

Selective COX-2 Inhibition Exerts No Negative Effects on Peripheral Blood Lymphocytes in Allergic Asthmatics

Guro Gafvelin^{a–c} Jeanette Grundström^{a, b} Maria Edin Grimheden^a
Sara Sánchez Vidaurre^a Kameran Daham^{b, d} Sven-Erik Dahlén^{b, e}
Barbro Dahlén^{b, d} Marianne van Hage^{a, b}

^aImmunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet and Karolinska University Hospital, and ^bThe Centre for Allergy Research, Karolinska Institutet, Stockholm, ^cTherapeutic Immune Design Unit, Department of Clinical Neuroscience, Karolinska Institutet, Solna, ^dDepartment of Medicine, Karolinska Institutet, Huddinge, and ^eInstitute of Environmental Medicine, Karolinska Institutet, Solna, Sweden

Key Words

Allergy · Asthma · T cell · Lymphocytes · COX-2 · Prostaglandin E₂

Abstract

Background: Selective inhibition of cyclooxygenase-2 (COX-2) reduces the production of prostaglandin E₂ (PGE₂), which can have both pro- and anti-inflammatory effects on allergic inflammation. Moreover, in vitro PGE₂ has been shown to affect inflammation through the modulation of lymphocyte responses. **Methods:** Sixteen subjects with mild allergic asthma were recruited to a two-period cross-over study: one treatment period with the selective COX-2 inhibitor etoricoxib and one without. Each treatment period ended with an airway challenge with the patient's relevant allergen. Antigen-specific proliferation with the major cat allergen, Fel d 1, was analysed in PBMCs. CD4+ T cells were phenotyped using flow cytometry, and mRNA expression of FOXP3 in anti-CD3-stimulated CD4+ cells were analysed. **Results:** No significant impact of in vivo inhibition of COX-2 was detected on the proportion of Th1, Th2, or Treg cells in peripheral blood. Likewise, the treatment had minor effects on the

stimulated expression of FOXP3 mRNA in CD4+ T cells. Proliferation of PBMCs to the major cat allergen Fel d 1 was slightly reduced by etoricoxib treatment in cat-allergic patients. **Conclusions:** Short-term treatment with the COX-2 inhibitor etoricoxib had a minor impact on T-cell responses, supporting its safe use also in subjects exposed to triggers of lymphocyte activation.

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Introduction

Allergic asthma is caused by an inappropriate T-helper type-2 (Th2) response to inhaled allergens and is characterized by chronic airway inflammation and bronchial hyper-responsiveness. In contrast to the general pro-inflammatory role of prostaglandin E₂ (PGE₂) in most tissues, current data support a protective role for PGE₂ in asthmatic airways [1]. Furthermore, PGE₂ has broncho-protective actions, reducing the allergen-induced release

Guro Gafvelin and Jeanette Grundström share first authorship.

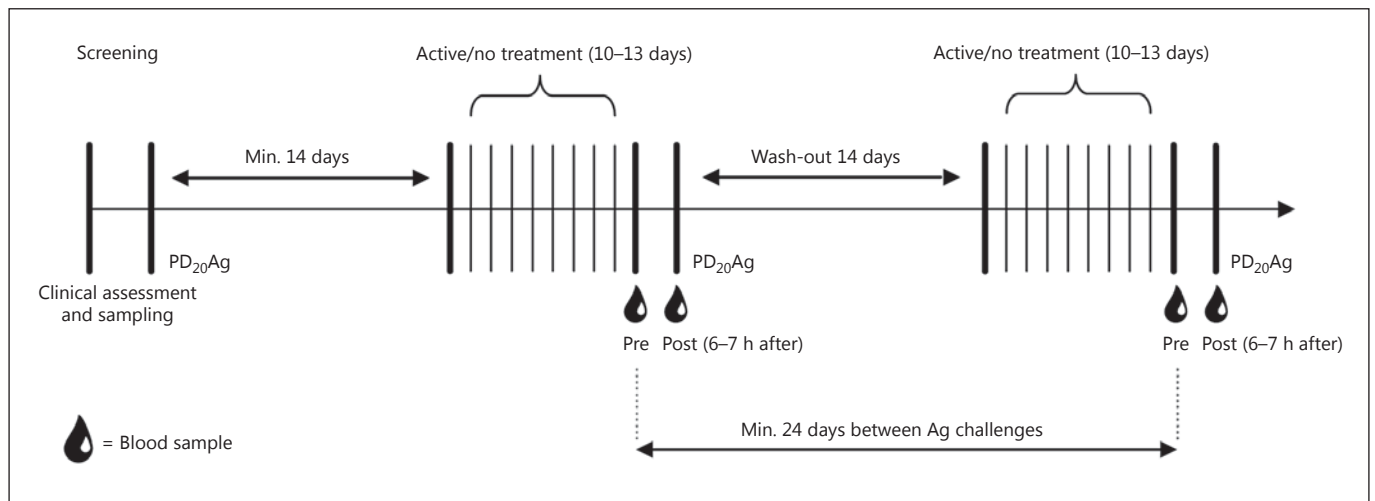


Fig. 1. Study design. The included patients were subjected to a two-period cross-over study with one active-treatment and one no-treatment period. A bronchial challenge test (PD₂₀Ag) was performed with the patients' specific allergen extract (cat dander or birch or timothy pollen).

of histamine, PGD₂, and cysteinyl leukotrienes in human airways in vitro and in vivo [2, 3]. The initial step in the biosynthesis of PGE₂ is catalysed by the cyclooxygenase enzymes COX-1 and COX-2.

The dual role of PGE₂ in inflammation is also reflected when studying lymphocyte-mediated responses. In humans, in vitro studies on peripheral blood mononuclear cells (PBMCs) have shown that PGE₂ promotes the production of IL-17 and Th17 cell expansion [4]. Kofler et al. [5] have recently shown that the expansion of Th17 cells is regulated by the repression of the PGE₂ receptor EP₂ on Th17 cells, mediated by the transcription factor RORC. PGE₂ has also been reported to suppress Th1 cytokines, but not Th2 cytokines, thus shifting the balance towards Th2 [6]. In addition, PGE₂ also affects Tregs (regulatory T cells), promoting their development, as indicated by the ability of PGE₂ to induce FOXP3 gene expression, and suppressing the activity of human CD4⁺ cells [7].

Selective COX-2 inhibitors are useful in the short-term treatment of acute episodes of pain or inflammation. However, asthmatic subjects are often advised to avoid non-steroidal anti-inflammatory drugs as they may cause bronchospasm in susceptible patients. In a recent clinical trial, however, it was documented for the first time that treatment with the selective COX-2 inhibitor etoricoxib had no negative effects on airflow obstruction, sputum eosinophilia, baseline lung function, or methacholine responsiveness in patients with mild allergic asthma [8]. The findings were consistent with previous evidence that

COX-2 selective inhibition is also tolerated by patients with clinically stable aspirin-exacerbated respiratory disease [9]. In view of the data suggesting the modulation of lymphocyte responses by COX-2 metabolites, we therefore performed a mechanistic study to investigate the allergen-specific effect of COX-2 inhibition on CD4⁺ cells in patients with mild allergic asthma. We analysed peripheral blood cells before and after the allergen challenge in the same patients as those participating in the main study.

Materials and Methods

Sixteen patients with mild allergic asthma were included in this two-period cross-over study (see online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000446960). The patients went through one period with etoricoxib treatment (90-mg tablets, Arcoxia®; MSD, Sollentuna, Sweden) and one period without. At the end of each period, blood was taken before and after the bronchial challenge with each patient's relevant allergen extract (Aquagen; ALK, Copenhagen, Denmark) (fig. 1). None of the patients had aspirin-exacerbated respiratory disease, chronic sinusitis, or nasal polyposis. Their blood eosinophil counts were in the normal range.

Th subsets and Tregs were analysed by flow cytometry on freshly prepared PBMCs by the surface expression of T-cell markers and the chemokine receptors CXCR3, CCR4, and CCR6, as previously described [10, 11]. Th1 cells were defined as CD4⁺CXCR3⁺ and Th2 cells as CD4⁺CCR4⁺CCR6⁺CXCR3[−] (gating strategy shown in online suppl. fig. S1). Tregs were analysed by the expression of CD25, FOXP3, and CD127 on CD4⁺ T cells.

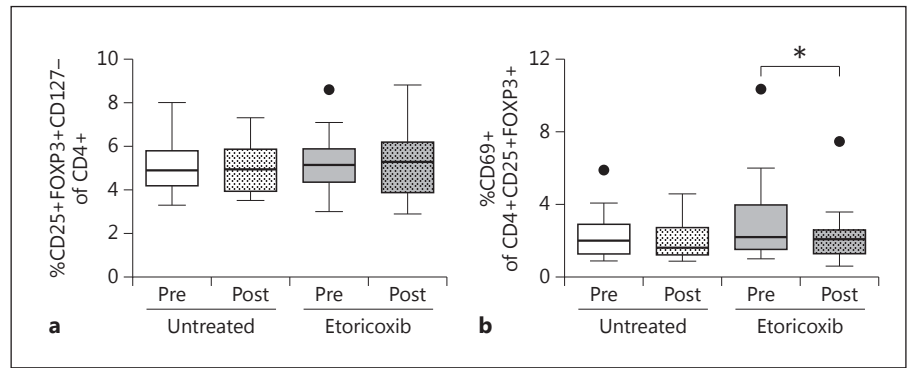


Fig. 2. Expression of markers for activation and regulatory cells in peripheral blood Th cells. Surface and intracellular antigens were labelled and analysed by flow cytometry. **a** Percentage of CD25+FOXP3+CD127- cells among CD4+ T cells. **b** Percentage of CD69+ cells among CD4+CD25+FOXP3+CD127- T cells. Box-

es represent the medians and 25th and 75th percentiles. Untreated: data from the no treatment period; etoricoxib: data from the etoricoxib treatment period; pre: before allergen challenge; post: after allergen challenge. * $p < 0.05$, Wilcoxon signed-rank test of matched pairs ($n = 16$).

Messenger RNA expression analysis was performed essentially as previously described [12] using primers for FOXP3 with EF-1 as the reference gene [13]. CD4+ cells were separated from PBMCs stimulated with anti-CD3 for 48 h using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). Amplification of cDNA was detected with SYBRGreen (Bio-Rad, Hercules, Calif., USA). The fold change in mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Stimulation of PBMCs with recombinant Fel d 1, the major cat allergen [14] (10 $\mu\text{g/ml}$) was performed on PBMCs from cat-allergic patients ($n = 9$). From all patients, PBMCs were stimulated with an influenza antigen (influenza vaccine, Vaxigrip®, diluted 360 $\times$; Sanofi Pasteur MSD, Lyon, France) or left unstimulated. The cells were cultured for 6 days, and proliferation was measured by ^3H -thymidine incorporation (PerkinElmer, Waltham, Mass., USA). For detailed information on methods and statistical analysis see the supplementary material.

Results

No difference in the proportion of CD4+CXCR3+ and CD4+CCR4+CCR6-CXCR3- cells between etoricoxib treatment and the untreated period was observed (online suppl. fig S2A, B). After the allergen challenge there was a significant increase of CD4+CXCR3+ cells during the untreated session ($p < 0.05$) but not during the etoricoxib treatment session (online suppl. fig. S2A). In addition, the percentage of Tregs was not affected by etoricoxib treatment or the bronchial allergen challenge (fig. 2a). The marker for recently activated T cells, CD69, was expressed on about 2% of the Tregs, and this low number of recently activated cells was essentially similar for all the sam-

pling time points (fig. 2b). The expression of CD69 in the etoricoxib period after the allergen challenge was lower compared to before the allergen challenge.

Next we investigated whether etoricoxib treatment affected the expression of FOXP3 mRNA in polyclonally stimulated CD4+ T cells. The FOXP3 mRNA expression was significantly higher in cells recovered before the allergen challenge during the untreated period compared to the etoricoxib period ($p < 0.05$; online suppl. fig. S3). Furthermore, the ratio of FOXP3 expression and the expression of the housekeeping gene EF-1 in unstimulated cultures was investigated, and no significant differences were observed in any of the study conditions (data not shown).

We also investigated allergen-specific proliferation in PBMCs from cat-allergic subjects ($n = 9$; online suppl. table S1) by stimulation with the major cat allergen Fel d 1. In the no treatment period of the study protocol, there was a significant increase in Fel d 1-stimulated proliferation after the allergen challenge compared to before ($p < 0.05$; fig. 3a). In contrast, no significant increase in proliferation was observed after the allergen challenge in etoricoxib-treated patients. In addition, the proliferation to Fel d 1 was significantly higher in the untreated patients compared to the etoricoxib-treated patients after the allergen challenge ($p < 0.01$; fig. 3a). For comparison, influenza-stimulated proliferation was investigated in all patients, and no differences in proliferation were noted throughout the study ($p > 0.05$; fig. 3b).

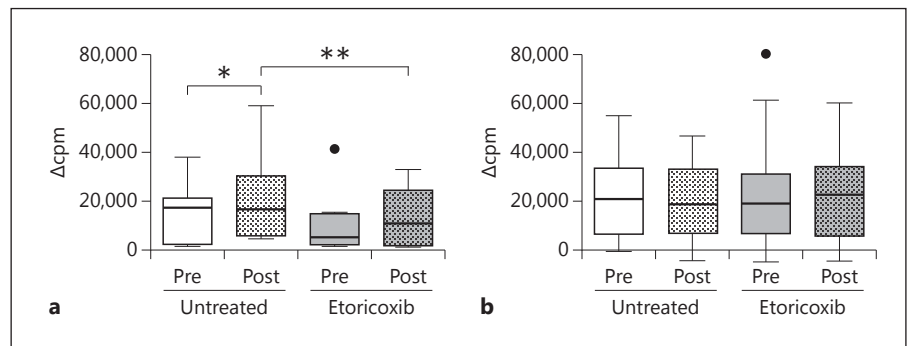


Fig. 3. Proliferation of PBMCs from cultures stimulated in vitro. Data are presented as net proliferation, Δcpm , where the baseline proliferation was subtracted from the stimulated proliferation. **a** PBMCs from cat-allergic patients were stimulated with Fel d 1 ($n = 9$). **b** PBMCs from all patients were stimulated with an influenza antigen ($n = 16$). Boxes represent the medians and 25th and

75th percentiles. Untreated: data from the no treatment period; etoricoxib: data from the etoricoxib treatment period; pre: before allergen challenge; post: after allergen challenge. * $p < 0.05$, ** $p < 0.01$, Wilcoxon signed-rank test of matched pairs; Fel d 1 stimulated ($n = 9$), influenza stimulated ($n = 16$).

Discussion

The present mechanistic study was designed to investigate the effect of COX-2 inhibition in vivo on peripheral blood cells from subjects with mild allergic asthma. The main study documented biochemically that there was complete inhibition of PGE₂ biosynthesis along the COX-2 pathway during the session when etoricoxib was given [8]. This proof of target engagement, therefore, provided a unique opportunity to evaluate the influence of complete COX-2 inhibition on lymphocyte function in vivo.

In vitro studies of the PGE₂ effect on Th cells suggest a down-regulatory effect of PGE₂ on IFN γ -producing Th1 cells [4, 6, 15] that can shift the immune response towards a Th2 profile. We found no difference in the proportion of CD4+CXCR3+ cells in PBMCs before the allergen challenge in the untreated compared to the treated period. However, there was a significant but small increase of CD4+CXCR3+ cells after the allergen challenge in the untreated period that was not seen after etoricoxib treatment. Thus, in vivo blocking of COX-2 seems to slightly inhibit circulating allergen-specific Th1 cells without affecting Th2 cells per se in peripheral blood. Since the analyses were performed on the whole Th populations, differences in the allergen-specific populations may have been masked.

The expression of FOXP3 mRNA in polyclonally stimulated PBMCs was slightly lower in CD4+ T cells before the allergen challenge after the etoricoxib treatment compared to the untreated period. This might indicate that

PGE₂ promotes the induction of Tregs, as also suggested by previous in vitro studies [7, 16]. However, the differences in mRNA expression were small. Furthermore, no change in the percentage of Tregs in freshly prepared peripheral blood cells was detected throughout the study, suggesting that any effects exerted on Tregs in this study were minor.

We found that the proliferation of PBMCs in cat-allergic patients in response to Fel d 1 was lower after the allergen challenge in the etoricoxib treatment period compared to the no treatment period. This seems to reflect a less activated cell status when COX-2 is inhibited, which renders the allergen-specific cells less responsive to stimulation. In contrast to these findings, an earlier study showed that allergen-specific CD4+ T-cell lines generated in vitro proliferate less in response to allergen when cultured in the presence of PGE₂ [17]. The proliferation of PBMCs with the influenza antigen did not change throughout the study despite most individuals having a memory response to the influenza antigen, indicating that the effect of COX-2 blocking is specific to the allergen used in the in vivo challenge.

In the present study all analyses were made on peripheral blood cells. When interpreting data obtained from PBMCs, it is important to bear in mind that, for example, Tregs and recently activated T-effector cells are recruited to the lung upon allergen challenge [12].

Our data implicate that treatment with etoricoxib has no major impact on peripheral blood T cells. A small effect was noted on the allergen-stimulated proliferation of PBMCs after etoricoxib treatment compared to no treat-

ment in cat-allergic patients after the in vivo allergen challenge. In contrast to previous in vitro studies, our study shows that short-term in vivo COX-2 inhibition appears to have minor effects on T cells in mild atopic asthma. Although some statistical differences were noted, the changes were in general small and probably not biologically relevant. Thus, the results on lymphocyte responses are in line with the clinical data of the study, where in vivo COX-2 inhibition did not have any negative effects on airflow obstruction, sputum eosinophilia, baseline lung function, or methacholine responsiveness [8]. Finally, because the treatment with etoricoxib inhibited biosynthesis of both PGE₂ and PGI₂ [8], the present study excludes a major in vivo role of these two main classes of COX-2 products for the currently evaluated lymphocyte responses.

Acknowledgements

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Supplementary material

Selective COX-2 inhibition exerts no negative effects on peripheral blood lymphocytes in allergic asthmatics

Guro Gafvelin^{a,b,c*}, Jeanette Grundström^{a,b*}, Maria Edin Grimheden^a, Sara Sánchez-Vidaurre^a, Kameran Daham^{b,d}, Sven-Erik Dahlén^{b,e}, Barbro Dahlén^{b,d} and Marianne van Hage^{a,b}

a. Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, and University Hospital, Stockholm, Sweden

b. The Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

c. Therapeutic Immune Design Unit, Department of Clinical Neuroscience, Karolinska Institutet, Solna, Sweden

d. Department of Medicine, Karolinska Institutet, Huddinge, Sweden

e. The Institute of Environmental Medicine, Karolinska Institutet, Solna, Sweden

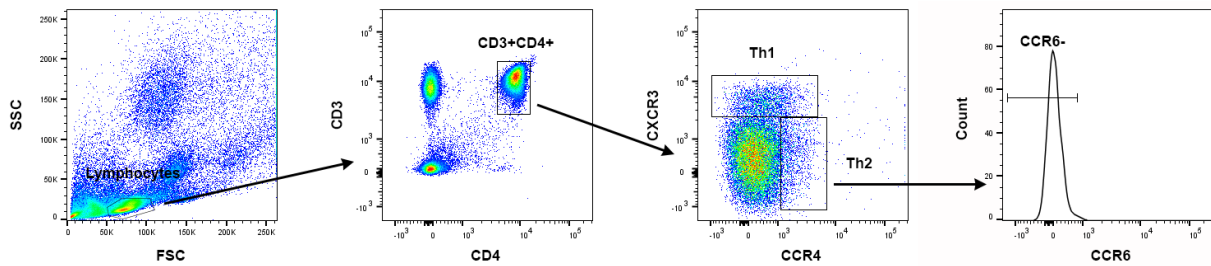
* Shared first authorship

Table S1: Demographic and serological characteristics of the study participants

Allergy:	Cat	Birch	Timothy
Number of patients	9	4	3
Sex (M/F)	5/4	3/1	3/0
Age, years (Median (Range))	32 (24-45)	29.5 (23-35)	44 (31-50)
Allergen-specific IgE, kU _A /L (Median (Range))	22 (1.6-66)	16.5 (4.5-46)	3.1 (0.76-14)
Total IgE, kU/L (Median (Range))	175 (53-300)*	113.5 (30-300)	69 (45-93)*
Blood eosinophils, cellsx10 ⁹ /L (Median (Range))	0.2 (<0.1-0.4)	0.1 (<0.1-0.3)	0.1 (<0.1-0.4)

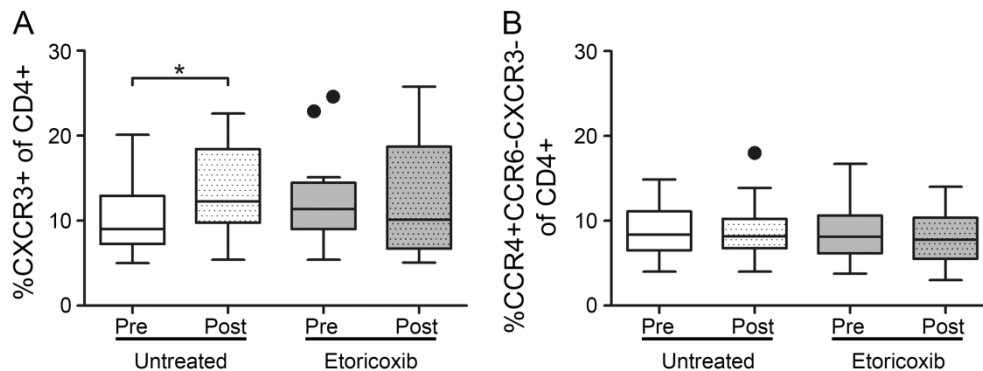
*One patient was not included as total IgE was not determined

Figure S1: Gating strategy for Th1 and Th2 cell subsets.



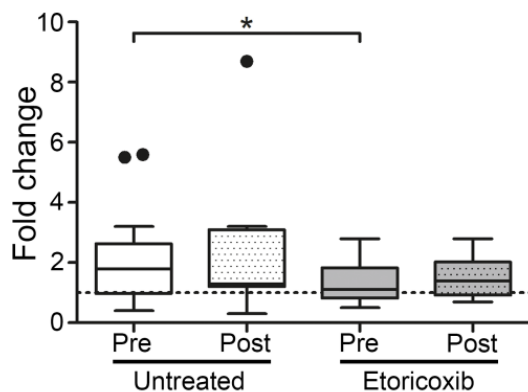
Cells were gated as lymphocytes based on forward and side scatter. Lymphocytes were further gated for CD3⁺CD4⁺ T-cells and those cells were gated based on the expression of CXCR3 and CCR4. Th1 cells were gated as CXCR3⁺ whereas Th2 cells were gated as CCR4⁺CXCR3⁻. Furthermore, Th2 cells were also gated as not expressing CCR6.

Figure S2: Expression of markers for Th1 and Th2 cells in peripheral blood.



Th1 was defined as CD4⁺CXCR3⁺ T-cells and Th2 was defined as CD4⁺CCR4⁺CCR6⁻CXCR3⁻ T-cells. **A**, percentage CXCR3⁺ of CD4⁺ cells. **B**, percentage CCR4⁺CCR6⁻CXCR3⁻ of CD4⁺ cells. Boxes represent the medians and 25th and 75th percentiles. Untreated = data from the no treatment period, Etoricoxib = data from the etoricoxib treatment period, Pre = before allergen challenge, Post = after allergen challenge. * $p < 0.05$ (Wilcoxon signed rank test of matched pairs, $n=16$).

Figure S3: FOXP3 mRNA expression.



The fold change expression of FOXP3 mRNA of CD4⁺ cells in anti-CD3 stimulated PBMCs was analysed with qPCR. Boxes represent the medians and 25th and 75th percentiles. Untreated = data from the no treatment period, Etoricoxib = data from the etoricoxib treatment period, Pre = before allergen challenge, Post = after allergen challenge. * $p < 0.05$ (Wilcoxon signed rank test of matched pairs, $n=16$).

Materials and Methods

Study participants

Sixteen non-smoking subjects with mild allergic asthma were recruited at the Lung and Allergy Clinic at Karolinska University Hospital, Huddinge. Patient demographic and serological characteristics are outlined in Table S1. This study was performed as an extension of the clinical NCT1002690 study and a detailed description of the patients has been published elsewhere (1). The study was approved by the local ethics committee, and all patients gave their written informed consent.

Study design

The study design has previously been described (1). Briefly, patients were subjected to a two-period, cross-over study with one 10-13 day period of treatment with a specific COX-2 inhibitor, etoricoxib (90 mg tablets, Arcoxia®, MSD, Sollentuna, Sweden), and one 10-13 day period without treatment (Fig. 1). An allergen inhalation challenge was performed on the last day of each treatment period with the relevant allergen extract (Aquagen, ALK, Copenhagen, Denmark) for each individual patient as described elsewhere (1). Nine patients underwent challenge with cat dander extract, 4 with birch pollen extract and 3 with timothy pollen extract (Table S1). The challenge was stopped when FEV₁ had fallen by at least 20% from baseline. Peripheral blood was collected in heparin tubes before and 6-7 h after challenge (Fig. 1). Peripheral blood mononuclear cells were isolated by density gradient centrifugation over Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). Cells that were not used immediately were frozen and kept at -150°C until use.

Flow cytometry

Markers for Th-cell subsets and Tregs were analysed on freshly prepared PBMCs by flow cytometry. Cell surface markers were stained with mouse-antibodies against CCR6 conjugated to PE (clone 53103, R&D Systems, Minneapolis, MN, USA), CD4 conjugated to ECD (clone SFCI12T4D11, Beckman Coulter, Brea, CA, USA), CD69 conjugated to Alexa Fluor 700 (clone FN50, BioLegend, San Diego, CA, USA), CD25 conjugated to FITC (clone M-A251), CD127 conjugated to PE (clone hIL-7R-M21), CXCR3 conjugated to PE-Cyanine5 (clone IC6/CXCR3), CCR4 conjugated to PE-Cyanine7 (clone 1G1), and CD3 conjugated to Pacific Blue (clone SP34-2), from BD Biosciences (San Diego, CA, USA). Intracellular staining for FOXP3 with rat anti-FOXP3 conjugated to Allophycocyanine (clone PCH101, eBioscience, San Diego, CA, USA) was performed according to the manufacturer's

instructions. Data were collected using a BD FACS Aria instrument (BD Biosciences) and analysed using FlowJo 7.6 software (Tree Star, Ashland, OR, USA).

***In vitro* culture of peripheral blood mononuclear cells**

Frozen PBMCs were thawed and cultures with 1×10^6 cells/ml in complete RPMI (RPMI supplemented with 10% BGS, 25 μ g/ml gentamicin, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (all from Gibco, Invitrogen Corporation, Paisley, UK) and 50 μ M β -mercapto-EtOH (Sigma-Aldrich, St. Louis, MO, USA)) were set up in 96 well culture plates. Stimulation of PBMCs from individual patients with the relevant allergen used for *in vivo* challenge was performed on cells from cat-allergic patients (n=9). From all patients, PBMCs were stimulated with influenza antigen (influenza vaccine, Vaxigrip®, Sanofi Pasteur MSD, Lyon, France) diluted 360x or left un-stimulated. Cat-allergic patients' PBMCs were stimulated with 10 μ g/ml of recombinant Fel d 1 (the major cat allergen, in house production (2)). The rFel d 1 protein preparation contained 50 ng lipopolysaccharide/mg protein as measured by limulus amebocyte lysate endochrome assay (Charles River Endosafe, Charleston, SC, USA). The cells were cultured for 6 days at 37°C, 5% CO₂. Then, 1 μ Ci of ³[H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to each well for the last 18 h of culture. Data is presented as Δ CPM where the baseline proliferation was subtracted from the stimulated proliferation.

Analysis of mRNA

Fresh PBMCs were cultured in 24-well plates in cRPMI, 3×10^6 cells/well, un-stimulated or in wells coated with an excess of anti-CD3 (OKT-3, in house production), 37°C, 5% CO₂ for 48 h. The cells were harvested, and separated into CD4⁺ and CD4⁻ cells using the CD4⁺ T Cell Isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). Thereafter the separated cell fractions were frozen in RLT-buffer (Qiagen, Hamburg, Germany) and stored in -80°C until preparation of RNA. RNA expression analysis was performed essentially as previously

described (3) using primers for FOXP3 with EF-1 as reference gene (4). Amplification of cDNA was performed in triplicates with SYBRGreen mix (Bio-Rad, Hercules, CA, USA) using a CFX96 detection system (Bio-Rad). The fold change in mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Data is presented as Tukey boxplots. Wilcoxon matched-pairs signed rank test was used for matched comparison of data between groups, and $p < 0.05$ was considered significant. All analyses were performed using Graphpad Prism 5 software (Graphpad Software Inc., San Diego, CA, USA).

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