

## Effect of cysteinyl leukotriene receptor antagonist on CD11b and CD23 expression in asthmatic children

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### Summary

**Background** Cysteinyl leukotrienes are potent pro-inflammatory mediators that contribute to the pathophysiologic features observed in allergic asthma. Inhibitors of leukotriene receptors represent novel therapy in asthma treatment. In addition to the protection from early asthmatic responses, these drugs have recently been shown to protect from late airway responses too.

**Methods** We studied the effect of treatment with an oral antagonist of cysteinyl leukotriene receptors on the increased expression of the low-affinity IgE receptor, CD23, on B cells, and of its ligands, CD11b and CD11c, on CD4<sup>+</sup> T cells and monocytes in peripheral blood of patients with allergic asthma. In this uncontrolled open-label study, 14 children with allergic asthma received montelukast, a cysteinyl leukotriene receptor antagonist, for a period of 6 weeks after demonstrating forced expiratory volume in 1 s (FEV<sub>1</sub>) of less than 80% of the predicted value. Samples of peripheral heparinized blood and sera were obtained before and after therapy completion. Three-colour immunofluorescence analysis was performed, and expression of CD11b and CD11c on CD4<sup>+</sup> T lymphocytes and monocytes as well as the expression of CD21 and CD23 on B cells were determined (*n* = 12). Peripheral blood eosinophil count, changes in FEV<sub>1</sub> and peak expiratory flow rate (PEFR), asthma exacerbations, and as-needed use of  $\beta$ -agonist were also monitored.

**Results** Montelukast improved FEV<sub>1</sub> and PEFR, and decreased peripheral eosinophil counts in all study patients. There was no significant change in the expression of CD21 and CD23 on B cells. The expression of CD11c on CD4<sup>+</sup> T cells and of both CD11b and CD11c on monocytes remained similar to the pretreatment expression. However, the percentage of CD11b<sup>+</sup>CD4<sup>+</sup> T lymphocytes significantly decreased after treatment with montelukast. This was accompanied by a significant decrease in the levels of total IgE.

**Conclusion** The capacity of 6-week montelukast therapy to reduce the percentage of CD11b CD4<sup>+</sup> T cells might be a mechanism leading to the immune response modulation on this T cell subset interaction with CD23-expressing B cells and subsequent down-regulation of IgE synthesis.

**Keywords** asthma, B lymphocytes, CD11b, CD11c, CD23, children, IgE, leukotriene receptor antagonist, montelukast, T lymphocytes

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### Introduction

The cysteinyl leukotrienes LTC<sub>4</sub> and LTD<sub>4</sub> are the most potent bronchoconstrictors so far described in human subjects, also contributing to airway inflammation in asthma [1, 2]. They are produced in the airway mucosa and submucosa by lipo-oxygenation of arachidonic acid released from cell membrane phospholipids following the activation of numerous cells that infiltrate airways in asthmatic patients, such as activated eosinophils, mast cells, basophils and monocytes. Cysteinyl leukotrienes are increased in bronchoalveolar lavage (BAL) fluid of most asthmatic patients, and their concentration further increases in BAL fluid from atopic

asthmatic patients after bronchial allergen challenge. The role of cysteinyl leukotrienes in the pathogenesis of bronchial asthma is further supported by several studies that have shown the protective effect of leukotriene-synthesis inhibitors and cysteinyl leukotriene receptor antagonists against a variety of bronchoconstrictor stimuli, e.g. exercise, allergens, aspirin and cold air [3–5].

Leukotriene receptor antagonists (LTRAs) are presently used as preventive treatment for persistent asthma in patients older than 2 years of age. Clinical studies in patients with mild to moderate asthma have demonstrated that monotherapy with LTRAs are able to improve airway function, asthma symptoms, use of as-needed medication, exacerbation rate and quality of life, without evidence of tolerance with prolonged use [6]. LTRAs attenuate the hallmarks of asthmatic inflammation, such as exhaled nitric oxide concentration and eosinophilia in the airway mucosa and

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peripheral blood. More recently, in addition to the protection from early asthmatic responses, these drugs have been shown to protect from late airway responses, too. In an animal model, the inhibition of leukotriene pathway with montelukast blocked the IL-2 induced late airway response and modulated cytokine expression after antigen challenge [3]. In asthmatic children, montelukast decreased serum concentrations of soluble interleukin 2 receptor (sIL-2R), IL-4, soluble intercellular adhesion molecule 1 (sICAM-1) and eosinophilic cationic protein (ECP) over a 6-week treatment period [4]. Another LTRA, pranlukast, was shown to suppress the production of IL-4, IL-5 and GM-CSF (granulocyte macrophage-colony stimulating factor) by peripheral blood mononuclear cells (PBMCs) under stimulation with specific antigens in patients with bronchial asthma [5].

The increased percentage of CD23, a low-affinity IgE receptor, in allergic diseases in man has stressed the role of this molecule in the regulation of IgE synthesis. The binding of CD23 to its ligands (CD21, CD11b and CD11c) could enhance the T-B cell interaction as well as activation of monocytes, leading to the regulation of IgE synthesis and release of inflammatory mediators, respectively. We have previously shown that monitoring the expression of CD23 and its counterparts in peripheral blood of allergic patients could be used to monitor the effect of allergen exposure or allergen immunotherapy [6–8]. Furthermore, increased expression of CD23 on B cells and of CD11b on CD4<sup>+</sup> T cells could be considered specific to allergic asthma, because children with intrinsic asthma showed no increase in the percentage of these cells.

Here we investigated whether 6-week treatment with an oral inhibitor of leukotriene receptors, montelukast, decreased the expression of low affinity IgE receptor, CD23, on B cells, and of its ligands, CD11b and CD11c, on CD4<sup>+</sup> T cells and monocytes in peripheral blood of patients with allergic asthma.

## Materials and methods

### Study subjects

This uncontrolled open-label study included 14 children with atopic allergic asthma (nine boys and five girls) aged 6–19 (median 11) years. All children had a history of bronchoconstriction with wheeze and chest tightness, positive skin test to aeroallergens, elevated total IgE levels ( $>150$  kU/L) and RAST to aeroallergens  $\geq$  class 3 (Table 1). At the time of the study, the patients had stable bronchial asthma without evident allergic manifestations. The symptom scores were graded according to the rating standards as follows: 0 = no symptoms, 1 = mild symptoms that did not interfere with daily activities, 2 = moderate symptoms that interfered with some activities and 3 = severe symptoms that interfered with many activities. The children received montelukast sodium (Singulair<sup>®</sup>, Merck Sharp & Dohme, Haarlem, The Netherlands) orally in a dosage of 10 mg/day for a period of 6 weeks. Montelukast is a selective and orally active leukotriene receptor antagonist that inhibits the cysteinyl leukotriene CysLT<sub>1</sub> receptor. Adverse effects, previously described by the manufacturer, such as pharyngitis, fever,

**Table 1.** Patient characteristics ( $n = 14$ )\*

| Characteristic                    | Data          |
|-----------------------------------|---------------|
| Age (years)                       | 11 $\pm$ 3    |
| Gender (%)                        |               |
| Male                              | 57            |
| Female                            | 43            |
| Pulmonary function                |               |
| FEV <sub>1</sub> (% of predicted) | 74 $\pm$ 12   |
| Morning PEFR (L/min)              | 281 $\pm$ 110 |

\*Data are presented as mean  $\pm$  SD.

FEV<sub>1</sub>, forced expiratory volume in 1 s; PEFR, peak expiratory flow rate.

sinusitis, diarrhea, nausea, etc. were not noticed in our group of children. Blood samples were obtained from the children after an informed consent provided by their parents. Total and specific IgE were determined using PRIST and RAST kits (Institute of Immunology, Zagreb, Croatia). Historical controls from gender- and age-matched children without evident allergic, immune or hematologic disorders were used for analysis of surface antigens for control purposes [6]. Samples of peripheral heparinized blood and sera, as well as eosinophil count, the measurement of lung functions (FEV<sub>1</sub> (forced expiratory volume in 1 s) and PEFR (peak expiratory flow rate)) and as-needed  $\beta$ -agonist use were obtained before and after therapy.

### Monoclonal antibodies

The following murine antibodies to human lymphocyte surface antigens were used: anti-CD4 (peridinin chlorophyll protein (PerCP)-conjugated), anti-CD20 (PerCP-conjugated), anti-CD23 (PE-conjugated), Leucogate<sup>®</sup> (anti-CD45 FITC/anti-CD14 PE) (all from Becton Dickinson, Heidelberg, Germany), anti-CD11c (FITC-conjugated), anti-CD11b (PE-conjugated) (Serotec, Oxford, UK) and anti-CD21 (FITC-conjugated) (Immunotech, Marseilles, France). In each experiment, FITC-, PE- and PerCP-conjugated isotype controls for determination of non-specific binding were included.

### Flow cytometry analysis

Using heparinized samples of peripheral blood, the standard procedure of triple, direct immunofluorescence staining of whole blood was performed as previously described [9]. Briefly, 50  $\mu$ L of heparinized blood were incubated in the dark at room temperature (RT) with 5  $\mu$ L of FITC-, PE- and PerCP-conjugated antibodies for 15 min. Erythrocytes were lysed by adding 2 mL of 10% FACS Lysing Solution (Becton Dickinson, San Jose, CA, USA) for 10 min at RT in the dark. After washing, the cells were resuspended in 0.5 mL of the staining buffer. In addition, blood was incubated with Leucogate (CD45 FITC/CD14 PE) using the same procedure to set the gate around lymphocytes (CD45<sup>+</sup>CD14<sup>-</sup>) and monocytes (CD45<sup>+</sup>CD14<sup>+</sup>) using the back-gating approach. Briefly, for analysis of lymphocytes, a fluorescence gate around the lymphocyte population expressing high levels of CD45 but low levels of CD14 was set. In the next step, a light-scatter gate was set to include  $\geq 98\%$  of the lymphocytes

included in the fluorescence gate. This approach enabled us to use four parameters (surface expression of CD14 and CD45, size and granularity of the cells) for a more accurate analysis of surface antigen expression throughout the study. Furthermore, for the analysis of CD11b and CD11c on CD14<sup>+</sup> monocytes, a fluorescence gate was set on the cells expressing high levels of both CD45 and CD14, followed by subsequent gating of the cells corresponding to monocytes.

Control suspensions were prepared by the same procedure, with fluorochrome-labelled normal murine immunoglobulins of the same isotype as the corresponding monoclonal antibodies.

Cell fluorescence of minimum 20 000 events was analysed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).

The collected data were analysed using CELLQuest software (Becton Dickinson) and expressed as percentage.

### Stimulation of PBMCs

For *in vitro* experiments, blood samples were obtained from a separate group of patients (three patients with extrinsic asthma, median 9 years, and three patients with intrinsic asthma, median 8 years). PBMCs were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as previously described [10]. Children with extrinsic asthma had positive skin tests to aeroallergens, elevated total IgE in serum higher than the geometric mean plus 2 SD of those in nonallergic children, RAST of classes 3–4, and peripheral blood eosinophilia. Patients with intrinsic asthma had a history of bronchial asthma only.

PBMCs ( $1 \times 10^6$ ) were cultured in RPMI 1640 growth medium supplemented with 10% heat-inactivated FCS (Gibco, Paisley, UK) with L-glutamine (2 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M) and gentamycin (1 mg/L), and kept at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

PBMCs were incubated for up to 48 h alone or with: (i) 10 µg/mL phytohemagglutinin (PHA, Sigma, St Louis, MO, USA), (ii) 10 ng/mL phorbol ester (PMA; Sigma) and (iii) 1, 10 and 100 ng/mL recombinant interleukin-4 (rIL-4; R&D Systems, Oxon, UK).

### Statistical analysis

The Wilcoxon test for paired data, and Mann–Whitney *U*-test for unpaired data were used. Median, upper quartile (UQ) and lower quartile (LQ) were used for descriptive statistics. The correlation was evaluated using Spearman rank correlation coefficient (rs).  $P < 0.05$  was considered significant.

## Results

Montelukast treatment significantly decreased the peripheral blood eosinophil count ( $P < 0.001$ , Fig. 1). This was accompanied by an increase in the morning PEFR and FEV<sub>1</sub>, measured at an outpatient clinic (Figs 2a and b). No correlation between the decrease in peripheral blood eosinophil count and either PEFR or FEV<sub>1</sub> was observed (data not shown). In most patients ( $n = 11$ ), the asthma was stable during the montelukast treatment, without as needed

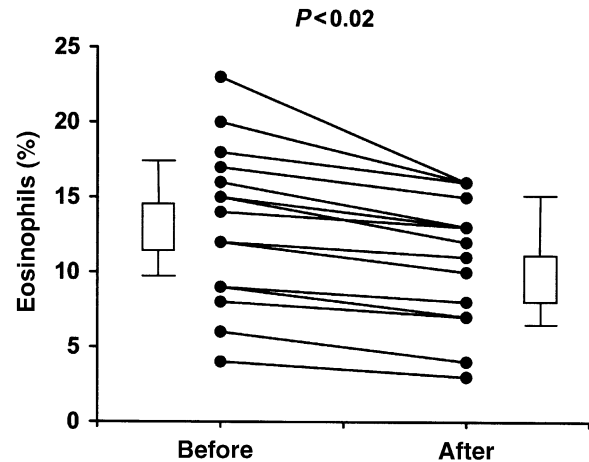


Fig. 1. Percentage of eosinophils before and after treatment with montelukast; median, upper and lower quartile before and after therapy are shown.

$\beta$ -agonist treatment, except for three patients who had mild symptoms that did not interfere with their activities, and they were successfully treated with  $\beta$ -agonists. There were no clinically relevant differences between these patients who required  $\beta$ -agonists and the rest of the patients included in the study. The level of total IgE decreased significantly after 6-week montelukast therapy ( $P < 0.002$ , Fig. 2c).

At the beginning of the study, the percentage of CD23<sup>+</sup> B cells was significantly higher in the extrinsic asthma group than in healthy children (historical controls) ( $P < 0.004$ , Fig. 3). Following 6-week treatment, the percentage of CD23<sup>+</sup> lymphocytes in the study group decreased, but not significantly ( $P > 0.05$ , before therapy median 78, LQ 71, UQ 81, after 6-week therapy median 73, LQ 65, UQ 79) and became similar to the percentage recorded in the control group of children.

The percentage of CD21<sup>+</sup> cells, which were mostly B lymphocytes, did not change in the patients receiving montelukast and was similar to the percentage measured in control subjects (data not shown).

Back-gating approach using monoclonal antibodies raised against CD45, a pan-leukocyte marker, and CD14, which is exclusively expressed on monocytes, enabled us to distinguish more precisely the CD11b expression on monocytes and T cells, since monocytes can also express CD4. With this approach we confirmed the percentage of CD4<sup>+</sup>CD11b<sup>+</sup> T cells to be significantly higher in the children with mild to moderate asthma than in the control group. Montelukast treatment significantly decreased the percentage of this T helper subset ( $P < 0.01$ , before therapy median 4, LQ 4, UQ 4, after therapy median 2.5, LQ 2, UQ 4.5) however, its peripheral blood level remained higher in asthmatic children as compared to controls even after 6-week montelukast therapy (Fig. 4). The expression of CD11c, which is mainly associated with monocytes, as well as the percentage of CD11b on these cells remained unchanged (data not shown).

In our study patients, lung function parameters (FEV<sub>1</sub> and PEFR) did not correlate with either of the measured parameters (CD23 on B cells, CD11b and CD11c on CD4<sup>+</sup> T cells and monocytes, IgE and peripheral blood eosinophilia; data not shown).

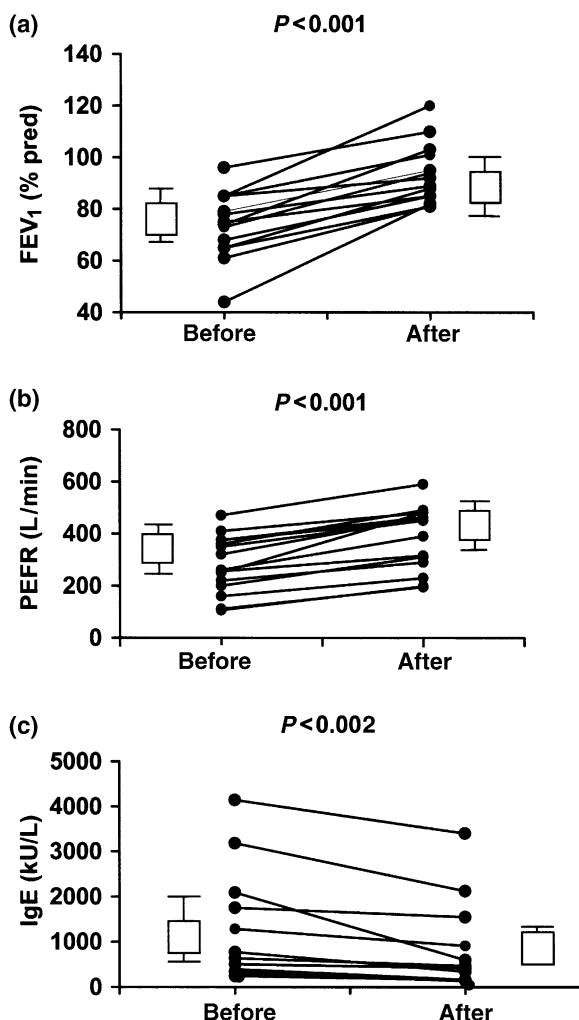


Fig. 2. FEV<sub>1</sub> (percentage of predictive value, a), PEF (peak expiratory flow, b) and serum level of IgE (kU/L, c) before and after treatment with montelukast. Each dot represents one patient; median, upper and lower quartile before and after therapy are shown.

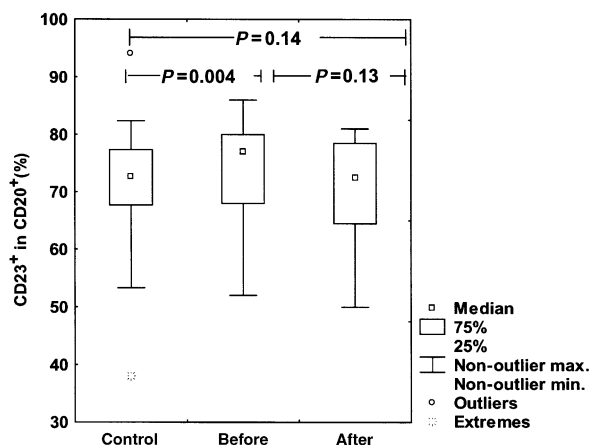


Fig. 3. Percentage of CD23<sup>+</sup> in CD20<sup>+</sup> lymphocytes in the control group, before and after treatment with montelukast.

We investigated whether the increased expression of CD11b on CD4<sup>+</sup> in patients with extrinsic asthma reflected the activation status of these cells or resulted from the effect of

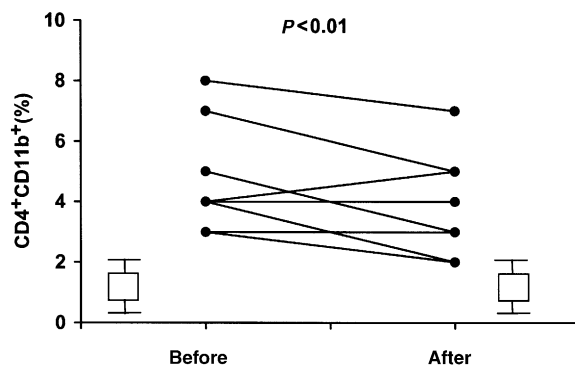


Fig. 4. Percentage of CD4<sup>+</sup> in CD11<sup>+</sup> lymphocytes in the control group, before and after treatment with montelukast. Each dot represents one patient; median, upper and lower quartile of CD11b CD4<sup>+</sup> cells in the controls are shown.

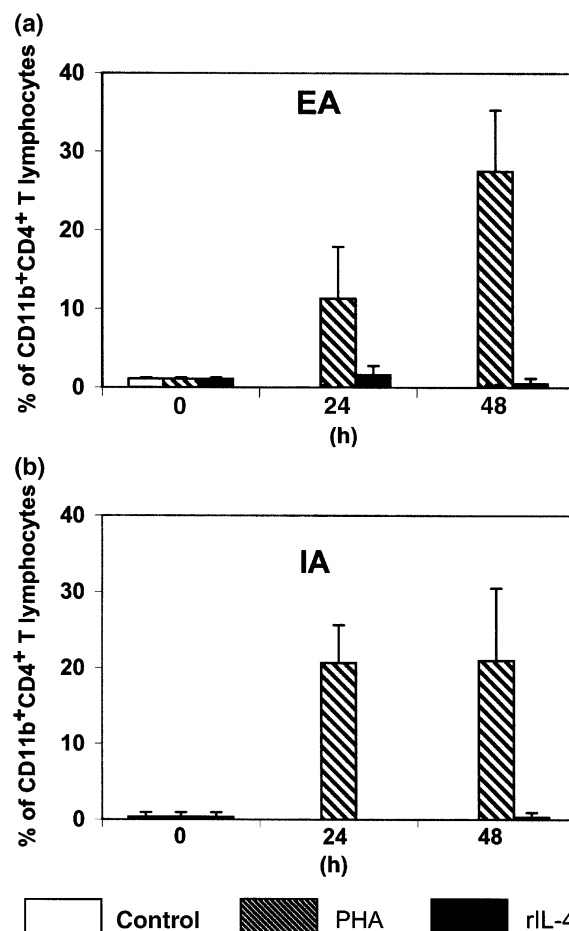


Fig. 5. Effect of rIL-4 and PHA on CD11b expression on CD4<sup>+</sup> from patients with extrinsic asthma (a) and intrinsic asthma (b) at 0, 24 and 48 h of cultivation.

cytokines involved in the Th2-type response, e.g. IL-4. Fig. 5a shows that PBMC from patients with extrinsic asthma further up-regulated CD11b on CD4<sup>+</sup> T cells, whereas rIL-4 had no effect. However, the same result was observed in the group of patients with intrinsic asthma (Fig. 5b).

## Discussion

To our knowledge, this study is the first to assess the possible effect of montelukast on a range of previously described markers of allergic disease in children with asthma. In previous studies, it has been shown that montelukast significantly improves asthma control, FEV<sub>1</sub>, eosinophil blood count and ECP. The latest literature data also show that, when compared to placebo, the 6-week administration of montelukast to children in a randomized, double-blind trial significantly decreased serum concentrations of IL-4 and sICAM. In the present study, we confirmed previous findings that montelukast improved lung function in patients treated with LTRAs. We also observed that this was accompanied by a significant decrease in total IgE levels. Although a role for leukotrienes in the regulation of IgE synthesis has been reported in animal models of allergic asthma [11], this has not yet been reported in humans. Our group and other authors have already reported that children with extrinsic allergic asthma have an increased percentage of CD23<sup>+</sup> lymphocytes in peripheral blood [7]. In the present study, the percentage of CD23 decreased, although not significantly, following the 6-week montelukast treatment and was similar to the values measured in historical controls.

There is ample body of data showing that IL-4 potently up-regulates the expression of CD23 on B cells. Although we did not measure the levels of IL-4 in patients receiving montelukast, one could argue that the observed decrease in CD23<sup>+</sup> B cells after 6-week treatment with montelukast, which was significantly lower than in the same patients before therapy and in age- and gender-matched control, could have resulted from this treatment. Indeed, it has been shown that endogenous leukotriene release by allergen-specific blood mononuclear cells can stimulate the synthesis of cytokines implicated in eosinophilia and IgE synthesis, such as IL-5 and IL-4, respectively [5]. Furthermore, IL-13 and IL-4 up-regulate the cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages, and a similar positive effect of IL-5 on eosinophils has also been shown [10, 12].

We also observed that, at least *in vitro*, IL-4 did not modulate the expression of CD11b on CD4<sup>+</sup> T lymphocytes. The only potent stimulus to up-regulate the expression of CD11b on these cells is cross-linking of CD3 molecule and activation with mitogens such as phytohemagglutinin [13]. Our *in vitro* data showed that upon culture with PHA, CD4<sup>+</sup> T cells up-regulated CD11b in patients with extrinsic and intrinsic asthma, whereas IL-4 had no effect. These results suggest that the modulation of CD11b expression on CD4<sup>+</sup> T cells observed in this study results from the effect of montelukast to dampen, probably indirectly, the activation of T cells. The absolute number of activated T cells bearing IL-2R correlates with eosinophilia in allergic patients and montelukast significantly decreases the level of the soluble form of IL-2R [14, 15].

This receptor is considered an important marker of T cell activation which could be shed from the surface of T cells. Increased levels of soluble IL-2 denote activation of T cell-mediated cellular immune response in asthma and also in other human diseases such as neoplasia [17, 18]. Therefore, changes in CD11b expression in allergic children could indicate activation of CD4<sup>+</sup> T cells, although the exact

mechanism is unknown. Furthermore, the increase in the percentage of CD11b-bearing helper T cells seems to be IgE-linked and disease-specific because the children with chronic, non-allergic obstructive pulmonary disease, i.e. intrinsic asthma, showed no increase in the percentage of CD11b<sup>+</sup>CD4<sup>+</sup> T cells. In a recent study, Nielsen et al. showed increased blood levels of CD11b<sup>+</sup> memory CD4<sup>+</sup> T cells in active rheumatoid arthritis [19]. However, in their hands, stimulation of PBMCs obtained from healthy donors with PHA showed that naive but not memory CD4<sup>+</sup> T cells up-regulate CD11b, indicating that CD11b-bearing memory CD4<sup>+</sup> T cells arise from specific rather than unspecific stimulatory events. The possibility that CD11b-expressing CD4<sup>+</sup> T cells have a regulatory function has also been suggested since antibodies to CD11b inhibited the anti-CD3-induced cell proliferation in T cell lines as well as IL-2 release [12]. However, these cell lines were obtained following repeated stimulation of peripheral blood T cells with PHA and irradiated mononuclear cells, so this phenomenon has yet to be confirmed by CD11b-expressing CD4<sup>+</sup> cells obtained from both healthy individuals and patients (i.e. rheumatoid arthritis or allergic patients).

In conclusion, our data point to a novel mechanism of montelukast action on anti-inflammatory response in children with allergic asthma. We have hypothesized that the decreased percentage of CD11b CD4<sup>+</sup> T cells modulates the immune response during their interaction with CD23-expressing B cells and subsequently leads to down-regulation of IgE synthesis.

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