/ α -CD28-induced levels of IL-4, IL-5 and IL-13 tended to decrease in the prechallenge situation, although these changes were not significant when compared with placebo (fig. 3a). Interestingly, the allergen-induced fold increase in IL-4 and IL-5 production was no longer observed (fold induction 1.31 ± 0.78 and $1.36 \pm 0.71\%$, $n = 4$; $p = 0.53$ and 0.47 , respectively), while after allergen challenge, the IL-13 production was even inhibited when compared with prechallenge values in the

Table 2. The effect of ciclesonide on absolute levels of α -CD3/ α -CD28-induced cytokine production

Cytokine, pg/ml	Placebo (t = 0)	Placebo (t = 6)	Ciclesonide (t = 0)	Ciclesonide (t = 6)
IL-4	52 $\pm$ 36	117 $\pm$ 105	37 $\pm$ 20	33 $\pm$ 13
+ fenoterol	44 $\pm$ 40	105 $\pm$ 107	28 $\pm$ 14	31 $\pm$ 19
IL-5	143 $\pm$ 61	262 $\pm$ 119 ^c	114 $\pm$ 47	118 $\pm$ 16
+ fenoterol	125 $\pm$ 68 ^a	253 $\pm$ 159	101 $\pm$ 24	86 $\pm$ 34
IL-13	83 $\pm$ 48	123 $\pm$ 110	47 $\pm$ 16	28 $\pm$ 8 ^c
+ fenoterol	68 $\pm$ 52	131 $\pm$ 137	37 $\pm$ 12	24 $\pm$ 9
IFN- γ	2,876 $\pm$ 1,626	2,469 $\pm$ 315	3,258 $\pm$ 2,094	2,612 $\pm$ 1,746
+ fenoterol	1,214 $\pm$ 801 ^a	727 $\pm$ 306 ^b	1,174 $\pm$ 643 ^a	943 $\pm$ 784

n = 5 for placebo and n = 4 for ciclesonide. t = 0: cytokine levels produced by T cells isolated before allergen challenge; t = 6: cytokine levels induced in T cells isolated 6 h after allergen challenge.

^a p < 0.05 when compared with absence of fenoterol.

^b p < 0.01 when compared with absence of fenoterol.

^c p < 0.05 when compared with t = 0.

ciclesonide treatment period (from 100 to 65 $\pm$ 17%; p < 0.05, n = 4) (table 2).

We next studied β_2 -AR regulation of the cytokines (fig. 3b). Given the wide range of the levels of cytokines secreted, the α -CD3/ α -CD28 secretion level before allergen challenge in absence of fenoterol was set to 100% (for absolute values of cytokine levels, see table 2). The production of IL-4, IL-5 and IL-13 was modestly but significantly inhibited by fenoterol before allergen challenge, from 100 to 81 $\pm$ 10%, 83 $\pm$ 12% and 75 $\pm$ 20%, respectively (p < 0.05, n = 5). This effect was no longer significant upon inhalation of the allergen, the relative cytokine productions being 87 $\pm$ 8%, 88 $\pm$ 10% and 93 $\pm$ 18% (p = 0.07, 0.17 and 0.60 for IL-4, IL-5 and IL-13, respectively) when compared with the absence of fenoterol. In contrast, the production of IFN- γ was strongly inhibited both before and after allergen challenge to 39 $\pm$ 9 and 28 $\pm$ 9%, respectively (p < 0.01), with no significant changes in the effect of fenoterol. These results on allergen-induced loss of β_2 -AR control over Th2-type cytokines are in line with our previous observations [22]. Treatment with ciclesonide did not significantly alter the effect of fenoterol on IL-4, IL-5, IL-13 or IFN- γ production, neither before nor after allergen challenge. Similar to placebo, fenoterol ability to inhibit IL-4, IL-5 and IL-13 was abolished after allergen challenge in the ciclesonide treatment period (the relative cytokine productions being 79 $\pm$ 28, 72 $\pm$ 22 and 83 $\pm$ 26%; p = 0.16, 0.38 and 0.29 for IL-4, IL-5 and IL-13, respectively), when compared with the absence of fenoterol. IFN- γ production

was strongly inhibited both before and after allergen challenge to 33 $\pm$ 5 and 18 $\pm$ 9%, respectively (p < 0.001 and <0.01), with no significant differences when compared with placebo. Thus, ciclesonide treatment does not improve β_2 -AR control over cytokine production. Since we previously observed that CCL17 is able to mimic the allergen-induced loss of β_2 -AR control over Th2-type cytokines in T cells [27], the absence of an effect on β_2 -AR control may be related to the inability of ciclesonide to block allergen-induced CCL17 release.

Effects of Ciclesonide Treatment on Signaling Pathways in T Cells

Chemokines can induce activation and membrane translocation of β ARK and β -arrestin, two molecules that are involved in multiple G-protein-mediated processes, including migration responses and receptor desensitization. We have previously observed that CCL17-induced membrane translocation of β ARK and β -arrestin may be involved in heterologous desensitization of the β_2 -AR in T cells [27]. Therefore, we tested whether β ARK and β -arrestin are translocated to the membrane in peripheral blood T cells upon allergen challenge. Before allergen challenge, β ARK (GRK2) was localized both in membrane and cytosol fractions. As control for the purity of the subcellular fractions, IL-1R was used as membrane marker and tubulin was used as cytosol marker. Interestingly, the membrane expression of β ARK was significantly increased upon allergen challenge, with an associated reduction in cytosol expression (fig. 4). A sim-

Fig. 4. Cellular localization of β ARK in peripheral T cells isolated from asthma patients before ($t = 0$) and 6 h ($t = 6$) after allergen challenge in the placebo (a) and 80- μ g ciclesonide treatment period (b). After T cell isolation, membrane and cytosol fractions were prepared. β ARK ($n = 5$) was detected by immunoblotting and related to the levels of IL-1R as membrane marker and tubulin as cytosol marker. Densitometry was performed, and the levels before allergen challenge were set to 100%. Mean $\pm$ SEM relative protein levels are depicted in the corresponding diagram. * $p < 0.05$ between the indicated values. Representative blots are shown.

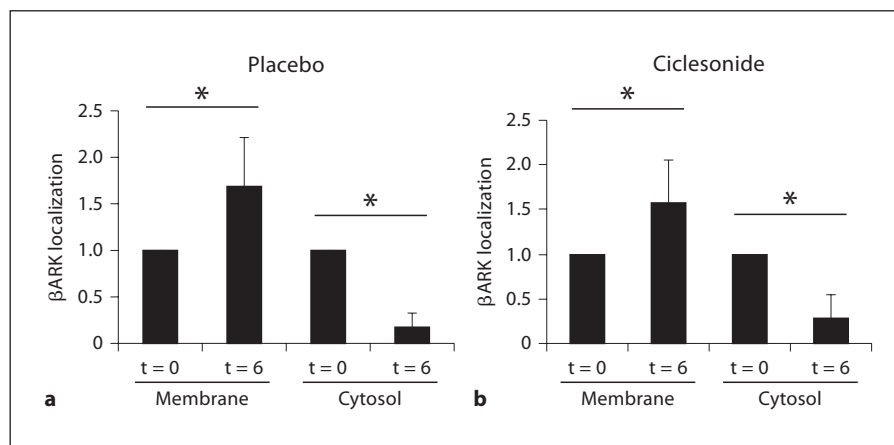
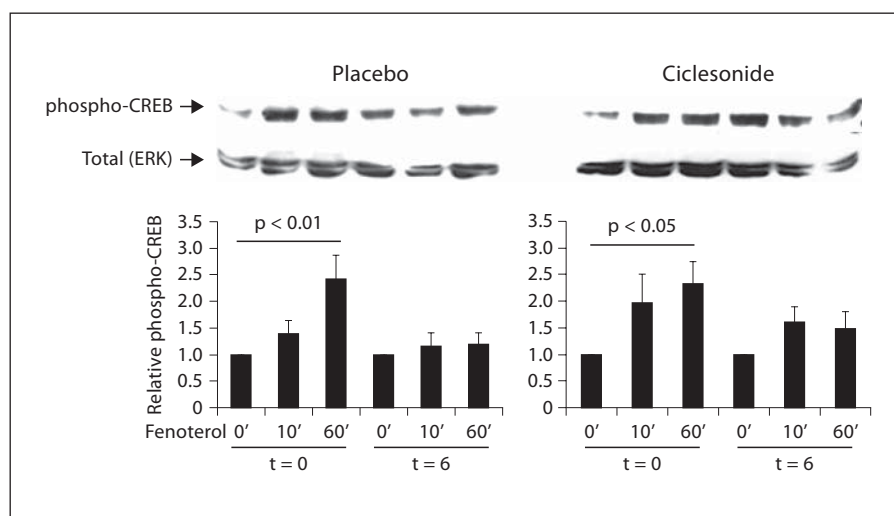


Fig. 5. Immunodetection of phospho-CREB in peripheral T cells isolated from asthma patients before ($t = 0$) and 6 h ($t = 6$) after allergen challenge in the placebo and 80- μ g ciclesonide treatment period. After stimulation of the T cells with fenoterol for 10 and 60 min, whole cell lysates were prepared. Phospho-CREB was detected by immunoblotting, densitometry was performed, and the levels were normalized for the total amount of protein (pan-ERK). The levels in the absence of fenoterol were set to 100%, and mean relative phospho-CREB values $\pm$ SEM are depicted in the corresponding diagram ($n = 5$). Significance is indicated between the values.



ilar pattern was observed for β -arrestin, although β -arrestin could not be detected in all of the patients, and the consistency of these findings requires further study (data not shown). In accordance with the lack of effect on CCL17 secretion, ciclesonide treatment did not prevent the allergen-induced membrane translocation of β ARK. Similar patterns of membrane and cytosol expression of β ARK (fig. 4) and β -arrestin (data not shown) were observed when compared with placebo.

Finally, to investigate changes in the ability of the β_2 -AR system to activate the AC/cAMP system, we studied the activation of cAMP downstream effector CREB. During placebo treatment, we observed that fenoterol induced a strong and significant increase in the phosphorylation of CREB in T cells isolated before allergen challenge (fig. 5). After allergen challenge, this effect was completely abolished, confirming impairment of β_2 -AR function. In agreement with the lack of effect of cicle-

sonide on β_2 -AR control over T cell activity, we observed no alterations in β_2 -AR activation of the cAMP-dependent pathway after treatment with ciclesonide. Similar to placebo, fenoterol induced an approximately 2.5-fold increase in CREB phosphorylation after 60 min of stimulation in T cells isolated before allergen challenge, while allergen challenge impaired this effect (fig. 5). Thus, ciclesonide treatment does not alter the ability of fenoterol to induce phosphorylation of CREB and does not prevent allergen-induced β_2 -AR desensitization.

Discussion

T-cell-mediated airway inflammation underlies the pathophysiology of allergic asthma. Recent evidence suggests that the C-C chemokine CCL17 may be one of the chemokines involved in the attraction of circulating T

cells to the airways. Ligand binding to C-C chemokine receptors is known to induce G_i-protein-mediated activation of GRKs, β -arrestin, Src/SOS-dependent signaling and cytoskeleton reorganization required for directional migration [40]. Recently, we have observed that CCL17 can induce GRK-dependent β_2 -AR desensitization in these cells [22, 27], which may contribute to the aberrant Th2 cell activity as observed in asthma [22]. Corticosteroids have proven effective in reducing Th2-mediated airway inflammation. However, the effect of ciclesonide, a steroid designed to act locally in lung tissue, on Th2-mediated airway inflammation in asthma and the release of Th2 cell-attracting chemokines has not been explored. In this study, we provide new evidence showing that Th2 cell-attracting chemokine CCL17 may play a role in the allergen-induced loss of β_2 -AR control over T cell activity. Furthermore, we demonstrate that treatment with a low dose of ciclesonide inhibits the late asthmatic response and reduces T cell migration as well as cytokine secretion after allergen challenge. This appears to be regulated independently from preventing the release of chemokines, e.g. CCL17, and improving β_2 -AR function in peripheral blood T cells.

A significant increase in CCL17 and CCL4 sputum levels was observed 24 h after allergen challenge. In contrast, none of the other chemokines increased. CCL4 has been described to act on CCR5 and CCR8, the latter being predominantly expressed on Th2 cells. Interestingly, part of the CCR4⁺ T cells in the asthmatic airways has been reported to coexpress CCR8 [11]. Eotaxin acts on CCR3, which is also preferentially expressed on Th2 cells. However, no allergen-induced increase in CCR3⁺ T cells has been observed. CCL5, CCL2 and CXCL9 are agonists of CCR5, CCR2 and CXCR3, respectively, which are all abundantly expressed on Th1 cells [13]. Although an increase in CXCL10 has been reported [19], in contrast to CCR4 and CCR8, reduced numbers of CXCR3⁺ T cells were observed in the asthmatic airways after allergen exposure [14]. Together, these and our data suggest that allergen challenge specifically induces release of Th2 cell-attracting chemokines, acting on CCR4 and CCR8.

In addition, we observed an allergen-induced loss of β_2 -AR control over CCR4-mediated T cell migration as well as α -CD3/ α -CD28-induced secretion of the Th2-type cytokines IL-4, IL-5 and IL-13. In contrast, the production of Th1-type cytokine IFN- γ was still under strict control of the β_2 -AR after allergen challenge. The specific loss of β_2 -AR control over Th2 cytokines might be explained by the specific allergen-induced increase in chemokines acting on Th2 cells. Allergen inhalation in-

duced similar changes in peripheral blood T cells as observed after in vitro exposure to CCL17 [22, 27], i.e. membrane translocation of β ARK and impairment of β_2 -AR-induced phosphorylation activation of CREB. This further supports that CCL17 may play a role in allergen-induced changes in T cell regulation in asthma.

Corticosteroids are known to reduce airway inflammation, but effects on chemokine release have remained largely unexplored. Both 40 and 80 μ g ciclesonide were effective in inhibition of the LAR in the larger multi-center trial [41, 42], whereas only 80 μ g of ciclesonide was able to significantly reduce the late asthmatic response in the patients included in the current study. Treatment with 80 μ g ciclesonide did not significantly reduce sputum levels of any of the chemokines measured, nor did it prevent the allergen-induced release of CCL17 and CCL4. Since we hypothesized that CCL17 release might be responsible for allergen-induced changes in β_2 -AR control of Th2 cells, this would also mean that ciclesonide treatment is unable to affect T cell β_2 -AR function. Indeed, after treatment with ciclesonide, no significant changes were observed in β_2 -AR downstream signaling and regulation of peripheral blood T cells. In addition, ciclesonide could not prevent the allergen-induced membrane translocation of β ARK, a molecule likely involved in TARC-induced β_2 -AR desensitization. This lack of effect of ciclesonide should not be considered fully definitive though, given the low numbers of patients tested in the current study. Previous studies have reported that ciclesonide and its pharmacologically active metabolite desisobutryl-ciclesonide are hardly detectable in the circulation, even at high doses of oral administration [29, 43]. Thus, despite the fact that corticosteroids are known to reverse downregulation of the β_2 -AR in lymphocytes [44–46], we did not expect ciclesonide to directly improve β_2 -AR function in peripheral blood T cells.

Although ciclesonide failed to have an effect on CCL17 release or β_2 -AR function in peripheral blood T cells, ciclesonide inhibited the migration response of T cells towards CCL17, most profoundly after allergen challenge. After allergen challenge, the migration response towards CCL17 was also slightly reduced in the placebo group. One of the explanations may be the allergen-induced efflux of a subset of CCR4⁺ T cells. The inhibitory effect of ciclesonide on CCL17-induced T cell migration may be one of the ways in which ciclesonide exerts its inhibitory effect on T(h2)-cell-mediated airway inflammation in asthma. We suggest that ciclesonide treatment, possibly in combined effect with allergen exposure, induces the release of (an) anti-inflammatory mediator(s) that sup-

press T(h2) cell activity. This is further supported by the finding that ciclesonide treatment abrogated the allergen-induced fold increase in the capacity of peripheral blood T cells to secrete IL-4 and IL-5 and even inhibited the capacity to produce IL-13 after allergen challenge. This inhibitory effect of ciclesonide on Th2 cell activity upon allergen challenge may contribute to the beneficial effect of ciclesonide on the LAR.

In summary, our results indicate that treatment with a dose of 80 µg ciclesonide for 7 days inhibits migratory capacity and activity of peripheral blood T cells after allergen challenge. This is regulated independently from

the prevention of allergen-induced release of chemokines and β_2 -AR desensitization in circulating T cells, but may contribute to the beneficial effects on airway inflammation in asthma.

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